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MINIREVIEWS

Role of inflammatory response in liver diseases: Therapeutic strategies

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Abstract

Inflammation and tumorigenesis are tightly linked pathways impacting cancer development. Inflammasomes are key signalling platforms that detect pathogenic microorganisms, including hepatitis C virus (HCV) infection, and sterile stressors (oxidative stress, insulin resistance, lipotoxicity) able to activate pro-inflammatory cytokines interleukin-1β and IL-18. Most of the inflammasome complexes that have been described to date contain a NOD-like receptor sensor molecule. Redox state and autophagy can regulate inflammasome complex and, depending on the conditions, can be either pro- or anti-apoptotic. Acute and chronic liver diseases are cytokine-driven diseases as several proinflammatory cytokines (IL-1α, IL-1β, tumor necrosis factor-alpha, and IL-6) are critically involved in inflammation, steatosis, fibrosis, and cancer development. NLRP3 inflammasome gain of function aggravates liver disease, resulting in severe liver fibrosis and highlighting this pathway in the pathogenesis of non-alcoholic fatty liver disease. On the other hand, HCV infection is the primary catalyst for progressive liver disease and development of liver cancer. It is well established that HCV-induced IL-1β production by hepatic macrophages plays a critical and central process that promotes liver inflammation and disease. In this review, we aim to clarify the role of the inflammasome in the aggravation of liver disease, and how selective blockade of this main pathway may be a useful strategy to delay fibrosis progression in liver diseases.

Key words: Caspase-1; Fibrosis; Hepatitis C virus; Inflammasome; Interleukin-1α; Interleukin-1β; Liver disease; Non-alcoholic fatty liver disease; NLRP3; Tumor necrosis factor-alpha

Core tip: Inflammasomes are newly recognized vital players in innate immunity. Several factors have been identified able to activate the NLRP3 inflammasome. Inappropriate activation of NLRP3 can contribute to the onset and progression of various diseases, particularly age-related diseases. It is well established that hepatitis C virus infection plays a critical role in the promotion of liver inflammation and disease, inducing the production of IL-1β and the activation of NLRP3. NLRP3 inflammasome gain of function aggravates liver disease, resulting in severe liver fibrosis and lately, hepatocellular carcinoma. In non-alcoholic fatty liver disease, the regulation of inflammation processes may prevent the progression of non-alcoholic steatohepatitis to fibrosis.

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INTRODUCTION

Hepatic inflammation is a common trigger of liver disease, and is considered the main driver of hepatic tissue damage, triggering the progression from non-alcoholic fatty liver disease (NAFLD) to severe fibrogenesis and, finally, hepatocellular carcinoma (HCC).

Liver diseases, whose etiology can be diverse, are becoming one of the most serious public health problems. The diseases usually occur in response to chronic hepatocellular injury caused mainly by the abuse of alcoholic intake, chronic infections such as those caused by the hepatitis C virus (HCV), bile duct damage, NAFLD or non-alcoholic steatohepatitis (NASH)[1]. NAFLD was defined for the first time in 1980[2] as an accumulation of fat (> 5%) in liver cells in the absence of excessive alcohol intake[3]. The disease affects more than 30% of the population of the western world, especially patients suffering from metabolic syndrome, obesity (76%), and type II diabetes (50%)[4]. The histological spectrum of NAFLD begins in a simple benign steatosis, evolving to a NASH. From this point, the consequent scarring and tissue replacement begins with type I collagen, developing fibrosis, cirrhosis and finally, in many cases complicating a HCC[3]. This pathogenesis, which is complex, is explained by two main impacts or damages to the liver tissue: on the one hand, a lipid accumulation in the hepatocytes, to which a second oxidative stress damage is added[5]. It is this second damage, which produces a lipotoxicit that triggers the inflammatory response by the release of danger-activated molecular patterns (DAMPS) and pathogenic-activated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). Finally, they activate the innate immunity that causes the hepatic inflammation, being able to aggravate the process of fibrosis, cirrhosis, causing HCC.

That is why the knowledge of the relationship between liver disease and uncontrolled activation of the immune response, resulting in aggravating liver inflammation of disease progression, can be crucial for the design of therapeutic strategies blocking the immune response uncontrolled.

PATHOGENESIS OF NAFLD

The pathogenesis of this disease is complex, and is explained by two major impacts or damage to the liver tissue: on the one hand, a lipid accumulation in the hepatocytes, to which a second oxidative stress damage is added[5].

Lipid accumulation in hepatocytes

The first of these is the abnormal accumulation of triglycerides in the hepatocyte, either due to a high intake of saturated fats and obesity, genetic deficiencies or insulin resistance (IR) due to hyperglycemia and hyperinsulinemia[6], what is known as metabolic syndrome. Hyperinsulinemia and increased hepatic glucose production induce the expression of the sterol regulatory element binding protein (SREBP-1c) and the carbohydrate response element binding protein (ChREBP), respectively, which activate in turn the transcription of most of the genes involved in the enzymatic machinery necessary for free fatty acids (FFA) synthesis, decreasing the beta oxidation thereof[7].

In addition, there are many mediating molecules such as peroxisome proliferator activating ligand receptors (PPAR), among which PPAR-γ, whose expression in conditions of liver damage is usually high, contribute to the accumulation of FFA[7]. The liver X receptor (LXR), another important mediator, activates genes involved in the synthesis of FFA, such as SREBP-1c and ChREBP, contributing to steatosis[8]. Finally, the AMP-activated protein kinase (AMPK) functions as a sensor of the energy levels of the cell, stimulating catabolic pathways such as mitochondrial beta oxidation, and inhibiting ATP-consuming processes, such as lipogenesis. All this is done by phosphorylating different proteins involved in these pathways, such as acetyl-CoA carboxylase (ACC), ChREBP and SREBP-1c, which end up being inhibited.

In the process of lipid accumulation, several molecules act as DAMPs and pathogen-activated molecular patterns (PAMPs), that is, molecules that trigger inflammation. These may include the fatty acids, adenosine triphosphate (ATP), uric acid or proteins derived from the extracellular matrix, among others. In addition, recent research suggests that in the liver tissue of humans and mice with steatohepatitis, cholesterol crystals are present within hepatocytes, and they can act as DAMPs.

Oxidative stress and liver inflammation

A second impact that explains the characteristic histological lesions of NAFLD is oxidative stress and lipid peroxidation[5,9]. As a result of liver damage and liver inflammation, the inflammatory cells and the hepatocyte itself release cytokines, such as TNF-α and reactive oxygen species (ROS). These mediators can cause peroxidation of plasma and mitochondrial membranes, which leads to cell death due to necrosis or apoptosis[5,9]. All this activates endothelial cells of the liver, which increase the expression of cytokines, and finally activates the hepatic stellate cells (HSCs), producing a phenotypic change, associated with the acquisition of pro-fibrogenic and pro-inflammatory functions[10].

Significant accumulation of triglycerides and cholesterol in VLDL and LDL particles, added to the oxidative stress developed in the hepatocytes, produces the oxidation of the cholesterol linked to LDL, generating particles of LDL oxidase (oxLDL) that constitute an important risk factor of the disease[11]. Recent studies show that oxLDL also contribute to the process of cellular inflammation and apoptosis.

ACTIVATION OF THE INNATE IMMUNE RESPONSE DURING NAFLD

Excessive lipids accumulation leads to hepatocyte damage, activating an inflammatory response that aggravates the progress of the liver disease, and which, in turn, feeds back the activation of the inflammation[12].

The immune response of the liver is produced by the action of immune cells such as Kupffer cells, monocytes, neutrophils, dendritic cells (DCs), natural killer cells (NK), and NK T cells (NKT), which initiate and maintain the hepatic inflammation through the production of cytokines and chemokines, especially TNF and interleukin (IL) -1β, as well as reactive oxygen species[13].

The triggering of hepatic inflammation, as noted above, is caused by the accumulation of infectious and non-infectious material, which is released during cell damage and is recognized by pattern recognition receptors (PRRs). These PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and several other receptors[14].

The endogenous molecules produced by cellular damage or stress result in an inflammatory response (DAMPs). These molecules are very diverse and do not have a common structure between them. In the pathology of the liver disease and liver inflammation, induced-inflammation particles are included, such as cholesterol crystals. These cholesterol crystals produce the development, not only of the atheroma plaques, but of the inflammation, being present in humans and mice with NASH[15] candidates are free fatty acids (FFA) that are released during liver damage. A specific mechanism by which they act is to activate TLR4, while fetuin-A, a 64-kDa protein specific for hepatocytes, is required. It has been proven that the reduction of fetuin-A in mice with high fat diet results in a decrease of inflammatory signalling mediated by TLR4 in adipose tissue. Normally, in patients with NAFLD, the expression of fetuin-A is high[16]. Palmitic acid (PA) is another molecule that causes liver inflammation, and it has recently been linked to the TLR2 receptor. The palmitic acid ligand of TLR2 induces the activation of caspase- Other 1 and the release of IL-1α and IL-1β, having an evident role in the liver inflammatory process[17].

Many of these DAMPs activate PRR present in immune cells, of which the TLRs are the best characterized. In liver disease, saturation of fatty acids produces inflammation in the hepatocytes, which increases the induction of caspase-1 activation and the release of IL-1β. This results in a release of more DAMPs from the hepatocytes, generating a feedback that amplifies the inflammatory response.

Among the various NLR receptors, the NLRP3 inflammasome is the best characterized and associated with a wider range of diseases including infections, autoinflammatory diseases and other autoimmune diseases. The NLRP3 inflammasome has the characteristic of forming a complex with apoptosis-associated speck-like protein to CARD (ASC), to activate caspase-1 and induce the maturation and secretion of important proinflammatory cytokines such as IL-1β and IL-18. Cytokines act directly against the damage and infection of the liver tissue[18].

Role of NLRP3 inflammasome in liver inflammation

Activation of the NLRP3 inflammasome is important in the inflammatory process, and occurs in two steps. The first includes the activation of TLRs by different molecules DAMPs and PAMPs (Figure 1). TLRs belong to the family of PRRs and their function is to maintain tissue homeostasis through the regulation of inflammatory responses. In the liver, TLRs are expressed in Kupffer cells, endothelial cells, DCs, epithelial biliary cells, HSCs and hepatocytes. Activated TLRs activate the cells and contribute to the release of cytokines that facilitate the progression of liver disease. There are at least 13 known TLRs, whose structure is characterized by having a leucine-rich repeat structure (LRR) in the extracellular domain and a Toll/IL-1 receptor (TIR) in its intracellular domain[19].

TLR4 has an interesting role in liver inflammation and fibrogenesis[20]. The Kupffer cells of the liver are the first to be attacked by the damage produced, and express TLR4 to which lipopolysaccharides (LPS) bind. This produces the activation of NF-kB, mitogen-activated protein kinase (MAPK)[21] extracellular signal-regulated kinase-1 (ERK1), p38, c-jun N-terminal kinase (JNK) and interferon regulatory factor 3(IRF3)[22], which finally triggers the production of proinflammatory cytokines and enhances IFN-β and STAT1 expression[23]. The proinflammatory stimulus facilitates hepatocyte damage, contributing to the secretion of profibrogenic

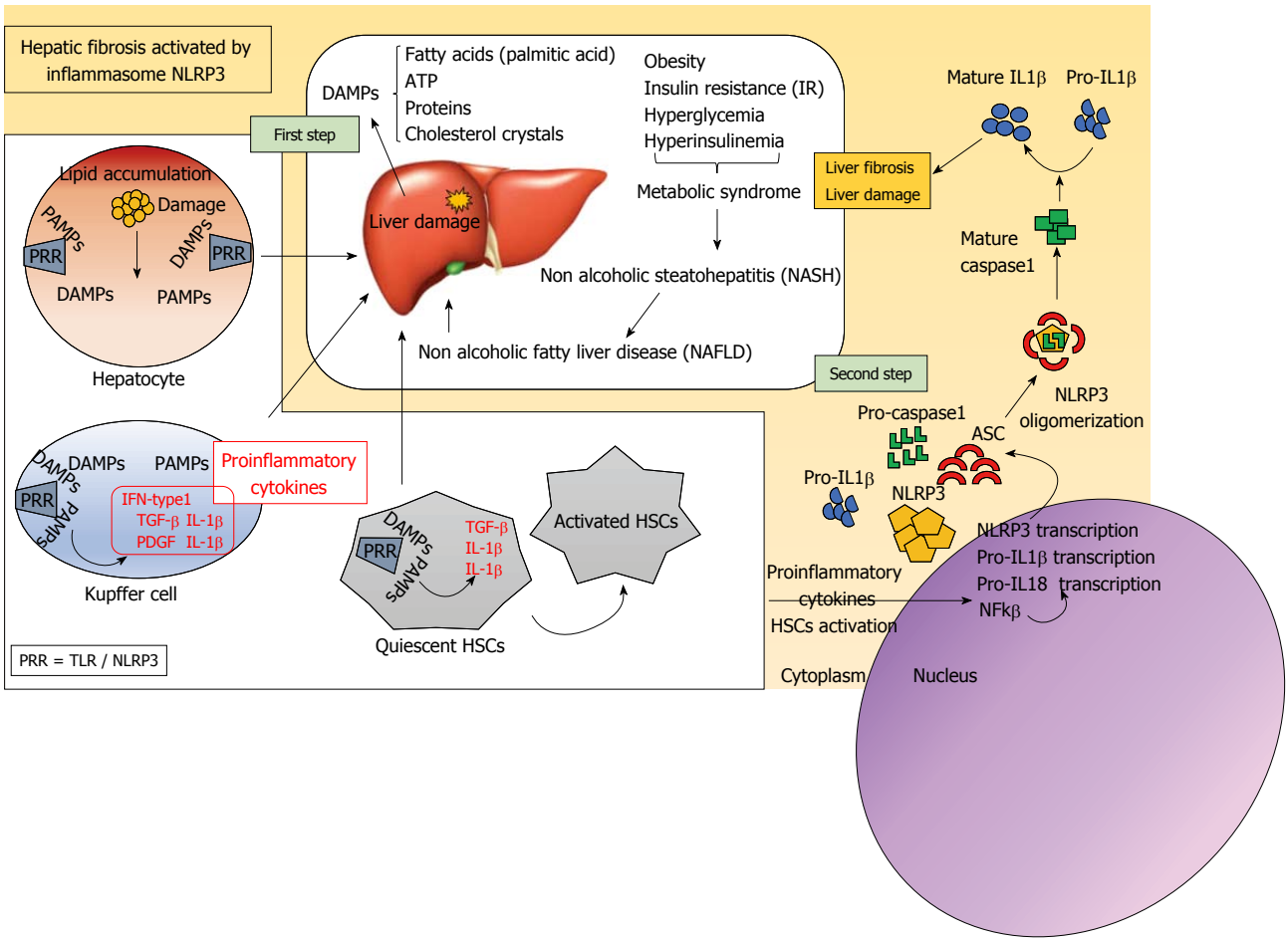

Figure 1 Hepatic fibrosis caused by the activation of the inflammasome-NLRP3 complex. The accumulation of lipids derived from the metabolic syndrome produces a non-alcoholic steatohepatitis (NASH) that progresses to a non-alcoholic fatty liver disease (NAFLD). The accumulated lipids, fatty acids, cholesterol crystals, among others, produce an alteration in the homeostasis of the liver cells, producing liver damage. Danger-activated molecular patterns (DAMPs) and pathogenic-activated molecular patterns (PAMPs) bind to and activate PRR receptors (TLRs, NLRPs, *etc*.) in hepatocytes, immune cells, hepatic stellate cells (HSCs), endothelial cells and DCs. Activated TLRs activate the cells and contribute to the release of cytokines that facilitate progression of liver disease. The proinflammatory stimulus facilitates hepatocyte damage, contributing to the secretion of profibrogenic cytokines such as transforming growth factor beta (TGF-β), promoting the activation of HSCs. This activation, in turn, up-regulates transcription of inflammasome-related components, including inactive NLRP3, pro-IL-1β and proIL-18. The second step of inflammation activation is the oligomerization of NLRP3 and subsequent assembly of NLRP3, ASC, and procaspase-1 into a complex. This triggers the transformation of procaspase-1 to caspase-1, as well as the production and secretion of mature IL-1β and IL-18, which produces liver damage and facilitates the progression of hepatic fibrosis.

cytokines such as transforming growth factor beta (TGF-β) and the platelet-derived growth factor (PDGF), promoting the activation of HSCs. This activation, in turn, up-regulates transcription of inflammasome-related components, including inactive NLRP3, pro-IL-1β and proIL-18[24,25].

The second step of inflammasome activation is the oligomerization of NLRP3 and subsequent assembly of NLRP3, ASC, and procaspase-1 into a complex (Figure 1). This triggers the transformation of procaspase-1 to caspase-1, as well as the production and secretion of mature IL-1β and IL-18[18].

NLRP3 signaling during liver inflammation

After NLRs overexpression, members of this family play an important role in the formation of intracellular multiprotein complexes called inflammasomes. The union of microbial components or activators of the inflammasome (DAMPs and PAMPs) that enter the cytoplasm are detected by these cytosolic NLRs, activating the inflammasome formation. The inflammasome consists of an NLR protein, the adapter molecule ASC and procaspase-1, which is an effector molecule[26]. The formation of this complex serves to activate cysteine protease caspase-1, which, in turn, produces the maturation of proinflammatory cytokines, including IL-1b and IL-18, and the proteolytic inactivation of IL-33[27].

In short, and specifically in NAFLD, the saturated fatty acids from the excess lipid in the liver represent harmful endogenous molecules that finally induce the activation of the inflammasome. In addition, hepatocytes exposed to these saturated fatty acids release signals that trigger the activation of immune cells. Activation of the inflammasome activates caspase-1 in Kupffer cells, inducing proinflammatory signaling and activation of HSCs. In this way, collagen

deposition occurs that triggers liver fibrosis[28] have shown that cholesterol crystals could be one pathway to activate the inflammasome in NASH. To test whether inflammasome blockade alters inflammatory recruitment, they used a drug called MCC950, which has already been shown to block NLRP3 activation, in an attempt to reduce liver injury in NASH. This drug partly reversed liver inflammation, particularly in obese diabetic mice.

Release of cytokines by immune cells

Inflammatory signaling of the liver is regulated by cytokines capable of activating effector functions in immune cells. Kupffer cells are the first to detect the presence of PAMPs and DAMPs through TLRs, activating the release of cytokines such as TNF-α, IL-1 and IL-6, as well as chemokines chemokine (C-X-C motif) ligand 13 (CXCL13), chemokine (C-X-C motif) ligand 8 CXCL-8 and Chemokine (C-C motif) ligand 24 (CCL24), which initiate an acute phase inflammatory response. These cytokines can produce apoptosis of hepatocytes, steatosis and inflammation, including the onset of a fibrosis process after interaction with HSCs *via* TGF-β. Cytokines can also activate hepatic sinusoidal endothelial cells that are ultimately involved in the recruitment of neutrophils, monocytes and NKT cells, being a feature of acute liver damage. Neutrophils can be activated, and their phenotype changed, releasing ROS, defensins and other chemokines that attract more neutrophils and monocytes. Monocytes can be differentiated into TNF-α, IL-1β, granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) macrophages, increasing the life expectancy of these neutrophils[29,30]. Finally, the inflammation resolves with the apoptosis of the neutrophils, being these apoptotic neutrophils signals involved in the onset of phagocytosis and in the increase of IL-10 and TGF-β, cytokines related to the end of the inflammatory response and the repair of the tissue[31].

Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal stem cells with immunonodulatory characteristics. MSCs are considered to act through multiple mechanisms to coordinate a dynamic, integrated response to liver inflammation and fibrosis, which prevents the progressive distortion of hepatic architecture. Management of inflammatory patterns is crucial also in case of potential treatment of liver diseases with stem cells[32]. Adult stem cells have gained in attractiveness over embryonic stem cells for liver cell therapy due to their origin, multipotentiality, and the possibility of autologous transplantation.

ACTIVATION OF HSCS AND HEPATIC FIBROSIS

Any of these necroinflammatory mechanisms described above can activate HSCs, the main ones involved in the process of hepatic fibrogenesis[33]. In normal liver, stellate cells are described as being in a quiescent state. Quiescent stellate cells represent 5%-8% of the total number of liver cells[34]. When the liver is damaged, stellate cells can change into an activated state. The activated stellate cell is characterized by proliferation, contractility, and chemotaxis. This state of the stellate cell is the main source of extracellular matrix production in liver injury[35].

The hepatic stellate cells are characterized by having in their cytoplasm small droplets of fat 1-2 microns in diameter that allow the storage of vitamin A, one of its main functions. Besides, they control intercellular communication through the release of mediators, and participate in the homeostasis of the MEC of the liver by the production of collagens and non-collagens, synthesis of metalloproteinases (MMP) that catabolize the components of the MEC, and synthesis of inhibitors of MPPs, also called tissue inhibitors of metalloproteinases (TIMP) that control the catalytic activity of MMPs to maintain a homeostasis of the MEC[36].

Therefore, after the chronic injury of the liver tissue, both the HSC and other cells producing the ECM undergo activation, a pathological process characterized by the loss of fat droplets, an increase in the number and size of the cells, and phenotypic transdifferentiation to proliferating, fibrogenic and contractile cells, which will be very similar to myofibroblasts. All this is mediated by different factors that induce cell proliferation, fibrogenic mediators such as TGF-β1 and IL-6, inducers of HSC contraction, such as endothelin-1, thrombin or angiotensin II and finally mediators of anti-inflammatory and anti-fibrogenic activity, such as IL-10 and interferon-γ (IFN-γ)[36].

Because of the activation of HSCs, phenotypic changes occur that affect the development of hepatic fibrosis, such as the production of collagen and non-collagen proteins of the MEC (collagen type I, type III, type IV, laminin, elastin, fibronectin and various proteoglycans) by the CEH[37].

CONCLUSION

Many evidences reported in the literature suggest that the activation of the NLRP3 inflammasome complex and the consequent generation of the acute inflammatory response in the liver, facilitates the progress of steatohepatitis to liver fibrosis, cirrhosis and finally, HCC development. However, recent studies show that the KO mice of NRLP6 and NLRP3 inflammasomes have a worse progression in the NAFLD/NASH disease. The absence of the inflammasome is associated with changes in the homeostasis of the intestinal microbiota, resulting in a strong hepatic steatosis and inflammation through TLR receptor agonists, such as TLR4, which allows the release of TNF-α which leads to the progression of NASH[38]. This demonstrates the complexity of the effects of the activation

of the inflammasome, which can generate an acute inflammation or, conversely, be protective. However and in general terms, this activation is usually proinflammatory in the liver, and its inactivation and absence in relation to the microbiota should be carefully studied. Recently, Pierantonelli *et al*[39] have shown that the progression of liver fibrosis is associated with the downregulation of NLRP3 in the gut which, together with the current evidence of a strong correlation between intestinal changes (including modification of microbiota composition) and liver disease, makes the role of NLRP3 in the intestine extremely attractive as a protective factor. Finally, MCC950 has been proven as an effective NLRP3 inhibitor, being able to reduce liver injury and inflammation.

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ORIGINAL ARTICLE

Basic Study

Preserved liver regeneration capacity after partial hepatectomy in rats with non-alcoholic steatohepatitis

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Abstract

AIM

To evaluate the liver regeneration capacity (LRC) after partial hepatectomy (PH) in experimental non-alcoholic steatohepatitis (NASH).

METHODS

Fifty-four female rats were fed a high-fat, high-cholesterol diet (HFCD, 65% fat, 1% cholesterol) or standard diet (STD) for 16 wk. A 70% PH was performed and the animals were euthanised before PH or 2 or 5 d post-PH. LRC was evaluated using: The total number of Ki-67 positive hepatocytes in the caudate lobe, N(Ki-67, lobe)

evaluated in a stereology-based design, the regenerated protein ratio (RPR), prothrombin-proconvertin ratio (PP), and mRNA expression of genes related to regeneration.

RESULTS

The HFCD NASH model showed significant steatosis with ballooning and inflammation, while no fibrosis was present. Mortality was similar in HFCD and STD animals following PH. HFCD groups were compared to respective STD groups and HFCD animals had a significantly elevated alanine transaminase at baseline ($P < 0.001$), as well as a significantly elevated bilirubin at day 2 after PH ($P < 0.05$). HFCD animals had a higher N(Ki-67, lobe) at baseline, ($P < 0.0001$), day 2 after PH ($P = 0.06$) and day 5 after PH ($P < 0.025$). We found no significant difference in RPR or PP neither 2 or 5 d post-PH. Expression of liver regeneration genes (*e.g.*, hepatic growth factor) was higher at both day 2 and 5 post-PH in HFCD groups ($P < 0.05$).

CONCLUSION

NASH rats had a preserved LRC after hepatectomy when compared to STD rats. The methods and models of NASH are essential in understanding and evaluating LRC.

Key words: Rat; Non-alcoholic fatty liver; Non-alcoholic steatohepatitis; Liver regeneration; Hepatectomy; Ki-67; Gene expression

Core tip: Liver regeneration capacity has been studied in different animal models of non-alcoholic steatohepatitis. This study is the first to use a high fat high cholesterol model which mimic the pathogenesis of human non-alcoholic steatohepatitis better than previous animal models. Liver regeneration capacity was evaluated using: (1) The total number of Ki-67 positive hepatocytes in the caudate lobe, evaluated in a stereology based design; (2) the regenerated protein content to describe the regenerated liver mass; and (3) the plasma concentration of coagulation factors as a marker of liver function. We found a preserved liver regeneration capacity in rats with non-alcoholic steatohepatitis, adding important knowledge to the subject.

Haldrup D, Heebøll S, Thomsen KL, Andersen KJ, Meier M, Mortensen FV, Nyengaard JR, Hamilton-Dutoit S, Grønbæk H. Preserved liver regeneration capacity after partial hepatectomy in rats with non-alcoholic steatohepatitis. *World J Hepatol* 2018; 10(1): 8-21 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/8.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.8

INTRODUCTION

The incidence of obesity and non-alcoholic fatty liver disease (NAFLD) is increasing worldwide, affecting approximately one-third of the general population[1]. Patients with NAFLD and especially non-alcoholic steatohepatitis (NASH) have a higher risk of developing primary hepatocellular carcinoma (HCC)[2]. Further, they have an increased risk of other cancers, for example colorectal carcinoma[3], which often metastasize to the liver[4]. Surgical resection of the liver tumor remains the gold standard treatment for both HCC and liver metastases from colorectal cancer[5]. Epidemiological studies have shown that liver resection is associated with increased morbidity and mortality in patients with NAFLD following liver resections[6]. It has been proposed that NASH livers are more vulnerable to surgical interventions because of decreased liver regeneration capacity (LRC)[7].

Previously, LRC has been studied in various rodent models of NAFLD/NASH generally based on the use of the methionine-choline deficiency diet (MCD)[7-12], choline deficiency diet (CDD)[13-16], simple high-fat diets (HFD)[17,18] and the genetic leptin-deficiency model[19-25]. These are widely accepted models of NAFLD/NASH, yet the animals lack many of the clinical and/or histopathological features related to human NAFLD/NASH. These previous studies of LRC have reported conflicting results, even when the same dietary models were used. In the MCD[7-12], CDD[13-16], HFD[17,18] and a high-fat model combined with fructose[26], decreased[9-14,18,26] as well as normal liver regeneration[7,8,15-17] have been demonstrated. Our group has previously studied a high-fat, high-cholesterol diet (HFCD) rat model[27,28] with features that closely resemble human NASH[29]. To our knowledge, HFCD models have never been used to study the LRC experimentally.

We studied partially hepatectomized, HFCD-fed rats, hypothesizing that rats with HFCD-induced NASH would have decreased LRC, as well as lower expression of genes related to regeneration. LRC was evaluated using: (1) The total number of Ki-67 positive hepatocytes N(Ki-67, liver) evaluated in a stereology-based design; (2) the regenerated protein ratio (RPR); and (3) plasma concentration of coagulation factors Ⅱ, Ⅶ, Ⅹ, prothrombin-proconvertin ratio (PP) before, and 2 or 5 d after hepatectomy.

MATERIALS AND METHODS

Animals

In total, 54 female Wistar rats (body weight 201-237 g; Taconic M and B, Ejby, Denmark) were housed at 21 ℃ ± 2 ℃ with a 12-h artificial light cycle. Three animals were housed in each cage with free access to tap water. All animals were allowed to acclimatize on a standard diet (STD) for a week followed by randomization and allocation. Then, half of the rats were fed STD and the other half HFCD ad libitum for 16 wk (Figure 1). Diets were obtained from Research Diets (NJ, United States). The STD (D14071501) consisted of the following energy sources: carbohydrates 67 g (70 kcal/100 kcal), fat 4 g (10 kcal/100 kcal), and protein 19 g (20 kcal/100 kcal)

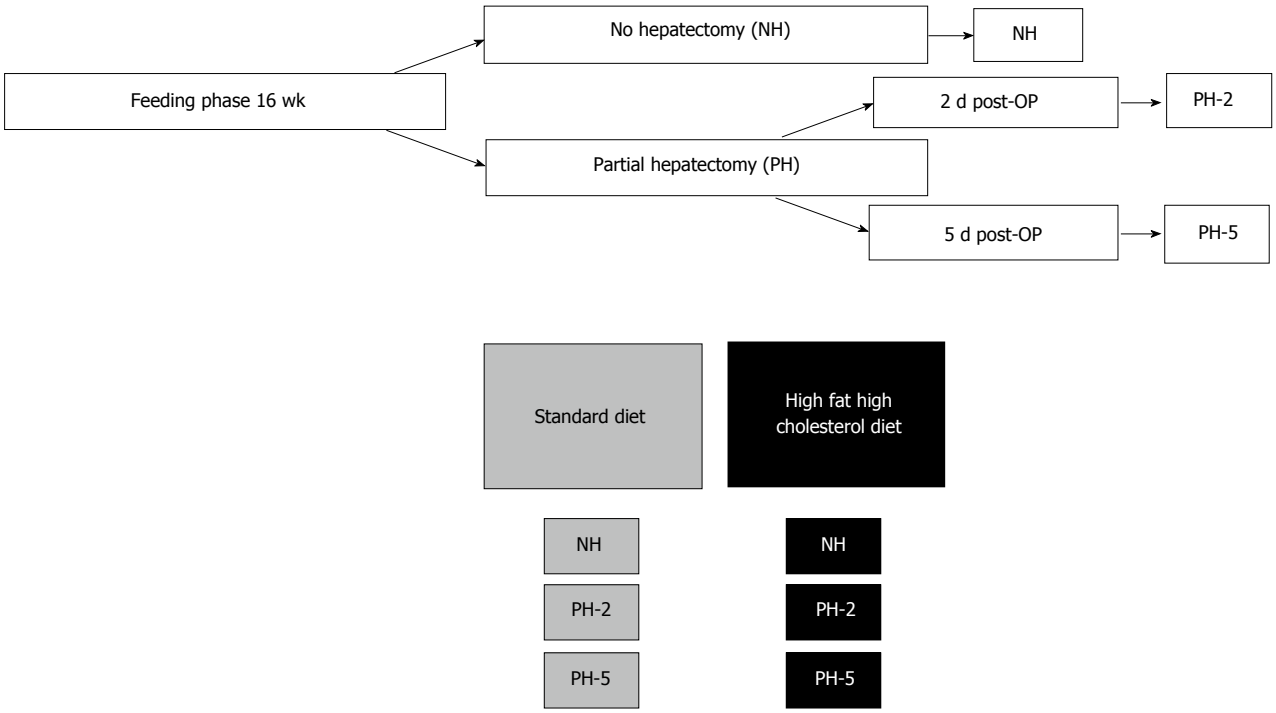

Figure 1 Overview of the groups, 9 animals in each group. PH-2: Partial hepatectomy and 2 d; PH-5: Partial hepatectomy and 5 d.

per 100 g diet. The HFCD (D14071502) consisted of carbohydrates 19 g (15 kcal/100 kcal), protein 27 g (20 kcal/100 kcal), 1 g cholesterol and fat 39 g (65 kcal/100 kcal) per 100 g diet, including 1% cholesterol and 0.25% cholate.

The study was performed in accordance with local and national guidelines for animal welfare and approved by the Animal Experiments Inspectorate (2014-15-2934-00997).

Design

After 16 wk, both STD and HFCD rats were randomly divided into the following groups of nine rats (Figure 1): (1) No hepatectomy before sacrifice (NH); (2) partial hepatectomy (PH), sacrificed two days post-surgery (PH-2); (3) partial hepatectomy, sacrificed five days post-surgery (PH-5).

The non-hepatectomized NH rats served as a "baseline" reference. The PH-2 and PH-5 animals underwent a partial hepatectomy as previously described[30]. Briefly, the abdominal cavity was opened with a longitudinal incision in the linea alba. The left lateral and median lobes were mobilized and ligated followed by a resection, resulting in a 70% reduction of the liver tissue. The abdominal wall was closed with a continuous 4.0 absorbent suture and the skin was closed with staples. The resected liver tissue was weighed after removal. To ensure minimal post-operative pain, Carprofen 5 mg/kg (Rimadyl; Pfizer Animal Health, Exton, United States) was administered prior to the surgical procedure and two days after hepatectomy. Following hepatectomy, rats were fed their initial diet (STD or HFCD) until euthanisation.

At euthanisation the animals were anesthetized with a subcutaneous injection of fentanyl/fluanisone 0.5 mL/kg (Hypnorm; Jansen Pharma, Denmark) and midazolam 2.5 mg/kg (Dormicum; La Roche, Switzerland) and body weights were registered. Blood samples were collected through the retrobulbar venous plexus. Blood for analysis of prothrombin-proconvertin ratio (PP) were collected through the vena cava caudalis. The liver was removed en bloc and weighed. Liver tissue was collected from the right liver lobe and immediately snap-frozen in liquid nitrogen and stored at 80 ℃ until use. The caudate liver lobe was immersion fixed in phosphate-buffered 4% formaldehyde for a total of 48 h before paraffin embedding. Prior to embedding, and 24 h into the fixation, the lobe was cut with a special designed razor tool to create 2.15 mm thick slabs of liver tissue. The tissue slabs were then put back in the grid in correct order, all facing the same way for stereological examination. The caudate lobe was used for the histological evaluation and the stereological Ki-67 evaluation. Euthanasia was then achieved by cervical dislocation. Euthanisation was carried out between 8 am and 13 pm.

Histology

All samples were stained with hematoxylin and eosin (HE) and Masson-trichrome (MT), using standard protocols. The degree of steatosis and the presence of NASH were evaluated by an expert liver pathologist using both the Kleiner and Bedossa criteria[31,32] examining 5 medium-power fields (20 x objective). Steatosis was classified as either: large droplet macrovesicular

steatosis (LDMS), small droplet macrovesicular steatosis (SDMS), mixed small and large macrovesicular steatosis (MXMS) or microvesicular steatosis (MVS).

Stereological quantitation

Ki-67 positive hepatocytes: We quantified the total number of Ki-67 positive hepatocytes using a stereological-based design. The paraffin embedded caudate lobes were cut in 3 μm thick slides and immunohistochemically stained with the anti-Ki-67 antibody (clone MIB-5, isotype IgG1; Dako, Denmark) using a standard (in-house) protocol. All Ki-67 stained slides were scanned as virtual images using an Olympus VS 120 slide scanner with a 20x oil lens (numerical aperture 0.85).

The image files were transferred to the newCAST software version 5.2.1 (Visiopharm, Denmark) for quantification, performed as previous described[30]. Briefly, the examiner was blinded to all slides. The software was then set up to perform systematic uniform and random sampling (SURS) of fields of view, an unbiased sampling method. An average of 60 fields was used per slide. A 2D unbiased counting frame for counting cell profiles per area covering 50% of the field of view was used when few positive hepatocyte profiles were visible; when many profiles were visible, a counting frame of 10% was used. Positive Ki-67-stained hepatocyte profiles were defined as a large (approximately 8 μm in diameter) oval cells with an obviously stained border and visible nucleus (Supplementary Figures 1-3).

To calculate the number of Ki-67 positive hepatocyte cell profiles per area the following formula was used:

$$Q_A(\text{Ki-67/lobe}) = [\Sigma Q(\text{Ki-67})]/[A(\text{frame}) \bullet P(\text{lobe})]$$

Q_A(Ki-67) is the number of Ki-67 positive hepatocyte cell profiles per mm^2 lobe, A(frame) is the area of the counting frame, and P(lobe) is the number of test points-a maximum of two per counting frame. Counted if the lower left or upper right corner is hitting lobe tissue.

Total number of Ki-67 positive hepatocytes in the caudate lobe: To account for the larger cell size of the HFCD livers the total number of Ki-67 positive hepatocytes in the caudate lobe, N(Ki-67, lobe), was estimated.

First, the number of Ki-67 positive hepatocytes per volume liver lobe was calculated; SURS was set up, and approximately 30 positive hepatocyte cell profiles were sampled and the diameter measured at 20 x magnification. The diameter was defined as the length of diameter perpendicular to the longest axis of the cell (Supplementary Figure 4). This was used in the following formula[33]:

$$N_V(\text{Ki-67/lobe}) = [Q_A(\text{Ki-67/lobe})]/[D(\text{cell}) + t(\text{section})]$$

N_V is the number of Ki-67 positive hepatocytes per mm^3 lobe, Q_A(Ki-67/lobe) is the number of Ki-67 positive cell profiles per mm^2 lobe, D(cell) is average diameter of the counted cells, *t*(section) is the thickness of the tissue sections (3 μm).

This is a model-based approach for number estimation biased by tissue shrinkage, projection effects, and deviations from model assumptions.

N(Ki-67, lobe) was then estimated; the volume of the caudate lobe was estimated based on the weight and the density of the rat liver. The density of the liver was set to 1.05 g/cm^3[34] and used in the following formula:

$$N(\text{Ki-67,lobe}) = N_V(\text{Ki-67/lobe}) \bullet V(\text{lobe})$$

N(Ki-67, lobe) is the total number of Ki-67 positive hepatocytes in the caudate lobe, N_V(Ki-67/lobe) is the number of Ki-67 positive hepatocyte profiles per volume lobe, V(lobe) is total volume of the caudate lobe.

Hepatic regeneration ratio: The hepatic regeneration ratio (HRR) was calculated for each animal, and defined as:

$$\frac{\text{liver weight per 100 g of body weight at euthanisation}}{\text{preoperative projected liver weight}^1 \text{ per 100 g of body weight}} = \text{hepatic regeneration ratio}$$

[1]Preoperative projected liver weight: Weight of resected liver at hepatectomy/0.7

Net regeneration: We calculated the net regeneration (NET), defined as:

$$\frac{\text{Liver weight at euthanisation}}{\text{preoperative projected liver weight/resected liver weight}} = \text{net regeneration}$$

Liver tissue analysis

Total protein analysis: The Pierce™ BCA total protein assay kit (Thermo Fisher Scientific, IL, United States) was used to measure the amount of total protein in the liver tissue. Prior to the total protein measurement, the tissue was homogenized in a lysis buffer as previously described[35], only, mortar and pestle was used in this study. The total concentration of protein was multiplied by the weight of the whole liver at euthanisation to determine the absolute amount of protein in the whole liver.

Regenerated protein ratio: The regenerated protein ratio (RPR) was calculated as follows:

$$\frac{\text{Absolute protein quantity at euthanisation}}{\text{Average absolute protein quantity of baseline reference group}} = \text{regenerated protein ratio}$$

RNA isolation and reverse transcription

We used a guanidinium thiocyanate-phenol-chloroform extraction protocol for RNA isolation as previously described[36]. The final RNA concentration was determined using a NanoDrop™ 2000 Spectrometer (Thermo Fisher Scientific). RNA concentrations were normalized to 1000 ng/μL and cDNA synthesized with High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific) on a polymerase chain reaction (PCR) Express Thermal Cycler (Thermo Hybaid, DE, United States), according to manufacturer's protocol.

Reverse transcriptase quantitative PCR (qPCR) was run on a 96-well StepOnePlus™ Real-Time PCR System Thermal Cycling Block (Thermo Fischer) using TaqMan Gene Expression Assays (Thermo Fisher Scientific). All assays contained the FAM dye. Samples were duplicated and we measured the mean cycle threshold (Ct) for each gene and standardized it to reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data was analyzed using the ΔΔ-Ct method as described by Livak *et al*[37]. The STD NH-group was set as a reference group with a fold change of 1 and the relative expressional levels were compared with this group for each gene. Selected genes are displayed in Supplementary Table 1.

Blood analysis

Routine analysis and PP measurements were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, on the day of euthanisation. Plasma samples were analyzed for alanine transaminase (ALT), bilirubin and albumin on a Cobas 6000 (Roche Diagnostics, Basel, Switzerland), using a routine protocol. PP was analyzed on a CS 2100i instrument (Sysmex, Siemens Siemens Healthcare). The remaining plasma and serum samples were stored at 80 ℃ until analysis. Serum samples were evaluated for the rat acute phase protein alfa-2-macroglobulin (α2M), by ELISA (Immunology Consultants Laboratory, OR, United States).

Statistical analysis

Statistical analyses were performed using STATA 11.0, graphs were drawn in Excel 14.4.1. Normality of data was checked by qq-plots. For continuous variables, comparisons were made using the ANOVA test for significance. *Post-hoc* comparisons were performed by Student's *t*-test. Categorical data were analyzed using Fisher's exact test. The qPCR data exhibited skewed distributions with variance heterogeneity. Therefore, these data were analysed using the nonparametric Kruskal-Wallis one-way analysis of variance on ranks test; when significant, post-hoc tests were performed using the Mann-Whitney rank sum test. Variables are expressed as means (± SD). Significance level was set at $P < 0.05$.

RESULTS

Animal characteristics

One HFCD animal died during surgery and one STD animal died of internal bleeding due to insufficent ligature of the right median lobe at day one after hepatectomy. Thus, none of the animals died from liver insufficiency or failure. At baseline, HFCD liver weight increased 2-fold compared with STD liver. HFCD animals had a significantly higher liver weight at all times ($P < 0.001$, Table 1).

Histology

All the HFCD livers showed marked steatosis (grade 3) (Figures 2-4). Prior to hepatectomy, steatosis was of SDMS type, however, changing to SDMS/MXMS during regeneration. None of the HFCD animals had evidence of fibrosis. According to the Kleiner criteria, nine had borderline NASH and 17 had NASH; with Bedossa criteria, 11 had NAFLD and 15 NASH (Table 2). No morphological abnormalities were observed in the liver tissues of the control animals.

Liver regeneration capacity [N(Ki-67, lobe), HRR, NET and RPR]

At baseline, a significant difference in Ki-67 liver proliferation index was observed between STD and HFCD animals ($P < 0.001$, Table 1); however, there was no difference between the HFCD and STD groups at either day 2 or day 5. Peak values were seen in the PH-2 groups.

The total number of Ki-67 positive hepatocytes in the caudate lobe [N(Ki-67, lobe)] was significantly higher in HFCD animals at baseline ($P < 0.0001$) and at day 5 ($P < 0.026$), while a trend was observed at day 2 ($P = 0.06$) (Figure 5A).

The hepatectomized HFCD rats had a lower hepatic regeneration capacity as determined by HRR than the STD rats ($P < 0.01$, all). However, Net regeneration (NET) showed no difference at day 2 and was significantly higher in HFCD animals at day 5 ($P < 0.018$).

No differences were observed in the regeneration of hepatic protein, as determined by RPR (Figure 5B). The HFCD groups had a higher amount of total protein in the liver at baseline and at day 5 after PH (both $P < 0.03$, Table 1).

Biochemistry (ALT, Bilirubin, PP, albumin and α2M)

At baseline, ALT was significantly higher in the HFCD than in the STD animals ($P < 0.001$, Figure 5C) whereas bilirubin was unchanged (Figure 5D). Peak ALT and bilirubin levels were seen two days after hepatectomy with HFCD animals having significantly higher levels than the STD group ($P < 0.05$, Figure 5C and D). However, when calculating ΔALT between baseline and day two, no difference was found between HFCD and STD animals (Table 1). Surprisingly, ALT levels were lower in the PH-5 HFCD group than in the non-hepatectomized HFCD group ($P < 0.001$, Figure 5C). Bilirubin was normalized at day 5 in both the HFCD and STD groups (Figure 5D).

We did not find any significant differences in PP levels between the groups (Table 1).

Table 1 Biochemistry and measurements

	Standard diet			High fat high cholesterol diet		
	NH (n = 9)	PH-2 (n = 9)	PH-5 (n = 8)	NH (n = 9)	PH-2 (n = 9)	PH-5 (n = 8)
Weight at euthanisation, g	294 ± 22	289 ± 18	305 ± 12	289 ± 21	271 ± 18[1]	290 ± 15
Liver weight at euthanisation, g	8.1 ± 0.6	6.1 ± 0.5	7.5 ± 1.3	15.4 ± 2.0[1]	9.0 ± 0.8[1]	12.3 ± 1.7[1]
Recected liver at PH, g	-	5.2 ± 0.3	5.0 ± 0.5	-	11.2 ± 2.0[1]	11.4 ± 0.7[1]
Hepatic regeneration ratio, %	1.0	0.82 ± 0.08	1.04 ± 0.17	1.0	0.61 ± 0.1[1]	0.77 ± 0.12[1]
Net regeneration (g)	-	3.8 ± 0.5	4.2 ± 1.2	-	5.3 ± 0.9	7.4 ± 1.6[1]
Total protein, g	0.83 ± 0.11	0.51 ± 0.08	0.69 ± 0.18	1.10 ± 0.13	0.60 ± 0.12	0.90 ± 0.14
Ki-67 Index, positive profiles, mm^2	1.6 ± 0.6	216 ± 83.2	27.2 ± 22.3	9.0 ± 3.2[1]	227.5 ± 80	31.7 ± 11.1
Spleen weigth at euthanisation, g	0.64 ± 0.12	0.83 ± 0.12	1.10 ± 0.16	1.25 ± 0.36[1]	1.04 ± 0.31	1.34 ± 0.44
Alanine transaminase, u/L	24.1 ± 5.2	64.9 ± 9.0	28.8 ± 13.4	79.9 ± 27.4[1]	130.1 ± 25.4[1]	45.8 ± 20.5
ΔAlanine transaminase, u/L	-	41 ± 9	5 ± 13	-	50 ± 25	-34 ± 21[1]
Albumin (g/L)	19.0 ± 1.9	14.7 ± 1.0	12.4 ± 1.5	15.1 ± 1.9[1]	12.2 ± 0.8	11.5 ± 2.3
α2-macroglobulin, μg/mL	18.1 ± 0.7	102 ± 12.6	98.2 ± 13	22.6 ± 3.4[1]	157.8 ± 40[1]	94.5 ± 64.5
Prothrombin-proconvertin ratio	0.32 ± 0.03	0.33 ± 0.04	0.32 ± 0.03	0.34 ± 0.03	0.38 ± 0.06	0.33 ± 0.03

Mean ± SD, [1]P < 0.05. NH: No-hepatectomy; PH-2: Partial hepatectomy and 2 d; PH-5: Partial hepatectomy and 5 d.

Table 2 Average scores of the Kleiner criteria and the final histological grading of both the Kleiner and Bedossa criteria

	High fat high cholesterol diet		
	NH (n = 9)	PH-2 (n = 9)	PH-5 (n = 8)
Kleiner criteria score			
Steatosis	3	3	3
Ballooning	0.6	0.7	0.5
Inflammation	2.7	1.6	0.8
Fibrosis	0	0	0
NAS score	6.2	5.2	4.3
Histological grading			
Borderline NASH/NASH, Kleiner criteria	1/8	4/5	4/4
NAFLD/NASH, Bedossa criteria	3/6	4/5	4/4

NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; NH: No-hepatectomy; PH-2: Partial hepatectomy and 2 d; PH-5: Partial hepatectomy and 5 d.

Plasma albumin was significantly lower in the HFCD animals (P < 0.05) at baseline and at day 2 after hepatectomy (P < 0.001) compared with STD animals, whereas no difference was observed at day 5 after PH. In the HFCD groups, albumin levels dropped after hepatectomy but were stable during regeneration (Table 1).

At baseline, α2M was significantly higher in the HFCD than the STD animals (P < 0.01, Table 1). α2M increased two days after hepatectomy with HFCD animals having significantly higher values than the STD group (P < 0.001, Table 1). The PH-5 HFCD group had a α2M value similar to PH-5 STD controls (Table 1).

mRNA expression of inflammatory genes

At baseline, the HFCD animals had increased tumor necrosis factor α (*TNFα*) and interleukin-6 (*IL6*) (Figure 6) gene expression when compared with STD animals, although interestingly, this difference was normalized during regeneration.

mRNA expression of fibrogenic genes

At baseline transforming growth factor β (TGFβ), connective tissue growth factor (CTGF) and Collagen 1α1 (COL1α1) mRNA expression were significantly higher in the HFCD group. At day 2, CTGF and COL1α1 mRNA expression were significantly higher in the HFCD group, while no significant difference was found when looking at TGFβ. At day five, only TGFβ was significantly higher when comparing HFCD and STD PH-5 groups (Figure 7) (P < 0.05).

mRNA expression of regeneration genes

At baseline, HFCD animals displayed a significantly higher mRNA expression of hepatocyte growth factor (HGF) and transforming growth factor α (TGFα), but no significant differences were seen in Proto-oncogene, tyrosine kinase (MET) and epidermal growth factor (EGF) mRNA expression. At day 2 and 5 HFCD groups had a higher expression of HGF, TGFα and EGF mRNA, while no significant difference was found when looking at MET compared with respective control groups (Figure 8) (P < 0.05).

DISCUSSION

We investigated LRC after a 70% PH in rats with HFCD-induced NASH. Surprisingly, we found a similar LRC when comparing HFCD and STD rats.

This is the first study to evaluate LRC in an HFCD-

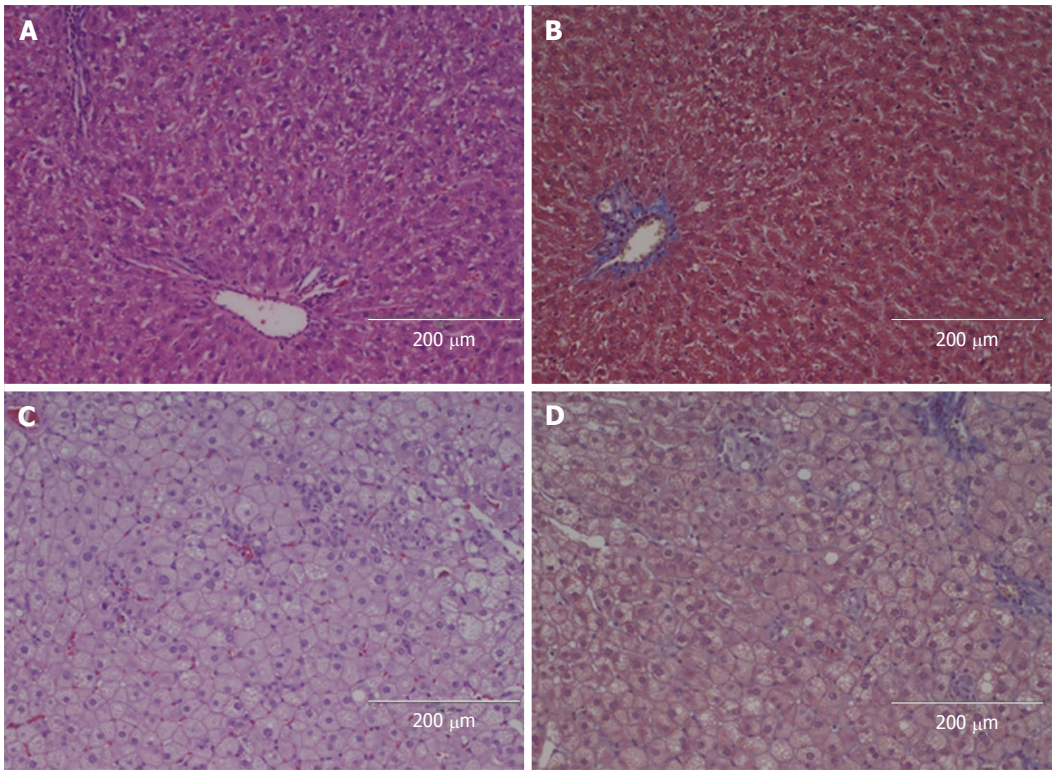

Figure 2 Examples of liver histology. A and C shows Hematoxylin and Eosin; B and D shows Masson Trichome; A and B are from an animal fed the standard diet and euthanised without hepatectomy; C and D are from an animal fed the high fat high cholesterol diet and euthanised without hepatectomy.

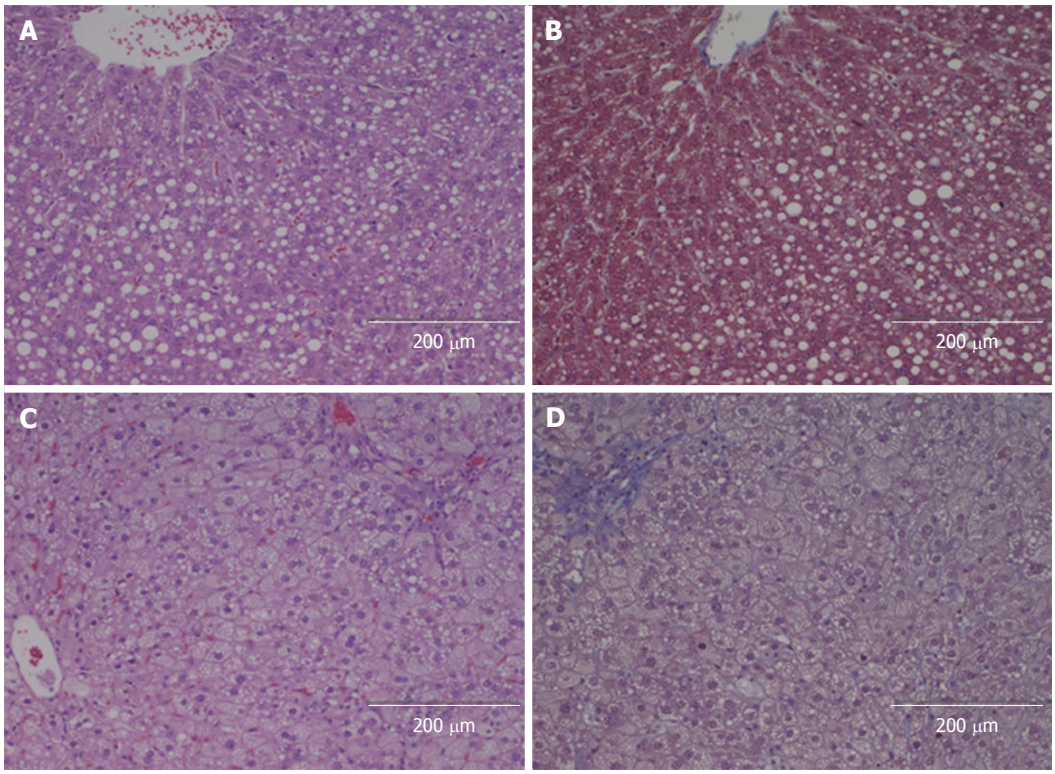

Figure 3 Examples of liver histology. A and C shows Hematoxylin and Eosin; B and D shows Masson Trichome; A and B are from an animal fed the standard diet and euthanised two days after hepatectomy; C and D are from an animal fed the high fat high cholesterol diet and euthanised two days after hepatectomy.

induced NASH model. Previous studies were conducted in other less suitable models. The MCD model potently induces NASH, but the rodents suffer severe weight loss and cachexia, in contrast to human NASH. In

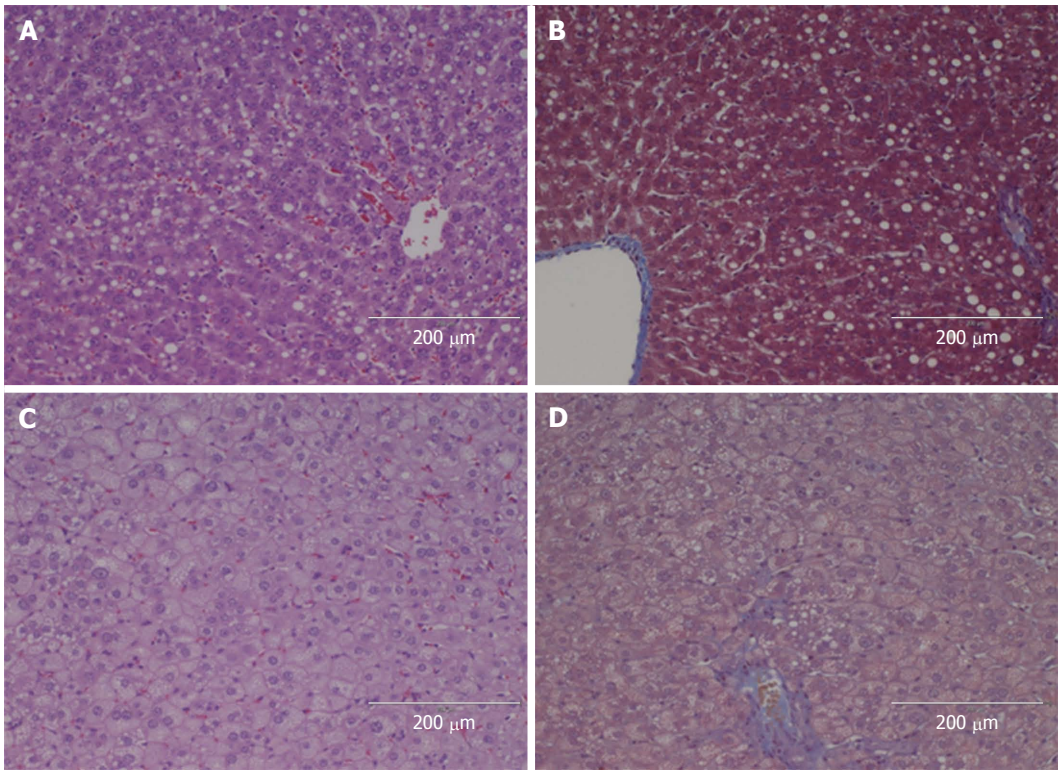

Figure 4 Examples of liver histology. A and C shows Hematoxylin and Eosin; B and D shows Masson Trichome; A and B are from an animal fed the standard diet and euthanised five days after hepatectomy; C and D are from an animal fed the high fat high cholesterol diet and euthanised five days after hepatectomy.

the CDD and HFD models only simple steatosis is induced[17,18,26,38-41]. Similarly, leptin-deficient rodents are gene-modified and thus do not reflect the etiological features of human NAFLD/NASH[29,42]. Our model successfully established NAFLD with significant steatosis and increased liver weight. NASH changes were present in the majority of HFCD animals with inflammation and ballooning. Prior to hepatectomy, the HFCD animals had elevated ALT levels with increased expression of genes related to inflammation and fibrogenesis, although none of the HFCD animals showed histological fibrosis. The type of steatosis changed from SDMS to SDMS/MXMS during regeneration, which might be explained by the fact that transient large fat droplet accumulation is a natural part of liver regeneration[43], as also seen in our STD animals.

As markers of liver injury, we observed elevated ALT and bilirubin levels two days post-hepatectomy in the HFCD groups compared with STD animals as previously found in MCD studies[9,11,12]. However, baseline ALT levels were already increased in the HFCD animals, and the relative ALT (ΔALT) increase following hepatectomy was similar in the HFCD and STD groups. A previous study using a steatosis-only HFD model found elevated ALT levels at day 1 after hepatectomy only in HFD animals compared to controls, but normal ALT levels both prior to hepatectomy and at day 3[26].

The Ki-67 liver proliferation index was similar between STD and HFCD animals during regeneration, however, this index does not take the larger cell size of the HFCD animals into account; which was the reason for estimating the total number of Ki-67 positive hepatocytes N(Ki-67, lobe).

N(Ki-67, lobe) was significantly higher in the HFCD animals at baseline and at day 5, with a trend on day 2. We used the same factor (1.05 g/cm^3) for both control and HFCD groups even though we expected the density to be lower in the HFCD livers due to fat accumulation. In adipose tissue, the density is approximately 0.9 g/cm^3[44]. This would probably decrease the total number of positive profiles by 5%-10% in the HFCD animals. Nonetheless, N(Ki-67, lobe) underlines the fact that the HFCD animals at the very least have a similar, if not higher, proliferative response during regeneration. Whether this is due to increased apoptosis, a delayed regenerative response or a higher regeneration remains elusive.

Other studies have reported both similar and opposing results using high-powered fields (HPF) when estimating proliferative indexes such as Ki-67. In rats fed a MCD diet for 5 wk, Veteläinen *et al*[12] found a decreased Ki-67 index at days 1, 2 and 3 after partial hepatectomy, while rats with simple steatosis after only one week of MCD diet had a normal Ki-67 index. Marsman *et al*[9] found a decreased Ki-67 index at day 1, but a normal or higher Ki-67 index between days 2 and 5 in MCD rats fed for 5 wk. Using a proliferating cell nuclear antigen (PNCA) labeling index (a marker of DNA synthesis), Tanoue *et al*[26] found normal PNCA index in HFD rats, whereas rats on a high fructose diet

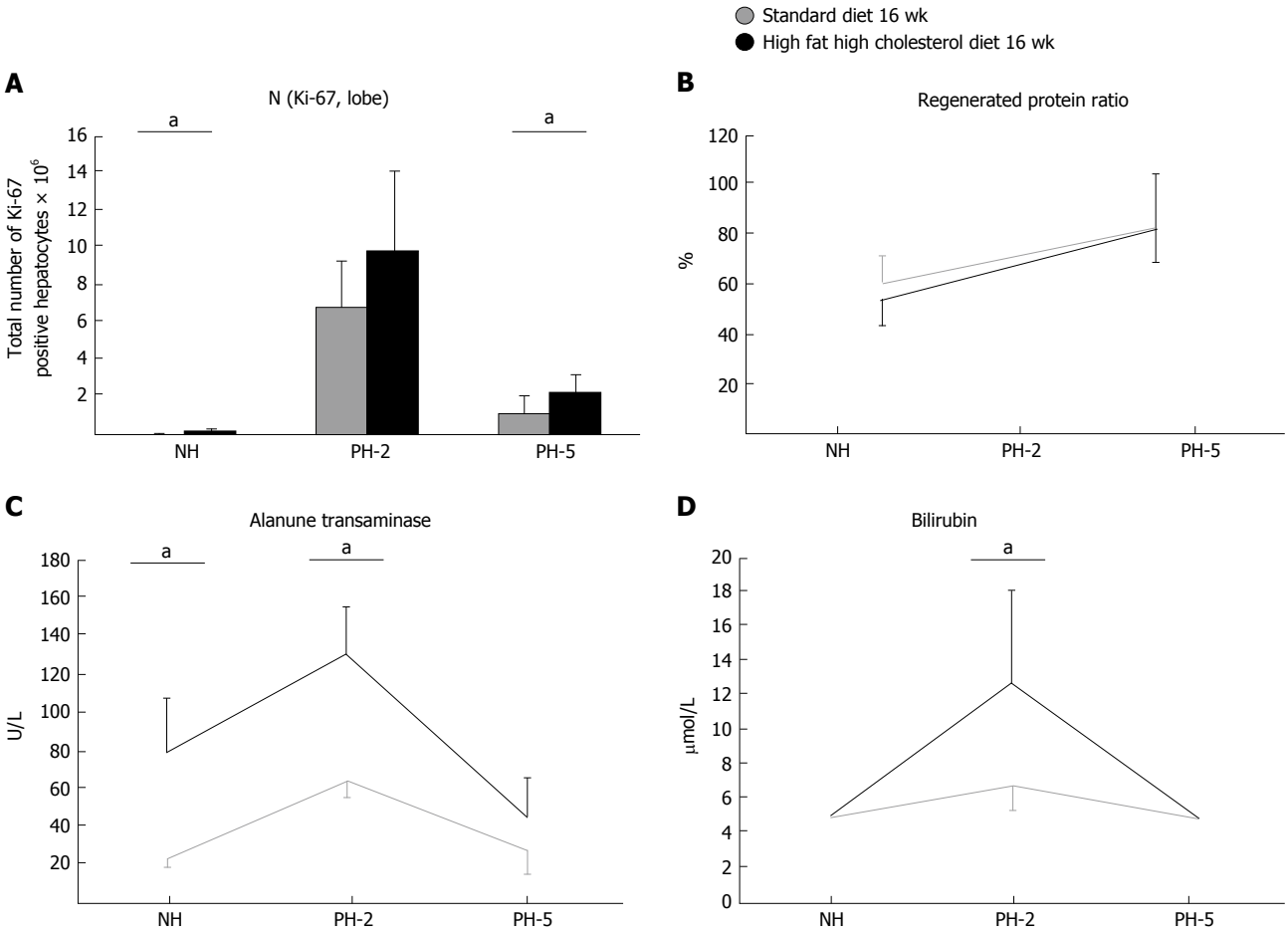

Figure 5 Total number of Ki-67 positive hepatocytes (A); regenerated protein ratio (B); Alanine transaminase (C) and Bilirubin (D). Means and standard derivation displayed. $^{a}P < 0.05$ compared to respective standard diet group. NH: No hepatectomy; PH-2: Partiel hepatectomy and 2 d; PH-5: Partiel hepatectomy and 5 d.

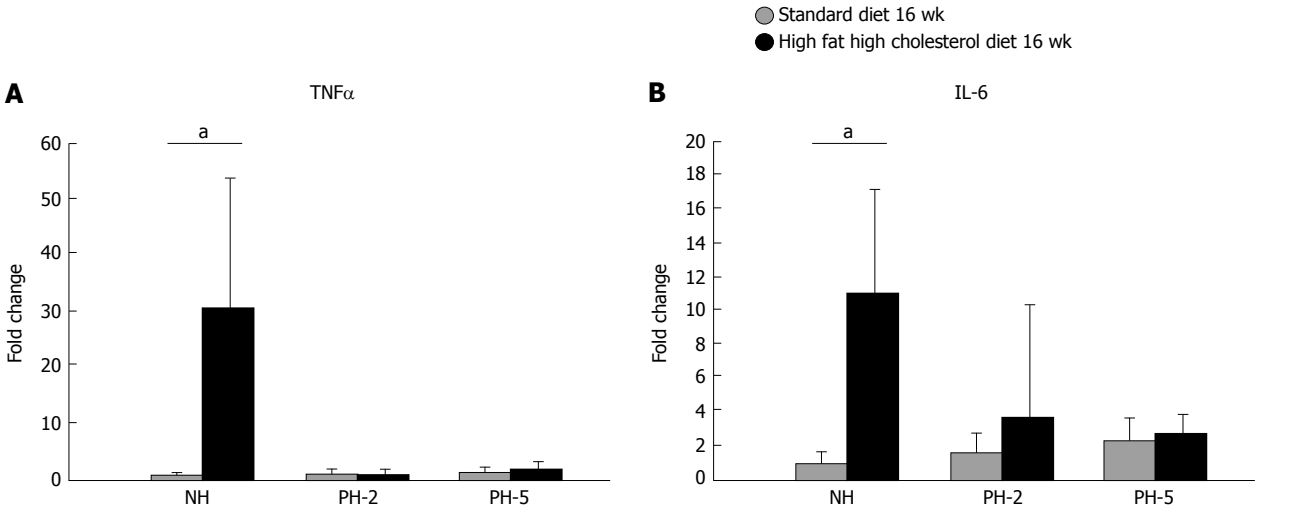

Figure 6 Relative mRNA expression. A: Tumor necrosis factor α (TNFα); B: Interleukin-6 (IL-6) standardized to glyceraldehyde 3-phosphate dehydrogenase. Means and standard derivation displayed. Statistical analysis made using the ANOVA test, $^{a}P < 0.05$ compared to respective standard diet group. NH: No hepatectomy; PH-2: Partiel hepatectomy and 2 d; PH-5: Partiel hepatectomy and 5 d.

had a decreased PNCA index, although they had better histological scores than HFD rats. They speculated that the etiology of steatosis had more impact on the proliferative index than the degree of steatosis.

Clearly, this diversity in published results indicates that the Ki-67 index is greatly influenced by factors in the experimental design, such as the study duration, composition of the diet and not just the histopathological findings. Also, the methods of evaluation are important. By using a stereology-based design instead of semi-

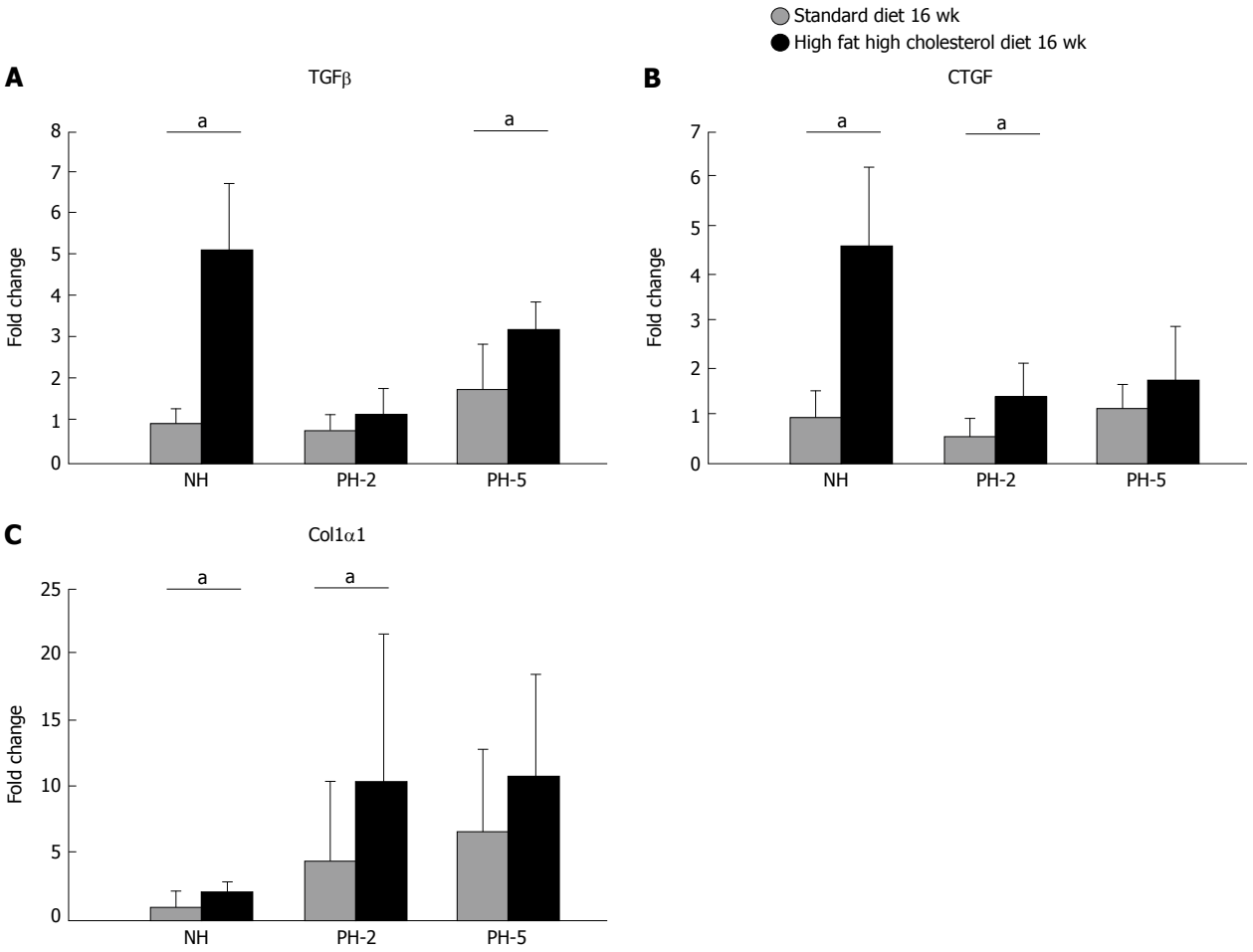

Figure 7 Relative mRNA expression. A: Transforming Growth Factor β (TGFβ); B: Connective Tissue Growth Factor (CTGF); and C: Collagen 1α1 standardized (Col1α1) to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Means and standard derivation displayed. Statistical analysis made using the ANOVA test, $^{a}P < 0.05$ compared to respective standard diet group. NH: No hepatectomy; PH-2: Partiel hepatectomy and 2 d; PH-5: Partiel hepatectomy and 5 d.

quantitative counting methods or counting HPF, we eliminated the selection bias of fields of view on obtained quantitative data. We sampled the whole caudate liver lobe, whereas previous studies have used single or multiple slabs from different liver lobes[9,12,26]. We believe that our stereological-based design is superior to HPF-based and semi-quantitative scoring systems since it eliminates several potential sources of error even though it is not perfectly unbiased.

Use of HRR and equivalent ratios have been widely reported in the literature of LRC in experimental NAFLD/NASH[7-10,12,14,17,18,20-22,24,39,45,46]. However, it is clearly problematic to use this measure, as the HRR is based on weight alone. Being fed a HFCD diet after PH, the HFCD liver will continue to accumulate fat. Using the HRR to compare steatotic livers with healthy livers, one must assume that the steatotic liver regenerates the same ratio of liver- and fat tissue as it has prior to hepatectomy.

In our study, we found a decreased LRC in HFCD rats looking exclusively at HRR. Looking at NET we found similar values at day 2 but at significantly higher value in HFCD animals at day 5. Both HHR and NET are measures that might be biased by fat accumulation. When considering other variables, such as the RPR, no difference was found between the two groups and this observation supports that the HRR value could be incorrect due to fat accumulation. To our knowledge, this study is the first to address this important issue - while previous studies have tended to use the outcome of the HRR uncritically[7-10,12,14,17,18,20-22,24,39,45,46].

Prothrombin-proconvertin ratio measures coagulation factors Ⅱ, Ⅶ and Ⅹ. Coagulation factors are exclusively synthesized in the liver and PP levels were therefore used as an indicator of hepatic protein synthesis. PP was not significantly affected in HFCD animals or at any time point following hepatectomy, which indicates that this specific metabolic liver function was already restored day 2 post-hepatectomy similar to findings during regeneration in healthy rats[47,48]. In contrast, albumin was decreased at baseline and at day 2 after hepatectomy in HFCD compared with STD animals; however, this difference disappeared at day 5.

For model evaluation and hepatectomy effects on liver inflammation and fibrosis, we measured the expression of genes related to inflammation (TNFα and IL-6) and fibrogenesis (TGFβ, CTGF, COL1α1). TNFα and IL-6 levels were elevated prior to hepatectomy

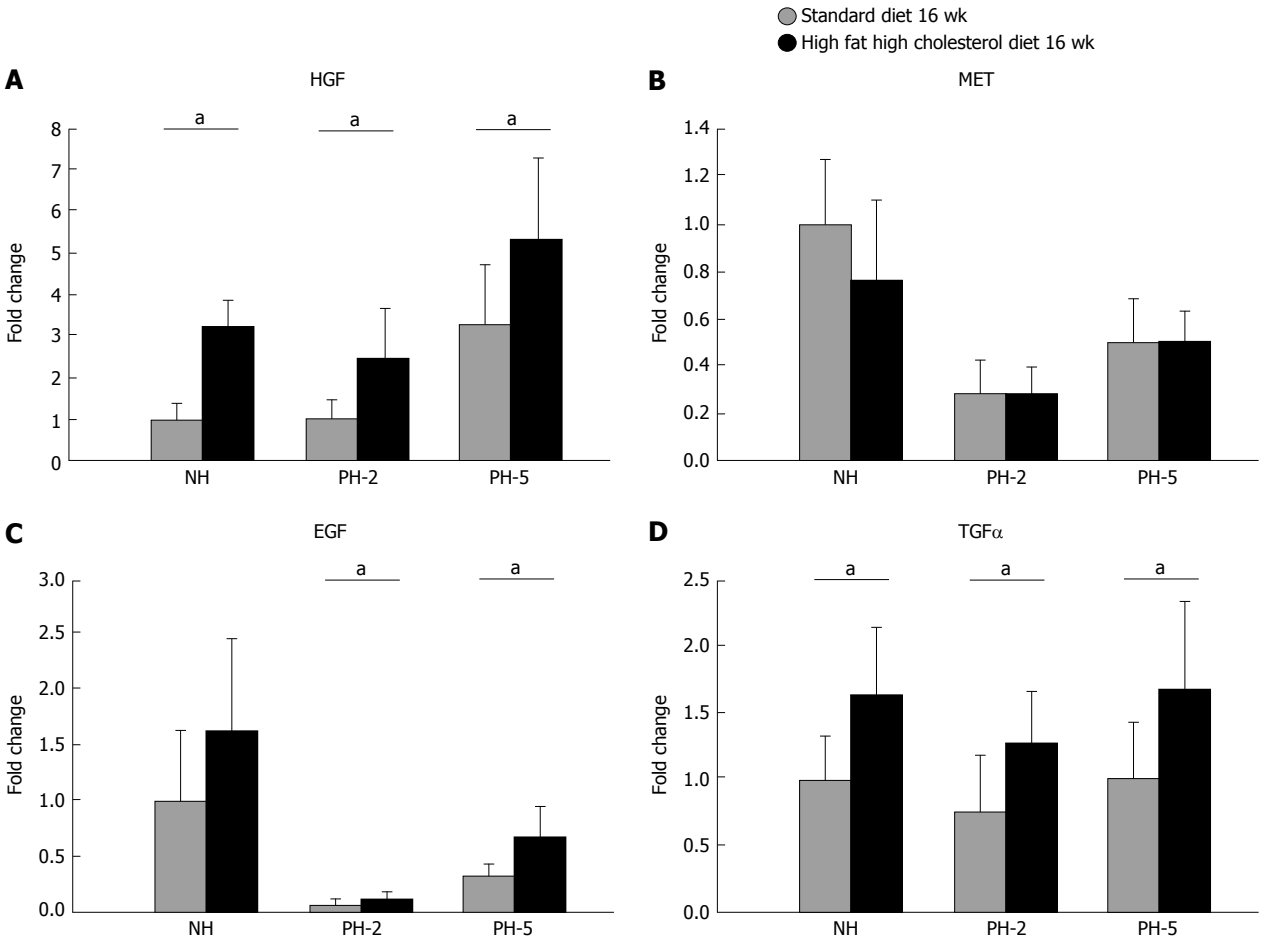

Figure 8 Relative mRNA expression. A: Hepatocyte growth factor (HGF); B: Proto-oncogene, tyrosine kinase (MET); C: Epidermal growth factor (EGF); and D: Transforming growth factor α (TGFα), standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Means and standard derivation displayed. Statistical analysis made using the ANOVA test, $^{a}P < 0.05$ compared to respective standard diet group. NH: No hepatectomy; PH-2: Partiel hepatectomy and 2 d; PH-5: Partiel hepatectomy and 5 d.

in the HFCD animals but no difference was observed between HFCD and STD groups after PH. Thus, it seems that the inflammatory process is on hold during regeneration.

Despite the absence of histological fibrosis, we demonstrated increased hepatic levels of pro-fibrogenic cytokines (COL1α1, CTGF and TGFβ) in HFCD animals indicating active fibrogensis although not yet visible in the histology.

We studied the pathways of MET and the epidermal growth factor receptor (EGFR), which are generally accepted as the main mitogenic pathways of liver regeneration[49]. MET binds it's ligand HGF, and EGFR binds several ligands, among others TGFα and EGF. Surprisingly, we did not find a decrease in the expression of the HGF/MET pathway in the HFCD animals. MET expression was unchanged and HGF expression higher at all time points in the HFCD animals compared to STD animals, which shows that this pathway is indeed not downregulated. HGF is proposed to have an anti-fibrotic effect[50], and we speculate that the elevation of HGF was a response to the on-going fibrogenesis in the HFCD animals. Also, we investigated the ligands for the EGFR pathway TFGα and EGF. In the HFCD groups, mRNA expression of both ligands was either higher or similar to STD groups in keeping with previous studies[51], indicating that this pathway is also not down-regulated.

The study has certain limitations. Liver regeneration is a process that commences immediately after liver injury and it would have been preferable to investigate liver regeneration as early as a few hours after PH as well as at day 1 after PH. Thus, early differences in HFCD animals may have been overlooked. Further, the evaluation of regeneration is compromised by the fat accumulation, which may disturb the results; however, no former study has addressed this important issue. The different measures of regeneration such as HHR, NET, RPR and N(Ki-67, lobe) are not flawless and have all limitations, but they leave the overall impression that the regenerative response in HFCD animals is at the very least, comparable to the STD animals.

In conclusion, we believe that the model and degree of NASH as well as methods of LRC evaluation are essential in understanding and evaluating LRC.

The HFCD induced significant steatosis and NASH

changes along with increased expression of pro-inflammatory and pro-fibrogenic genes. However, we found that the HFCD rats had a preserved liver regeneration as assessed by total number of Ki-67 positive hepatocytes, RPR and PP, which was supported by the gene expression of growth factors during regeneration.

ARTICLE HIGHLIGHTS

Research background

Epidemiological studies showed that liver resections are associated with increased morbidity and mortality in patients with non-alcoholic fatty liver disease (NAFLD)/ non-alcoholic steatohepatitis (NASH). It has been suggested that NASH livers are more vulnerable to surgical interventions because of decreased liver regeneration capacity (LRC). LRC has been studied in different animal models of NAFLD/NASH. However, these models may have significant limitations. Some models induce NASH but with severe weight loss, while other models induce simple steatosis only, further, genetic modified models may not reflect the etiological features of human NASH. In the present study we used a high fat high cholesterol diet (HFCD) rat model, which mimic human NASH better than previous models.

Research motivation

This is the first study of LRC in rats with NASH induced by a HFCD. Previous experimental NAFLD/NASH studies showed contradictory findings with decreased LRC or unchanged LRC, even when the same animal models were used. Clearly, the model and methods of evaluation may significantly influence the results and conclusions. For future treatment strategies of liver resections, it is important to understand whether the LRC of NAFLD/NASH livers is compromised.

Research objectives

The aim of the present study was to evaluate LRC in rats with NASH induced by a HFCD. Authors the methods of evaluation and the chosen model of NAFLD/NASH significantly influences the results and further research on the subject should be aware of this.

Research methods

Rats were fed a high-fat, high-cholesterol diet (65% fat, 1% cholesterol) or standard diet (STD) for 16 wk. After the feeding phase 1/3 of the animals were euthanised immediately and served as a baseline reference. The remaining 2/3 of the animals underwent 70% partial hepatectomy (PH) and the hepatectomized animals were euthanised either 2 or 5 d post-PH. The degree of steatosis and the presence of NASH were evaluated by an expert liver pathologist using both the Kleiner and Bedossa criteria. LRC was evaluated using: the total number of Ki-67 positive hepatocytes in the caudate lobe, N(Ki-67, lobe) evaluated in a stereology-based design, the regenerated protein ratio (RPR), prothrombin-proconvertin ratio (PP), and mRNA expression of genes related to regeneration. The study is the first to use a stereology based design to evaluate cell proliferation. The authors believe this design superior to former methods of evaluation. The study is also the first to address that future research should be cautious using the regenerated liver weight only to evaluate LRC. The NASH liver weight is biased by fat accumulation and when using the liver weight only one cannot account for whether the NASH liver regenerates fat- or liver tissue. Thus, we estimated the total protein concentration in the livers and used this to describe the regenerated liver mass. Biochemical tests were used as markers of liver injury. The data was analyzed using STATA. Normality of data was checked by qq-plots. For continuous variables, comparisons were made using the ANOVA test for significance. *Post-hoc* comparisons were performed by Student's *t*-test. Categorical data were analyzed using Fisher's exact test. The qPCR data exhibited skewed distributions with variance heterogeneity. Therefore, these data were analysed using the non-parametric Kruskal-Wallis one-way analysis of variance on ranks test; when significant, post-hoc tests were performed using the Mann-Whitney rank sum test.

Research results

The HFCD NASH model showed significant steatosis with ballooning and inflammation, while no fibrosis was present. Mortality was similar in HFCD and STD animals following PH. Further, HFCD animals had significantly elevated markers of liver injury after PH. HFCD animals had a higher N(Ki-67, lobe) at baseline, day 2 after PH and day 5 after PH. However, we found no significant difference in RPR or PP neither 2 or 5 d post-PH. Expression of liver regeneration genes was higher at both day 2 and 5 post-PH in HFCD groups. Authors evaluated LRC at day 2 and 5 after PH; however, it would have been interesting also to evaluate the very early stages of liver regeneration including time points as early as a few hours after PH and at day 1 after PH. Further, it would be of interest to investigate this rat model after more prolonged HFCD diet treatment when fibrosis may be more pronounced and if this decreases LRC. In addition, finding and identifying relevant new and better methods of LRC evaluation may ease the interpretation of the results.

Research conclusions

The novel finding is that in a HFCD NASH model without fibrosis authors observed preserved LRC. The etiology and methods of evaluation is of great importance when evaluating LRC in animal models. Further, the fat accumulation in the NAFLD/NASH liver is a bias when estimating LRC and it needs to be addressed in future studies. In animal models the etiology of NAFLD/NASH and methods of evaluation is of significant importance in understanding LRC. Seemingly, NASH without fibrosis induced by a HFCD does not decrease LRC. HFCD induced NASH without fibrosis does not compromise LRC in rats following hepatectomy. HFCD induced NASH without fibrosis does not compromise LRC in rats following hepatectomy. When evaluating LRC the fat accumulation of the liver must be addressed, thus we have used both a stereological design to evaluate cell proliferation and measured the total protein concentration in the liver as a marker of regenerated liver mass. Prior to the study hypothesized LRC to be decreased, but in contrast we found a preserved LRC. It is too early to draw conclusions for clinical practice, but this study adds insight to the subject. Speculating, the reasons for increased morbidity and mortality in patients with NAFLD/ NASH following liver resections should be sought elsewhere than in decreased LRC.

Research perspectives

Identifying and/or optimising relevant animal models of NAFLD/NASH as well as methods of evaluation for LRC. Using a pure stereological design for evaluation of cell proliferation, as this is perfectly unbiased. Using different markers LRC and being aware of the potential bias fat accumulation brings when evaluating LRC based on liver weight alone.

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ORIGINAL ARTICLE

Basic Study

Bioengineered humanized livers as better three-dimensional drug testing model system

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Abstract

AIM

To develop appropriate humanized three-dimensional *ex-vivo* model system for drug testing.

METHODS

Bioengineered humanized livers were developed in this study using human hepatic stem cells repopulation within the acellularized liver scaffolds which mimics with the natural organ anatomy and physiology. Six cytochrome P-450 probes were used to enable efficient identification of drug metabolism in bioengineered humanized livers. The drug metabolism study in bioengineered livers was evaluated to identify the absorption, distribution, metabolism, excretion and

toxicity responses.

RESULTS

The bioengineered humanized livers showed cellular and molecular characteristics of human livers. The bioengineered liver showed three-dimensional natural architecture with intact vasculature and extra-cellular matrix. Human hepatic cells were engrafted similar to the human liver. Drug metabolism studies provided a suitable platform alternative to available *ex-vivo* and *in vivo* models for identifying cellular and molecular dynamics of pharmacological drugs.

CONCLUSION

The present study paves a way towards the development of suitable humanized preclinical model systems for pharmacological testing. This approach may reduce the cost and time duration of preclinical drug testing and further overcomes on the anatomical and physiological variations in xenogeneic systems.

Key words: Acellularization; Repopulation; Drug testing; Humanized liver; Bioengineering

Core tip: Liver is the central organ for absorption, distribution, metabolism, excretion and toxicity (ADMET) of pharmacological drugs and molecules. Available *in vitro* and *in vivo* preclinical models deals with several limitations including xenogeneic barrier, lack of natural humanized liver architecture and functional responses. Bioengineered humanized livers developed in present study can overcome on such limitations. This humanized liver model system provides better platform which could be used more efficiently to screen the ADMET of several pipeline drugs and other pharmacological molecules. This approach could reduce the time and cost of the total drug screening experiments as compared to the animal models. It provides enhanced dose response relationship by using drug concentrations relative to human exposure. Ease of *ex-vivo* access of cellular and molecular responses in humanized liver model system during pharmacological screening also offers high-throughput studies to determine the cellular response networks and toxicity pathways.

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INTRODUCTION

Drug testing has been one of the most critical challenges faced by the pharmaceutical companies with approximately 90% failure due to unpredictable adverse events which remain unidentified in preclinical phase[1]. The average time to introduce one drug to market is approximately 8.5 years from the time of clinical testing to Food and Drug Administration (FDA) approval which has 21.5% of clinical success rate imposing about $2 billion cost per drug[2]. Since many years, animal models have been gold standard and the most preferred choice of drug testing to understand the underlying mechanisms of various human pathologies. However, the marked biochemical variations, anatomical complexities and physiological responses limit the bio-mimetic outcomes of drug testing. To overcome these hurdles, patient specific stem cells have been employed to recapitulate the human pathologies *in vitro* to evaluate cellular process which is also termed as "disease-in-a-dish"[3].

Human primary hepatocytes culture system gives closest representation of human liver physiology[4]. However, the source of tissue along with phenotypic variations represent major limitations[5]. In addition, suspension culture of primary human hepatocytes offer the maximum drug incubation time for 4-6 h thereby requiring high dose of drugs to identify the cellular toxicity. Whereas, the monolayer cultures of human primary hepatocytes allows drug toxicity study for 4-72 h, but the drug metabolism capacity of such cultures represent severe downregulation which negatively impact the correlation with clinical outcomes[6]. In addition, the conventional two-dimensional (2D) cell culture systems do not complement the higher order processes which further neglect the crucial stimuli for cellular organization and function. These drawbacks of conventional cultures have been because tissue specific functions are dependent on several crucial factors other than only cell autonomous system which includes extracellular microenvironment with soluble factors, physical strength and extra-cellular matrix (ECM)[7]. Conventional cultures lack these crucial factors for proper cell to cell and cell to microenvironment interactions.

The leveraging tissue-engineering strategies to stabilize the functions of primary human hepatocytes within the xenogenic liver scaffolds provides unique model which can be utilized for better predicting human drug responses, pharmacokinetics and metabolic synchronization similar to human system[8,9]. Hence, the present study was designed to bioengineer humanized livers using more efficient technology of whole xenogenic liver acellularization and human hepatic stem/progenitor cells repopulation. This technology provides biomimetic natural organ scaffold with highly intact native ECM, vascular networks and mechanical strength. Repopulated cells in these acellularized whole liver scaffolds are organized in natural manner and perform high level of bio-mimetic liver functions better than conventional 2D culture systems.

In present study, the structural and functional

advantage of humanized livers has been evaluated by testing the metabolism of six cytochrome P-450 (CYP) probe substrates (phenacetin, diclofenac, S-mephenytoin, dextromethorphan, nifedipine and testosterone). CYP has been considered the most common drug metabolizing enzymes which are profusely expressed in liver apart from lungs, kidney, intestine and brain etc. The expression level of these CYPs changes according to the physiological conditions and disease status[10]. Hence studying the behavior of these CYPs against different kinds of substrates in humanized liver could provide better choice for identifying the pharmacokinetics and pharmacodynamics of drugs. This technology offers tremendous potential option for pre-clinical pharmacological drug testing which can reduce the cost, time and unpredictable adverse events.

MATERIALS AND METHODS

Spontaneously aborted 10 wk gestation aged human fetuses (n = 2) were collected from local maternity hospitals after taking written informed consent from their parents. The study was approved by the Institutional Ethics Committee of Deccan College of Medical Sciences, Hyderabad. The study was conducted according to the ethical and regulatory guidelines of Indian Council of Medical Research (ICMR), India.

Establishing the technology for efficient acellularization of xenogenic liver

The whole liver was harvested by laparotomy from male Wister rats (n = 10, average body weight = 180-200 g) having intact hepatic artery and portal vein. The rats were obtained from National Institute of Nutrition (NIN), Hyderabad, Telangana, India. Harvested rat liver was initially perfused with heparinized phosphate buffered saline (100 U/mL) through portal vein with the help of 22G (gauge) intravenous catheter. Following to this, 3.8% of Sodium Citrate solution was infused to completely remove the red blood cells from liver. Afterwards a sequential perfusion was performed through main hepatic artery using different concentration gradients of Sodium dodecyl sulphate (SDS) at 30 Hg pressure and with flow rate of 1 mL/min for 16 h. After obtaining the complete acellularized whole liver scaffold, distilled water was run for 10min followed by Triton-X-100 (1.0%) perfusion. Completely acellularized rat liver scaffolds (ALS) were preserved in distilled water containing antibiotic and antimycotic solutions and stored at 4 ℃ until further use.

Characterization of acellularized liver scaffold

Identifying the residual nucleic acids: Acellularized xenogenic liver scaffolds were first characterized for the absence of nucleic acid contents in tissue lysate and flow through after 16 h of acellularization. Briefly; the lysate of acellularized and native liver was prepared by digestion with 0.1% papain solution, 1 mmol/L EDTA, 7.0 mmol/L cysteine and 1 mol/L NaCl in 1 × PBS at 60 ℃ for 48 h in an incubator shaker and residual nucleic acid content was quantified using spectrophotometric analysis at 260 and 280 nm. The ratio of 260/280 nm sample optical density was calculated to compare the presence of nucleic acid content.

Immunohistochemical staining: Further immunohistological staining was performed for the ALS using H and E staining. The presence of intact ECM components within ALS was determined using immunofluorescence staining for collagen, fibronectin and laminin. Briefly; ALS was fixed in 4% paraformaldehyde (PFA) and further used for the preparation of 3-5 μm thin sections which were stained using specific primary and secondary antibodies and analyzed under the microscope. Parallel analysis was also performed using native rat liver sections for comparison.

Ultra-structure analysis: The ultra-structure analysis of ALS was performed using scanning electron microscopy (SEM). Section were prepared and fixed in 2.5% (v/v) Gluteraldehyde in 1 × PBS and further subjected to dehydration using graded series of ethanol (50%, 75%, 80%, 95% and 100%) for 15 min each and dried in a HCP-2 critical-point dryer using CO_2. The cross sections were mounted and subjected for SEM analysis using JOEL-JSM 5600 SEM at RUSKA Lab's College of Veterinary Science, SVVU, Rajendranagar, Hyderabad, India[11].

Sterilization of ALS: Sterilization of ALS was performed to preserve the functional homology of liver matrix. Briefly; the ALS were perfused with 0.1% peracetic acid (PAA) for 30 min in laminar chamber at room temperature and further exposed to ultra-violet (UV) light for 30 min.

Mechanical strength: ALS was subjected to mechanical strength analysis following to sterilization procedure using mixed tensile strength, suture retention strength and compressive strength assays[12]. All the mechanical properties of ALS were compared with the native liver without acellularization.

Vascular integrity analysis

Methylene blue dye was infused into the acellularized liver through main hepatic artery to check the integrity of liver vascular system. The microvasculature and the surface capsule integrity was evaluated further by increasing the infusion rate using peristaltic pump.

Derivation and immunomagnetic enrichment of human hepatic progenitor cells

Human hepatic cells were isolated from 10 wk gestation aged spontaneously aborted human fetal livers following the protocol as described in our earlier studies[13,14].

The enrichment of human hepatic progenitor cells (hHPCs) was performed by magnetic activated cell sorting (MACS) using epithelial cell adhesion molecular (EpCAM) antibody tagged with the iron nanoparticles (MiltenyBiotec)[15]. EpCAM+ve enriched cells were termed as hHPCs which were further tested for their viability and counted using hemocytometer. Human HPCs were further characterized for the expression of other liver cell specific pluripotent markers using immunofluorescence and molecular analysis. Human HPCs with more than 90% viability were used for repopulating the ALS.

Humanization of acellularized rat liver scaffold

Post-sterilization ALS was transferred to a perfusion chamber kept in a CO_2 incubator having inlet and outlet for the flow of culture media. Initially, Dulbecco's Modified Eagle's Medium (DMEM)-F12 medium supplemented with 10% Fetal Calf Serum (FCS), 0.0036 μg/mL insulin, 10 ng/mL Epidermal Growth Factor (EGF), 1 × antibiotics and antimycotics was perfused through cannula connected with the main hepatic artery. Following to this, 12 × 10^6 EpCAM+ve enriched hHPCs were resuspended in 5 mL of human hepatic maturation medium and infused into ALF through hepatic artery at flow rate of 1 mL/min. Recellularized liver was incubated for three hours in static culture. The flow through before and after incubation was collected to determine the cells repopulation efficiency. After static culture, continuous fluidic culture was established by supplying culture media to the repopulated liver at flow rate of less than 0.5 mL/min with the help of a peristaltic pump. The perfusate was collected after 24, 48 and 72 h of culture and used for DNA quantification and release of lactate dehydrogenase (LDH).

Characterization of humanized liver

SEM: SEM analysis of repopulated liver scaffold (RLS) was performed using the standard protocol described by Bozzola and Russell (1998)[11]. The humanized liver tissues were fixed in 2.5% (v/v) gluteraldehyde in 1 × PBS and the ultra-structures of these tissues were documented using JOEL-JSM 5600 SEM at RUSKA Lab's College of Veterinary Science, SVVU, Rajendranagar, Hyderabad, India.

Immunofluorescence staining: The immunofluorescence staining of repopulated hHPCs in ALS was identified using specific antibodies for Glucose-6-phosphatase catalytic subunit (G6PC) and albumin (ALB). 4',6-diamino-2-phenylindole (DAPI, Sigma) was used as counterstain for nuclear components. Stained sections were imaged using inverted fluorescence microscope (Carl Zeiss, Germany).

Histology: Histological analysis of RLS was performed using H and E staining of liver tissue microsections and compared with the native liver.

Functional analysis: The functional response of humanized livers was identified by quantification of albumin and glucose-6-phosphatase catalytic subunit (G6PC) liver enzyme in culture supernatant at different time point's post-repopulation.

Drug treatment and metabolism study

Six of the commonly used CYP probe substrates such asphenacetin (100 μmol/L) specific to CYP1A2[16], diclofenac (25 μmol/L) specific to CYP2C9[17], S-mephenytoin (5 μmol/L) specific to CYP2C19[18], dextromethorphan (50 μmol/L) as a substrate of CYP2D6[19], nifedipine (5 μmol/L) as a substrate of CYP3A4 and testosterone (120 μmol/L) as a substrate of CYP3A4[20] were used to evaluate cellular metabolism in humanized livers in comparison to 2D-cultures. One hundred microlitres of each test compound (diluted in DMSO water) was infused in repopulated humanized livers to maintain final volume of DMSO below 0.2% (v/v). The drug metabolism time was set for two hour post-treatment in CO_2 incubator at 37 ℃ temperature, 5% CO_2 and 95% humid atmospheres. Each of the treatment condition was performed in triplicates in two separate cohort studies. The reaction was terminated using 2 mL of acetonitrile containing 1 mg/mL celecoxib as internal control. The supernatant from each treatment group was transferred in sterile glass tubes and the contents were dried using steam of nitrogen with the help of multivap evaporator set at 40 ℃ (N-evap, Orginomation, Berlin, MA, United States). The residue was reconstituted in 200 μL mobile phase (A:B, 1:1). One hundred microlitres of this reconstituted solution was injected in High-performance liquid chromatography (HPLC) for further analysis.

HPLC analysis

HPLC analysis of drug metabolism was performed as earlier described by Rao *et al*[21] 2003. Briefly; HPLC system containing water alliance separation module attached with a water photodynamic array detector was set at detection range of 190-400 nm. A C18, 3V column (GL Sciences, Inc, Japan) was used for the analysis. A tertiary mobile phase gradient system containing three different types of solutions (solution A: 0.01 mol/L ammonium acetate having pH 5 and acetonitrile 90:10, solution B: 0.01 mol/L ammonium acetate having pH 5 and acetonitrile 5:95, and solution C: 0.01 mol/L ammonium acetate having pH 5 and methanol 5:95). The total run time was 40 min with gradient flow. Analysis was conducted by estimating the peak area at individual UV-spectra with the integration of the peak area counts obtained from the internal standards. The area counts of each test compound were divided by the area counts of internal standard within the same analytical run to find the area ratio. This calculated area ratio was used to determine the percentage depletion of the parent

compound after 2 h of metabolism in 3D-humanized liver as compared to 2D-culture system.

Statistical analysis

The data were expressed as mean ± SEM. Each experiment was performed in triplicate in two separate cohort studies to maintain the reproducibility. During metabolism studies, area of the drug was divided by the area of internal standard to calculate the area ratio. The area ratio obtained at 0 h was considered as 100% and at 2 h was calculated to get the metabolic stability of test compounds. Drug metabolism in each group was estimated using the substrate depletion approach[22] with the formula: Percentage substrate remained in test sample = (ratio of substrate in test sample/ratio of substrate in control sample) × 100. One way and two way ANOVA was performed using Graph Pad Prism (version V) to identify the statistical significance among multiple groups. $P < 0.05$ was set as statistical significance for all the variables in different groups.

RESULTS

Bioengineering humanized liver using acellularization and repopulation technology

Humanized livers were bioengineered based on the acellularization and human HPCs repopulation technology as demonstrated in Figure 1. This strategy involves the compete removal of cellular components from the total liver through perfusion with acellularization reagents. The continuous flow of acellularization reagents at fixed speed and pressure is maintained with the help of peristaltic pump.

Characterization of liver tissue scaffolds pre and post-acellularization

The optical characterization of liver tissues post-acellularization showed absence of liver parenchyma and non-parenchyma cells. The solid and red color whole liver became translucent post-acellularization while retaining intact vascular networks (Figure 2). Further expression and distribution analysis before and after acellularization of whole liver showed intact ECM proteins. More specifically, the immunohistochemistry of liver key ECM proteins collagen type 1 and fibronectins showed complete preservation of liver ECM components post-acellularization. In addition, laminin staining showed intact lining of liver vasculature representing the intact network of vascular tree within the liver post-acellularization.

Ultra-structural characterization of ALS: SEM analysis of ALS showed intact 3D-architecture and retention of micro-structures of liver specific ECM proteins. The key structural components such as organ vasculatures were well maintained and distributed throughout the scaffold. The prints of liver cells could be easily recognized in parenchyma region of the liver scaffolds which were surrounded by the network of ECM proteins (Figure 3A). Overall, these observations confirmed the intact three-dimensional anatomy and ultra-structures of liver post-acellularization.

Vascular-tree imaging: Methylene blue dye infusion through main hepatic artery in ALS confirmed the intactness of liver vasculature through gradual distribution from major artery to distant smaller arteries. The vasculature dying also demonstrated the intactness of all three vascular systems named portal, arterial and biliary. The dye infusion first colored the liver parenchyma and finally reached to the central venous system which showed the complete retention of intact perfusion polarity within the ALS (Figure 3B).

Residual DNA content in ALS: The liver perfusate showed high quantity of dsDNA (Figure 3C) whereas complete reduction of dsDNA in ALS was identified (Figure 3D).

Mechanical properties of ALS post-sterilization: The retention of preserved mechanical properties of ALS was determined post-sterilization with PAA and UV and further compared with the fresh liver as control. Three different types of mechanical characterization using tensile strength test, suture retention test and compressive strength analysis revealed that all three mechanical properties of ALS were preserved similar to the control (Figure 3E-G).

Humanized liver repopulated with human HPCs

The intact vasculature and liver capsule post-acellularization offers the development of neo-humanized liver system. Herein, the humanized liver was achieved through repopulation of human HPCs into completely ALS at day 7. Optical images of humanized liver showed revival of liver tissues with well intact capsule and liver architecture (Figure 4A). The repopulation efficiency was calculated to be > 80% at day 7 post-repopulation.

SEM analysis of humanized liver

Ultra-structural analysis of humanized liver using SEM showed that human liver cells are well engrafted and proliferated in parenchyma and around the vascular spaces. Liver parenchyma was completely surrounded by the human liver cells at day 7 which suggests that the cells were migrated beyond the ECM barrier to reach the acellularized sinusoidal spaces (Figure 4B).

Immunohistochemical analysis of humanized liver

H and E staining was performed to characteize the cellular arrangement in humanized liver tissue at day 7 post-repopulation (Figure 4C). The staining revealed proper arrangement and distribution of cells at defined locations as observed by SEM analysis.

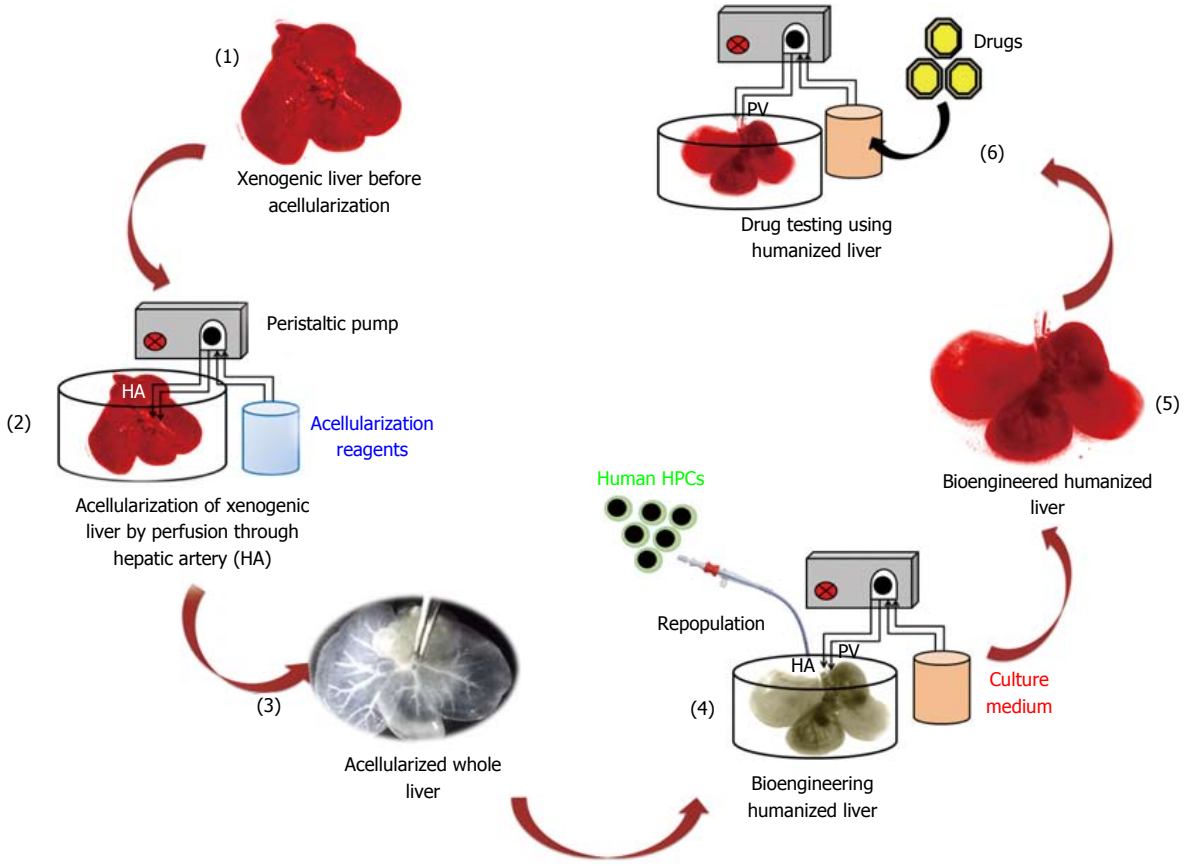

Figure 1 Schematic study plan. Representation of bioengineering technology to generate humanized livers using acellularization and human HPCs repopulation strategy for the development of three-dimensional platform for drug testing.

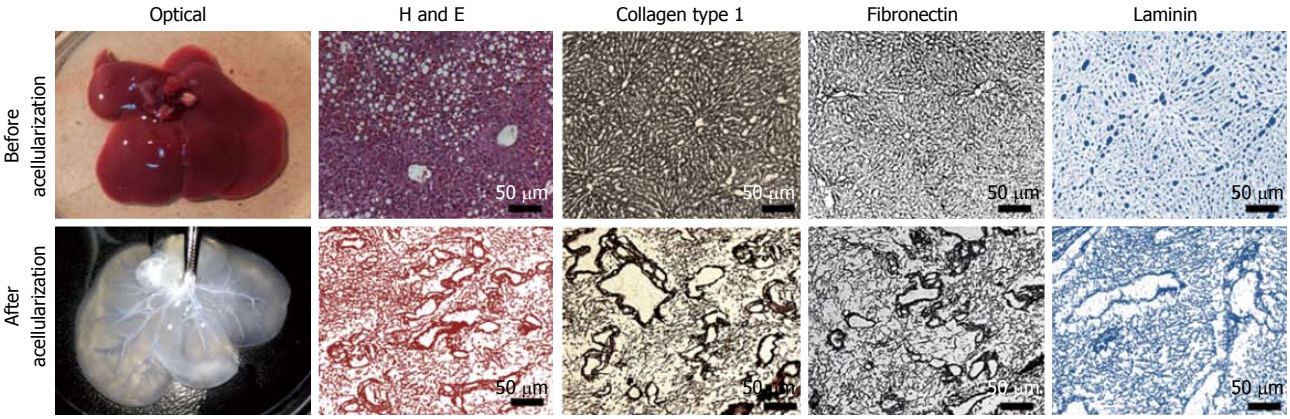

Figure 2 Xenogenic liver before and after perfusion acellularization. Macroscopic appearance (optical) of acellularized liver showing removal of hematopoietic, liver parenchyma and non-parenchyma cells while retaining the vascular tree. Histological comparison of native and acellularized liver. The expression and distribution of liver extracellular matrix proteins such as collagen type 1 was seen in the parenchymal space as well as around the blood vessels. Fibronectin staining demonstrated conserved meshwork in sinusoidal spaces and biliary ducts post-acellularization. Laminin staining showed intact vasculature throughout the liver scaffold (magnification: 10 ×).

Further the functional analysis of cells engrafted within the humanized liver was identified using immunocytochemuical staining for albumin (Figure 4D) and key liver cell enzyme G6PC (Figure 4E). These investigations confirmed that the repopulated human liver cells are viable and functional. The percentage cell apoptosis post-repopulation was determined by the TUNEL staining of humanized liver tissue microsections before (negative) and after humanization at day 7 (RL/d7). The analysis revealed < 10% cells were apoptotic after 7 d of repopulation (Figure 4F).

Nuclear content in humanized liver

The quantity of nuclear contents in humanized liver showed that < 25% dsDNA were present in liver perfusate during repopulation at day 7 which may

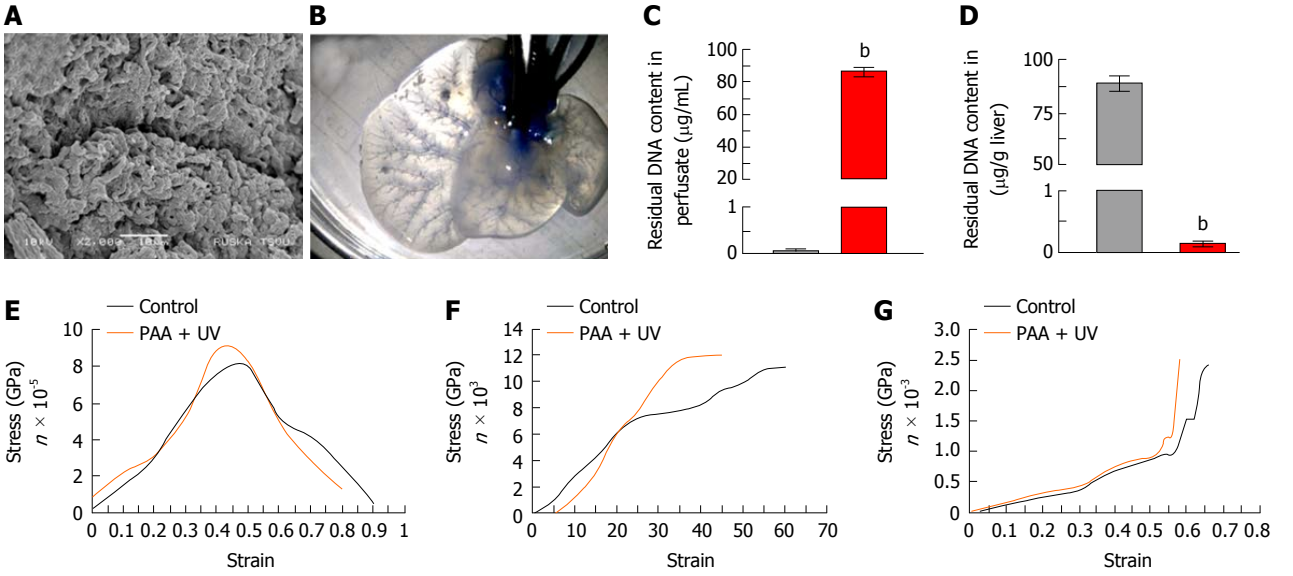

Figure 3 Characterization of acellularized liver scaffold. A: Ultra-structural characterization of acellularized xenogenic liver showing acellularity in the scaffold with preserved three-dimensional microanatomy of the portal tract surrounded by lobular structures. The parenchymal spaces were found enriched with connective tissue fibres arranged with honeycomb-like structures which are an exceptionally preserved anatomy of connective tissues which structures the hepatocyte-free spaces; B: Methylene blue dye infusion showing intact vasculature post-acellularization; Residual DNA content in liver perfusate (C) and in liver tissues (D) before (black bar) and after acellularization (red bar) ($^{b}P < 0.0001$); E: Mixed tensile strength; F: Mixed suture retention strength; G: Mixed comprehensive strength plots showing higher degree of retention of mechanical properties post-acellularization.

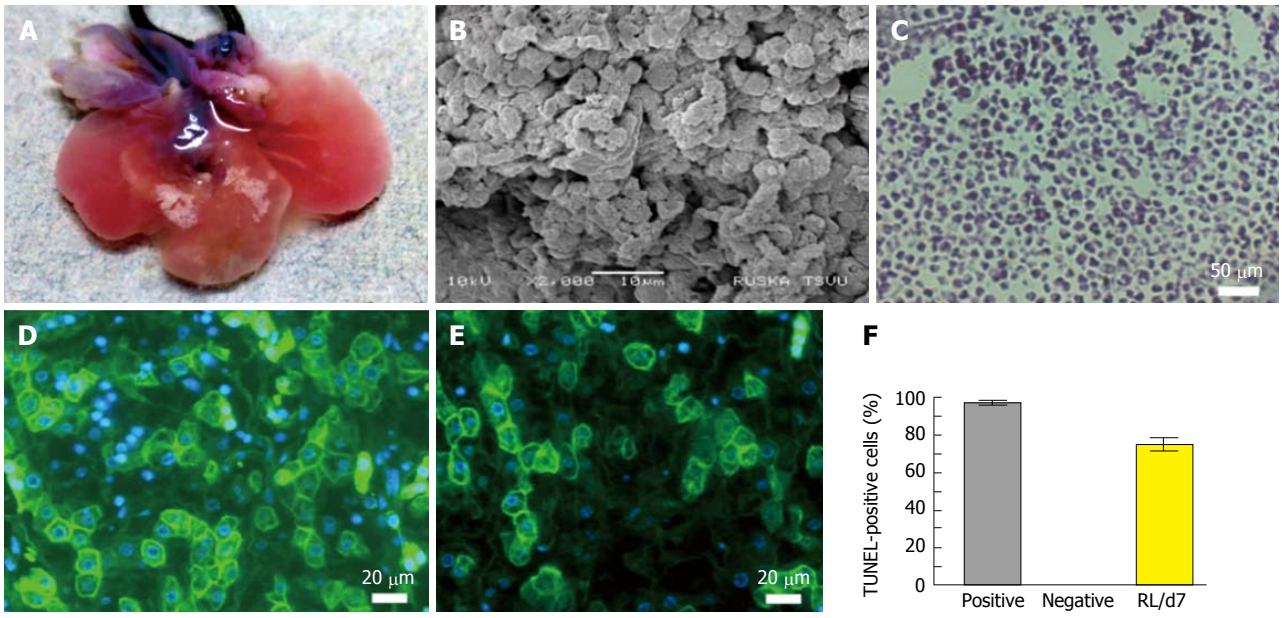

Figure 4 Characterization of humanized liver. A: Optical image of humanized liver after 7 d of repopulation with human HPCs into a customized bioreactor; B: SEM image of humanized liver at day 7 showing cellular proliferation and natural arrangements; C: H and E staining of thin micro-section of humanized liver (Magnification: 10 ×); Albumin (D) and G6PC (E) immunofluorescence staining (green) representing the functional activity of repopulated human liver cells (Magnification: 20 ×); F: TUNEL staining of repopulated cells at day 7 (RL/d7) showed significantly high expression as compared with the negative control. SEM: Scanning electron microscopy.

include 10% of apoptotic DNA as observed by TUNEL assay (Figure 5A). Humanized liver tissue extract also showed almost similar quantity of dsDNA per gram of humanized liver tissue as compared to the fresh liver tissue (Figure 5B).

Functional characterization of humanized liver

The integrated cellular function of humanized liver was identified by estimating the albumin secretion and LDH released from human hepatic cells at different time points of repopulation and compared with the 2D-culture system. Albumin estimation in liver perfusate revealed extensively increased albumin secretion by the liver cells in humanized liver along with the time and was comparatively higher than the 2D-cultured cells (Figure 5C). LDH released from the cells in humanized liver

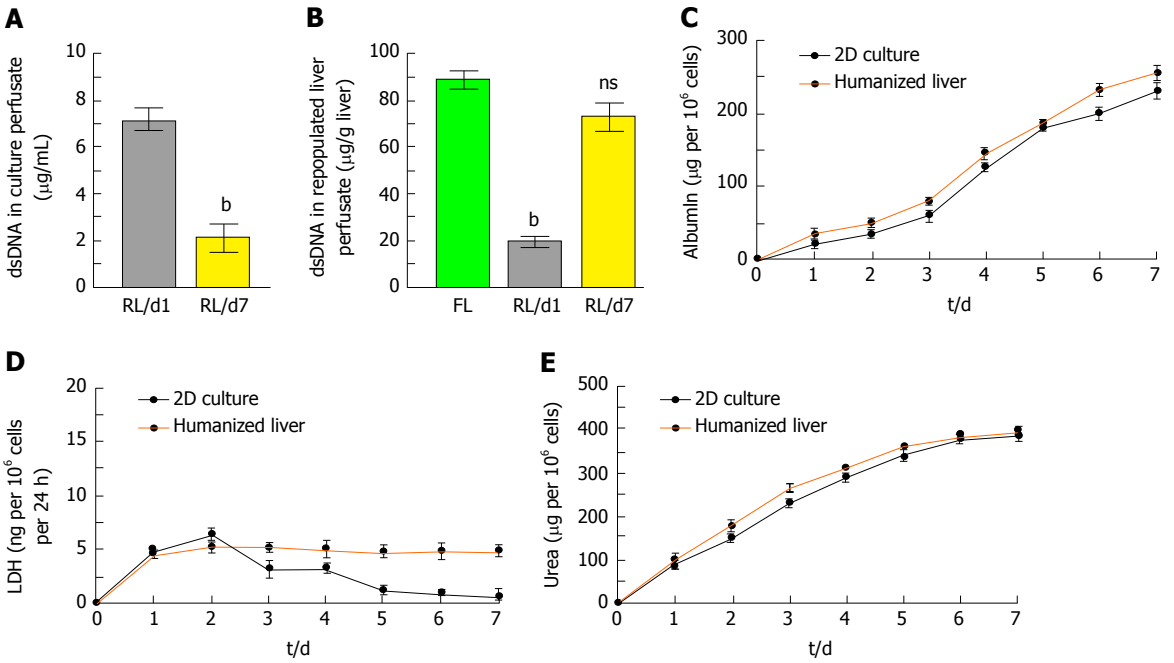

Figure 5 Functional assessment of humanized liver. A and B: Quantification of double stranded DNA (dsDNA) in (A) liver perfusate showing reduction in quantity at day 7 whereas (B) in humanized liver post-repopulation showed reciprocal relationship which was significantly high ($^bP < 0.001$) at day 7 as compared to day 1 and was almost similar to the fresh liver (FL); C-E: The rate of (C) albumin secretion (D) lactate dehydrogenase release and (E) urea synthesis in humanized livers at different time points showing improved response as compared to the conventional 2D-cultures.

was quite stable as compared to 2D-culture system which represents the stability and well established synchronization in liver cells within the scaffold (Figure 5D). Furthermore, urea synthesis, one of the functional characteristic of mature liver cells also demonstrated progressively higher degree response in humanized livers as compared to 2D-culture system (Figure 5E).

Metabolism of CYP substrates in humanized liver

The drug metabolism study performed in humanized livers for six well-known CYP substrates (Figure 6A) using substrate depletion assay showed that humanized liver metabolites the CYP substrates better than the 2D-cultured cells. The percentage retention of examined six CYP substrates was significantly lower in humanized livers than the 2D-culture system. Complete depletion was observed for nifedipine and testosterone in both humanized liver as well as 2D-culture system. Whereas, the depletion rate was quite high for dextromethorphan (> 20%, $P < 0.001$), diclofenac (> 10%, $P < 0.01$), mephenytoin (> 25%, $P < 0.001$) and phenacetin (> 10%, $P < 0.01$) in humanized liver as compared to the 2D-culture system (Figure 6B). These results clearly suggest that humanized liver could be better *in vitro* three-dimensional drug testing model system to optimize the dose for safety evaluations prior to clinical applications.

DISCUSSION

Human liver play significant role in drug metabolism and toxicological response. Therefore drug-induced liver toxicity has been a major concern for the development of acute liver failure and post-market drug withdrawal due to the absence of suitable humanized preclinical model system. Animal models have been the gold standard platform to identify the toxicological effects of pharmacological drugs/molecules. However, species difference always does not allow predictive outcome similar to human system[23]. Hence, several *in vitro* models of human livers have been developed to complement the animal model system. The most widely used *in vitro* models include human liver specific cell lines such as HepG2, Hep 3B and SNU-398. However, these cell lines lack expression of several molecular cues for drug targeting. Currently, human stem cells have been considered the most suitable cell types for such studies.

Among the various choices of stem cells, induced pluripotent stem cells (iPSCs) have been proposed as the best choice for *in vitro* drug testing. These cells are generated by reprogramming of somatic cells into pluripotent nature by inducing OSKM Yamanaka transcription factors[24]. The major advantages of iPSCs are its highly proliferative nature, ease of accessibility and less/or no ethical constraints[25]. However, the preclinical and clinical applicability of iPSCs has been limited due to reprogramming obstacles, financial hurdles, reprogramming inefficiencies, and genetic instability[26,27]. One of the examples of failing iPSCs pre-clinical and clinical applicability was demonstrated by ophthalmologist Masayo Takahashi in collaboration with Shinya Yamanaka where they claimed for the regeneration and improvement in vision post-trans-

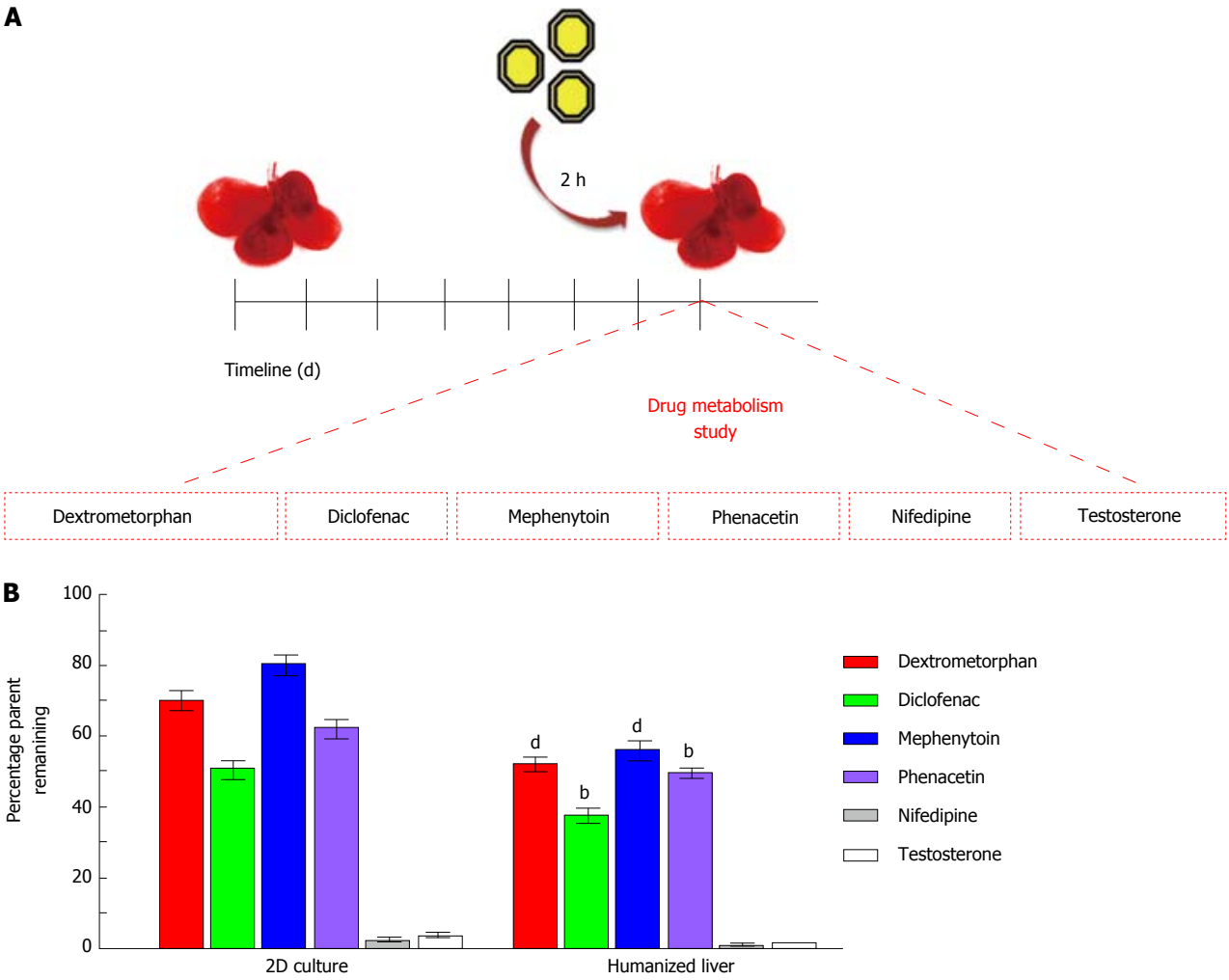

Figure 6 Drug metabolism studies using 3D humanized liver system. A: Schematic representation showing the timeline of humanized liver development and drug metabolism study; B: Bar graphs showing the rate of cellular metabolism of six cytochrome P-450 probe substrates in humanized liver as compared to 2D-culture system ($^{b}P < 0.01$, $^{d}P < 0.001$).

plantation of iPSCs-derived retinal pigment epithelial (RPE) sheets in patient suffering with age related muscular degeneration. This trial was halted due to unexplained mutations in transplanted RPE and the patient's iPSCs which concluded that several crucial safety assays need to be established before considering the pre-clinical applicability of iPSCs[28,29].

Alternative to iPSCs, human HPCs have been proposed as better cell type for drug testing model development. However, the source and isolation technique has been challenging to obtain enriched homogenous population of primary hHPCs. Our group has reported well established approach to isolate human fetal hepatic progenitor cells[30,31]. However, due to the ethical concerns alternative adult sources are needed to be identified. Our earlier studies have demonstrated tremendous clinical beneficial effects in the field of stem cells transplantation specifically in patients with acute[32,33] and chronic liver failure[13,34-36] and metabolic syndrome[37]. In addition to this, various other groups have also demonstrated significant role of stem cells in liver regeneration[38,39].

Development of humanized organs has always been a challenging area in regenerative biology. Discovery of stem cells has given a potential hope to regenerate the diseased organs or tissues in human body. Since then, various strategies have been tried to evolve humanized organs and/or tissue using stem cell technology for *in vitro* discoveries and *in vivo* transplantation studies. However, very limited success has been achieved as far as *ex-vivo* development of whole functional humanized organs/tissues is concerned. Due to the enormous potential of humanized tissues/organs in pharmacological studies, several investigations have been focused to generate biomimetic humanized organs which is an urgent need to replace the conventional 2D/or 3D *ex-vivo* systems and animal models to reduce the investigatory and economic burdens towards the preclinical evaluation of drugs.

Over the past decades, micro culture technology has emerged to probe the biomedical mechanisms and functions[40]. However, the natural 3D system is critical to bridge the preclinical and clinical studies more effectively. The most popular 3D-model system named organoid culture exhibit more complexity in structure and function than the 2D cultures which

results in several challenges in systemic assessment of pharmacological interventions. Furthermore, the batch to batch diversity in complexity, size, morphology, 3D-arrangement of cells and more importantly the protocol variability represent major challenges to overcome.

The very recent preclinical technology named "human organs-on-chip" do not mimic with the full complexity of human liver functions and show limited pharmacokinetic recapitulation and can't exhibit the clinically relevant processes[41-43]. Hence, identifying a more clinically relevant 3D-humanized model system can streamline and expedite the drug evaluation process. Given the limitations of currently available preclinical models, human metabolites and their downstream effects often go undetectable until the human clinical trials which is the most costly and risky phase of drug development[44]. Despite these significant advances, several crucial issues related to drug absorption, distribution, metabolism, excretion and toxicity (ADMET) indicates lack of sufficient predictability in drug evaluation models. To avoid such higher failure rate in late-stages of drug testing processes, more appropriate humanized platform is highly desirable to generate better preclinical outcome. Our earlier effort was to generate such platforms using various bioengineering technologies in different organs[45-47]. However, drug metabolism studies in humanized liver remain to be studied.

The bioengineered humanized model developed in this study provides natural system for above described assumptions which could be more practical approach to replace the earlier developed models including animals. In addition to the natural architecture, presence of human primary hepatocytes provides activities of human liver metabolic enzymes to identify the real pharmacokinetics of drugs. As CYP is the most common group of enzymes found in liver for the clearance of drugs, it has been proposed better pathway to study the drug metabolism[48]. Another important role of CYPs has been its quantitative variability during the drug metabolism. Hence identifying CYP mapping could provide important information about the drug metabolism either by a single or multiple isoforms of CYPs. FDA guidance requires more than 25% clearance from the CYP mediated liver metabolism prior to conduct human trial on a particular drug[49]. The metabolism of six CYP substrates in present study using bioengineered liver system could provide better platform for future drug metabolism studies as a replacement of animal models as unique pre-clinical model system.

Humanized liver model system could be ideal choice for drug metabolism studies using tissue specific 3D-architecture, proper cell to cell and cell to ECM interactions which make them one of the best model systems to predict the drug responses. The 3D-architecture of this model provides *in vivo* like context and also eliminates the species differences. This system allows biomimetic humanized preclinical outcomes by allowing natural drug delivery and distribution. In summary, bioengineered humanized livers could be more suitable option for determining drug safety and efficacy in human mimetic preclinical model system. This unique biomimetic platform can produce better outcome during disease modeling and ADMET studies.

ARTICLE HIGHLIGHTS

Research background

The present study offers a new platform for drug metabolism studies in 3D-biomimetic humanized model system.

Research motivation

This approach can provide more realistic outcome of drug metabolism in human cells under organ specific biological and mechanical cues.

Research objectives

The real-time pharmacokinetics of drug absorption, distribution, metabolism, excretion and toxicity can be identified in natural humanized system using cytochrome P-450 probes.

Research methods

This unique system offers several advantages over the conventional models of drug metabolism studies such as comparatively less cost and time is required for the maintenance and care of the cultures than animal studies.

Research results

Smaller quantities of chemicals are required for *ex-vivo* drug testing.

Research conclusions

The cellular response networks and toxicity pathways can be easily determined against drug exposure.

Research perspectives

Enhanced dose-responsive relationships can be identified relative to human exposure.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Risk factors for hepatic steatosis in adults with cystic fibrosis: Similarities to non-alcoholic fatty liver disease

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Abstract

AIM

To investigate the clinical, biochemical and imaging characteristics of adult cystic fibrosis (CF) patients with hepatic steatosis as compared to normal CF controls.

METHODS

We performed a retrospective review of adult CF patients in an academic outpatient setting during 2016. Baseline characteristics, genetic mutation analysis as well as laboratory values were collected. Abdominal imaging (ultrasound, computed tomography, magnetic resonance) was used to determine presence of hepatic steatosis. We compare patients with hepatic steatosis to normal controls.

RESULTS
Data was collected on 114 patients meeting inclusion criteria. Seventeen patients (14.9%) were found to have hepatic steatosis on imaging. Being overweight (BMI > 25) (P = 0.019) and having a higher ppFEV1 (75 *vs* 53, P = 0.037) were significantly associated with hepatic steatosis. Patients with hepatic steatosis had a significantly higher median alanine aminotransferase level (27 *vs* 19, P = 0.048). None of the hepatic steatosis patients had frank CF liver disease, cirrhosis or portal hypertension. We found no significant association with pancreatic insufficiency or CF related diabetes.

CONCLUSION
Hepatic steatosis appears to be a clinically and phenotypically distinct entity from CF liver disease. The lack of association with malnourishment and the significant association with higher BMI and higher ppFEV1 demonstrate similarities with non-alcoholic fatty liver disease. Long term prospective studies are needed to ascertain whether CF hepatic steatosis progresses to fibrosis and cirrhosis.

Key words: Cystic fibrosis liver disease; Hepatic steatosis; Non-alcoholic fatty liver disease

Core tip: Our retrospective cohort study of cystic fibrosis (CF) patients with hepatic steatosis demonstrates that hepatic steatosis in CF is associated with a higher body mass index as well as a higher percent predicted forced expiratory volume in 1 s, as compared to normal CF controls. None of our patients with hepatic steatosis exhibited evidence for advanced liver disease. Our findings are novel and demonstrate similarities between hepatic steatosis in CF and adult non-alcoholic fatty liver disease and future prospective studies are required to determine whether this steatosis may evolve into cirrhosis.

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INTRODUCTION

Cystic fibrosis (CF) is the most common fatal autosomal recessive disease in Caucasians. The majority of clinical manifestations of CF are due to a mutation of the CF transmembrane receptor (CFTR) resulting in defective chloride transport[1]. Outside of the classic pulmonary manifestations of CF, involvement of other organ systems such as the hepatobiliary and gastrointestinal system is common[2]. With improving care and increasing life expectancy, CF liver disease (CFLD) has arisen as a major cause of morbidity and mortality for CF patients. CFLD is now considered the third leading cause of death in CF patients after lung disease and transplantation complications[3]. Due to varying definitions of CFLD, its prevalence in adults has been reported to be between 3%-37%[4-6].

Biliary cirrhosis is the classic phenotypical manifestation of CFLD and is directly attributed to the underlying CFTR defect. However, the spectrum of hepatobiliary disease in CF patients is wide and ranges from asymptomatic elevations in aminotransferases, to end-stage cirrhosis and portal hypertension. Hepatic steatosis detected on imaging or biopsy is the most common hepatic manifestation, with a prevalence rate of 20%-60%[7,8]. While steatosis has classically been considered a benign condition in CF patients, the relationship between hepatic steatosis and the ultimate development of fibrosis and cirrhosis remains unclear[8,9]. In light of the increasing awareness of non-alcoholic steatohepatitis (NASH) as a major cause for cirrhosis there have been calls for the reconsideration of the importance of this clinical entity in CF patients[10].

Due to the fact that steatosis has classically been considered a benign lesion, patients with isolated steatosis are often excluded from studies on CFLD[11-13]. There has been little dedicated study of the risk factors for steatosis and the clinical characteristics of CF patients that exhibit this lesion. To better characterize patients with hepatic steatosis and ascertain the clinical characteristics and risk factors associated with this finding, we conducted a cross-sectional study of adult CF patients in an academic outpatient setting.

MATERIALS AND METHODS

Patients

Patients enrolled at the University of Florida Adult Cystic Fibrosis Center during the year 2016 with a confirmed diagnosis of CF who were at least 18 years of age and had at least 1 year of complete follow up were eligible for inclusion in this cross-sectional analysis. Demographic, clinical, radiographic and laboratory data on patients eligible for inclusion were retrospectively collected. Patients with incomplete clinical, laboratory and/or radiological data were excluded. All patients with laboratory or imaging findings to suggest hepatic abnormalities underwent testing for chronic liver diseases including viral hepatitis, Wilson disease, autoimmune hepatitis, primary sclerosing cholangitis and alpha-1-antitrypsin deficiency. Patients found to have any of the previous diseases were excluded from the analysis. Patients with known CF liver disease based on well-accepted criteria by Debray were also excluded[8].

Definitions

Diagnosis of CF was confirmed by the combination of clinical symptoms and an elevated sweat chloride

⩾ 60 mmol/L or the presence of two disease-causing mutations in CF transmembrane conductance regulator gene (*CFTR*). *CFTR* mutation testing was performed by amplification of selected regions of the *CFTR* gene, followed by detection of wild-type and mutant sequences. Chronic pseudomonas colonization was defined as detection within a period of 6 mo of a minimum of three positive *P. aeruginosa* cultures, with at least 1 mo between the positive cultures[14]. Patients were considered pancreatic insufficient if they demonstrated clinical symptoms and fecal elastase values less than 200 μg/g[15]. All patients at our clinic undergo annual 2 h oral glucose tolerance testing (OGTT) and a diagnosis of CF related diabetes (CFRD) was established if the patient met standard diagnostic criteria outlined by the American Diabetes Association[16]. History of alcohol consumption was patient reported and documented in the medical chart. Patients were considered to have "any alcohol use" if they reported any amount of alcohol intake in the past 2 years. Significant alcohol intake was defined as > 21 drinks per week in men and > 14 drinks per week in women over a 2-year period[17]. Hyperlipidemia was based on documentation in the medical chart and/or elevations of total cholesterol, LDL or fasting triglyceride levels above standard laboratory cut-offs.

Imaging criteria for determination of hepatic steatosis

Patients were considered to have hepatic steatosis on ultrasound if their liver demonstrated increased echogenicity as compared to the right kidney and impaired visualization of diaphragm and intrahepatic vessels[18,19]. Low hepatic attenuation on CT as compared to the spleen, or decreased T2 signal intensity on MRI were also considered to represent steatosis[20]. All previously mentioned imaging studies, have been independently validated with good sensitivity and specificity for the detection of hepatic steatosis in comparison to biopsy[21-23].

Testing for other forms of liver disease

Non-invasive markers of liver disease: We calculated the scores of three non-invasive biomarkers of hepatic fibrosis including AST-to-platelet ratio index (APRI), fibrosis-4 index (FIB-4) and the AST-to-alanine aminotransferase (ALT) ratio (AAR) (see supplementary files for formulas). These scoring systems have been heavily evaluated for use in chronic hepatitis C, hepatitis B and NASH[24-26]. Recently, criteria for the evaluation of CFLD that include the use of these non-invasive markers have been developed[27], thus we have included these scores in our analysis.

Statistical analysis

Normally distributed data are presented as proportions (mean ± SD) and for variables not conforming to a normal distribution as median and interquartile range (IQR). Two-sample comparisons were by Fisher's exact and χ^2 tests as appropriate. For proportions, student's *t* test was used for normally distributed variables and Mann-Whitney *U* test for other variables. Shapiro-Wilk test was used to determine normality of continuous variables. A two-sided *P*-value of < 0.05 was used to indicate statistical significance in all analyses. STATA version 13.0 (Statacorp, College Station, TX, United States) was used for statistical analysis.

RESULTS

Basic demographics

Of the 143 adult CF patients evaluated for inclusion, 114 met inclusion criteria. Of the 112 patients with known mutations, 57 had a homozygous *ΔF508* mutation, 47 had a heterozygous *ΔF508* mutation and 11 had other mutations. Median age at time of study was 29 years (IQR 24-35), median BMI was 20.9 kg/m^2 (19.3-24.9) and median percent predicted FEV_1 ($ppFEV_1$) was 57 (36-76). Ninety-two patients were pancreatic insufficient, 80 patients were chronically colonized with Pseudomonas aeruginosa, 47 had CF related diabetes mellitus (CFRD) and 26 had a history of childhood meconium ileus.

Imaging findings

Three imaging modalities (abdominal ultrasound, CT imaging, MR imaging) were used to evaluate and establish the presence of hepatic steatosis as described in the methods section. Ten patients were found to have steatosis based on ultrasound, 6 patients based on CT and 1 patient through MR imaging. Two patients demonstrated borderline splenomegaly with a spleen span of 13 cm[8]. None were found to have hepatomegaly or signs of portal hypertension.

Clinical features of patients with and without hepatic steatosis

Seventeen patients (14.9%) were found to have hepatic steatosis on imaging. The clinical characteristics of patients with hepatic steatosis as compared to those without are illustrated in Table 1. Eight of the 17 patients (47%) with hepatic steatosis were overweight with a BMI > 25 kg/m^2. Only being overweight (*P* = 0.019) and having a higher $ppFEV_1$ (75 *vs* 53, *P* = 0.037) were significantly associated with hepatic steatosis. When BMI was analyzed as a continuous variable, the significant association between higher BMI and hepatic steatosis persisted (22.3 *vs* 20.7, *P* = 0.010).

There was no significant association of hepatic steatosis with gender, age at time of study, homozygous or heterozygous ΔF508 genotype or a childhood history of meconium ileus. There was also no association with hypertension, hyperlipidemia, CFRD or any alcohol use. None of our patients had a history of significant alcohol use. None of the patients with hepatic steatosis had CF liver disease based on criteria proposed by Debray *et al*[8]. There was no association between pancreatic

Table 1 Demographics of our patient sample

Feature	All subjects	Data by hepatic steatosis		
		Hepatic steatosis	No steatosis	*P* value
No. of patients	114	17	97	
Male/female	58/56	9/8	49/48	0.854
Median age at time of study (IQR)	29 (24-35)	27	29	0.981
Median BMI (IQR)	20.9 (19.3-24.9)	22.3	20.7	0.010[a]
Underweight (BMI < 18.5)	19	2	17	0.557
Overweight (BMI > 25)	28	8	20	0.019[a]
Genotype				
ΔF508/ΔF508	57	11	46	0.216
ΔF508/other	44	6	38	0.714
Other	11	0	11	0.140
Unknown	2	0	2	
ppFEV$_1$	57 (36-76)	75	53	0.037[a]
Chronic pseudomonas colonization	80	14	66	0.234
Pancreatic insufficiency	92	15	77	0.394
Replacement dose	2011 (1334-2405)	1897	2012	0.610
Meconium ileus	26	4	22	0.939
CFRD	47	4	43	0.108
Hypertension	5	1	4	0.561
Hyperlipidemia	12	1	11	0.665
Any alcohol use	42	8	34	0.344
On CFTR modulator therapy	29	6	23	0.312

[a]*P* < 0.05. IQR: Interquartile range; BMI: Body mass index; ppFEV1: Percent predicted forced expiratory volume in 1 s; CFRD: Cystic fibrosis related diabetes mellitus; CFTR: Cystic fibrosis transmembrane conductance regulator.

Table 2 Comparison of biomarkers between patients found to have hepatic steatosis and those without steatosis on imaging

Biomarker	Data by hepatic steatosis		*P* value
	Hepatic steatosis (*n* = 17, 15%)	No hepatic steatosis (*n* = 97, 85%)	
AST	23 (20-29)	21 (16-26)	0.284
ALT	27 (19-36)	19 (13-32)	0.048[a]
ALP	103 (75-120)	99 (73-150)	0.793
Platelets	279 (244-311)	270 (207-342)	0.764
Total bilirubin	0.3 (0.3-0.4)	0.4 (0.2-0.5)	0.022[a]
INR	1 (1-1.1)	1 (1-1.1)	0.350
Albumin	3.7 (3.5-4.2)	4.2 (3.8-4.4)	0.034[a]
LDL	78.5 (44-89)	63.5 (45-81)	0.424
HDL	36.5 (31-42)	45 (36-56.5)	0.091
Triglycerides	78.5 (65-96)	80.5 (62-114.5)	0.756
Total cholesterol	124.5 (93-152)	133 (103-162.5)	0.819
HbA1c	6.5 (5.8-7.1)	6.1 (5.5-6.7)	0.097

[a]*P* < 0.05. AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; INR: International normalized ratio; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; HbA$_{1c}$: Hemoglobin A$_{1c}$.

insufficiency and the presence of hepatic steatosis and there was no statistically significant difference in daily pancreatic enzyme replacement dosing between the two groups. There was also no significant association between being on CFTR modulator therapy and hepatic steatosis.

Laboratory values and non-invasive biomarkers of liver disease in patients with and without hepatic steatosis

The laboratory values and non-invasive biomarkers of liver disease of patients with hepatic steatosis as compared to those without are illustrated Tables 2 and 3, respectively. Patients with steatosis had a significantly higher median ALT level (27 *vs* 19, *P* = 0.048), lower total bilirubin (0.3 *vs* 0.4, *P* = 0.022) and lower albumin (3.7 *vs* 4.2, *P* = 0.034). There was no significant difference between total cholesterol, LDL, HDL or triglyceride in the two groups. There was a trend towards a higher HbA1c level in hepatic steatosis patients (6.5 *vs* 6.1, *P* = 0.097). In terms of non-invasive biomarkers of liver disease, only the AAR was significantly lower in patients with hepatic steatosis (0.79 *vs* 1, *P* = 0.017). There were no significant differences in APRI or FIB-4 scores.

Table 3 Comparison of non-invasive biomarkers of hepatic fibrosis between patients found to have hepatic steatosis and those without steatosis on imaging

Biomarker	Data by hepatic steatosis		*P* value
	Hepatic steatosis (*n* = 17, 15%)	No hepatic steatosis (*n* = 97, 85%)	
APRI	0.28 (0.14-0.27)	0.19 (0.12-0.32)	0.579
FIB-4	0.49 (0.35-0.67)	0.57 (0.36-0.82)	0.629
AAR	0.79 (0.65-1.08)	1.00 (0.82-1.33)	0.017[a]

[a]*P* < 0.05. APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis 4 score; AAR: Aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio.

DISCUSSION

In this cross-sectional study of 114 adult CF patients, 14.9% of patients were found to have hepatic steatosis. None met widely accepted criteria for CF liver disease[8]. Hepatic steatosis was found to be significantly asso-

ciated with a higher BMI as well as higher ppFEV$_1$. Patients with steatosis had a significantly higher ALT level and a significantly lower AAR value. There was no association of hepatic steatosis with hypertension, hyperlipidemia, alcohol use or CFRD.

While CFLD manifestations such as focal and multilobular cirrhosis have been well described, hepatic steatosis in CF adults has not been well characterized in the literature. In our cohort, a higher BMI was significantly associated with hepatic steatosis and a significant proportion (47%) of our patients with hepatic steatosis were overweight with a BMI > 25 kg/m^2. While the association between obesity and steatosis in non-alcoholic fatty liver disease (NAFLD) has been well-described[28], this has not been previously reported in patients with CF. Only one study in predominantly pediatric CF patients reported no association between overweight BMI and steatosis, however did not specifically include data to support that conclusion[29]. We believe that our findings may indicate a possible similarity between hepatic steatosis in CF adults and other forms of adult liver disease such as NAFLD. While it has been suggested that steatosis in CF patients may be related to alcohol use[7,9], none of our patients consumed significant amounts of alcohol. Even when considering any amount of alcohol use, we found no significant difference between patients with and without hepatic steatosis.

To further delineate possible similarities with NAFLD, we investigated the association between hepatic steatosis and classic risk factors for NAFLD including hypertension and hyperlipidemia[30]. We found no significant association with either. However, we note that our study cohort is relatively young with a low prevalence of both conditions. Another classic risk factor for NAFLD is insulin resistance and associated diabetes mellitus. We did not find a significant association between hepatic steatosis and CFRD in our cohort. While multiple authors have hypothesized that insulin resistance and CFRD are possible risk factors for hepatic steatosis in CF patients[7,31,32], our study is the first note a the lack of such an association in adult patients with hepatic steatosis.

Early studies of CFLD associated the finding of hepatic steatosis with severe malnutrition[33] while others have associated it with essential fatty acid deficiency[34]. However, it has been noted in later studies that many cases occur in patients with excellent nutritional status[7]. In our cohort, there was no significant association of steatosis with pancreatic insufficiency and the mean daily pancreatic enzyme replacement dose was similar between the two groups. This, in addition to our findings and regarding BMI above, do not support overt malnutrition as a risk factor for steatosis. Interestingly, we also found a significantly higher ppFEV$_1$ in our hepatic steatosis group. Multiple studies have demonstrated that better nutritional status has been linked to improved pulmonary function and ppFEV$_1$[35]. We believe that the higher BMI demonstrated in the steatosis group reflected better nutritional status and associated improved ppFEV$_1$. Another possibility, although less likely, is that patients with overall less severe pulmonary disease and better ppFEV$_1$ at baseline were able to maintain adequate nutrition and caloric intake leading to a higher BMI and ultimately associated hepatic steatosis.

Other risk factors for hepatic steatosis have been suggested in the literature, such as high levels of circulating cytokines in the setting of chronic infection as well as chronic antibiotic therapy[7,36]. We however found no association between chronic pseudomonas colonization (and indirectly the associated chronic antibiotic use) and hepatic steatosis. In addition, we also demonstrated a lack of association between gender or childhood meconium ileus and hepatic steatosis, both of which are classic risk factors for CFLD[37]. None of our hepatic steatosis patients met criteria for classic CFLD and none had imaging findings concerning for portal hypertension or cirrhosis. This supports the fact that hepatic steatosis in CF adults is likely phenotypically and pathophysiologically distinct from classic CFLD and possibly shares similarities with NAFLD.

Serum activities of ALT, AST and alkaline phosphatase have previously been shown to correlate with liver fibrosis in CFLD but not steatosis[29]. In one series 57% of those with steatosis detected on ultrasound had an associated elevation in aminotransferases[38]. In our cohort we found that those with hepatic steatosis only had a significantly higher ALT level as compared to those without. We found no difference in calculated non-invasive biomarkers of fibrosis including APRI and FIB-4 scores. The median AST-to-ALT ratio (AAR) in hepatic steatosis patients was < 1 and was significantly lower than patients without steatosis. This likely reflects the overall predominance of significant ALT elevation in comparison to AST elevation in our steatosis cohort. It is unclear whether this pattern is specific to CF patients with steatosis and would require validation in larger cohorts. An AAR value of $\geqslant$ 1 has been found to be predictive of cirrhosis in chronic viral hepatitis and NASH[39-41], thus routine monitoring of AAR for increasing values may be worthwhile during long term follow up of CF patients with hepatic steatosis to monitor for possible progression to fibrosis and cirrhosis.

Our study has several limitations. Our relatively small sample size and single center analysis may limit generalizability. However, we note that the University of Florida Health System is a major referral center in the southeastern United States, which increases the external validity or our results. The retrospective nature of our study only allows us to ascertain associations without determination of causality. Finally, the lack of histopathological analysis of our hepatic steatosis patients may be a relative limitation. However, it has been well-established that the clinical utility of liver biopsy is quite limited due to the patchy nature of liver

disease in CF patients and liver biopsy is not routinely recommended in patients with CFLD.

Future studies may incorporate liver biopsy into their design, as well as other means of detecting insulin resistance in patients with steatosis such as homeostatic model assessment (HOMA). There have also been studies indicating significant differences in the blood levels of fatty acids and serum phospholipids between patients with CFLD and controls[42]. It would be of interest for future studies to compare such levels between patients with steatosis and controls.

In summary, in this cross-sectional analysis of adult CF patients we demonstrate a significant association between higher BMI and hepatic steatosis as detected by abdominal imaging. A trend towards higher Hba1c was also noted in patients with hepatic steatosis. We hypothesize that hepatic steatosis in adult CF patients shares similarities with NAFLD. Future, long-term prospective studies are needed to ascertain whether adult hepatic steatosis progresses to fibrosis and cirrhosis.

ARTICLE HIGHLIGHTS

Research background

Hepatic steatosis is increasingly recognized in patients with cystic fibrosis (CF) on imaging. Patients often do not demonstrate associated laboratory abnormalities or abnormal physical findings. Whether hepatic steatosis represents a manifestation of classic CF liver disease is unknown. The risk factors for such a manifestation are also unknown.

Research motivation

To describe the clinical characteristics of CF patients with hepatic steatosis and to describe risk factors for the condition as compared to patients with hepatic steatosis.

Research methods

A retrospective cohort study compares cases with hepatic steatosis to controls.

Research results

Our study demonstrates that CF patients with hepatic steatosis demonstrate a higher body mass index (BMI) as well as improved pulmonary function reflected by higher forced expiratory volume as compared to normal controls. These findings indicate that patients with hepatic steatosis were relatively healthier and had an improved nutritional status as compared to controls.

Research conclusions

To our knowledge, this study is the first retrospective study dedicated to characterizing hepatic steatosis in adults with CF. The authors found patients with hepatic steatosis to have a higher body mass index as well as better pulmonary function. The authors did not find any patients with frank liver disease. The findings indicate similarities to non-alcoholic fatty liver disease. Whether this finding evolves into cirrhosis will need to be determined with longer prospective studies.

Research perspectives

CF patients with hepatic steatosis should be followed closely to determine the evolution of their disease. Caution should be exercised by providers since this lesion may exhibit similarity to non-alcoholic fatty liver disease which is now known to progress to cirrhosis in a sub-set of patients. Future, long-term prospective studies of CF patients with hepatic steatosis are needed to identify how frequently patients progress to cirrhosis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Fatty liver disease, an emerging etiology of hepatocellular carcinoma in Argentina

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Abstract

AIM

To investigate any changing trends in the etiologies of hepatocellular carcinoma (HCC) in Argentina during the last years.

METHODS

A longitudinal cohort study was conducted by 14 regional hospitals starting in 2009 through 2016. All adult patients with newly diagnosed HCC either with pathology or imaging criteria were included. Patients were classified as presenting non-alcoholic fatty liver disease (NAFLD) either by histology or clinically, provided that all other etiologies of liver disease were ruled out, fatty liver was present on abdominal ultrasound and alcohol consumption was excluded. Complete follow-up was assessed in all included subjects since the date of HCC diagnosis until death or last medical visit.

RESULTS

A total of 708 consecutive adults with HCC were included. Six out of 14 hospitals were liver transplant centers (n = 484). The prevalence of diabetes mellitus was 27.7%. Overall, HCV was the main cause of liver disease related with HCC (37%) including cirrhotic and non-cirrhotic patients, followed by alcoholic liver disease 20.8%, NAFLD 11.4%, cryptogenic 9.6%, HBV 5.4% infection, cholestatic disease and autoimmune hepatitis 2.2%, and other causes 9.9%. A 6-fold increase in the percentage corresponding to NAFLD-HCC was detected when the starting year, *i.e.*, 2009 was compared to the last one, *i.e.*, 2015 (4.3% *vs* 25.6%; $P < 0.0001$). Accordingly, a higher prevalence of diabetes mellitus was present in NAFLD-HCC group 61.7% when compared to other than NAFLD-HCC 23.3% ($P < 0.0001$). Lower median AFP values at HCC diagnosis were observed between NAFLD-HCC and non-NAFLD groups (6.6 ng/mL *vs* 26 ng/mL; $P = 0.02$). Neither NAFLD nor other HCC etiologies were associated with higher mortality.

CONCLUSION

The growing incidence of NAFLD-HCC documented in the United States and Europe is also observed in Argentina, a confirmation with important Public Health implications.

Key words: Hepatocellular carcinoma; Etiology; Fatty liver; South America

Core tip: Despite the increasing incidence of non-alcoholic fatty liver disease (NAFLD) and NAFLD related hepatocellular carcinoma (HCC) in developed countries, information related with the burden of NAFLD-HCC in developing countries as those in South America is lacking. In this multicenter cohort study from Argentina including patients with HCC, while HCV and alcoholic related cirrhosis were the most frequent causes of HCC between 2009 and 2016, NAFLD-HCC had a 6-fold increased during the same period. This changing scenario was observed without precluding any specific etiology of liver disease. NAFLD might become one of the first HCC related causes in the coming decades; an issue to be consider with effective prevention strategies.

Piñero F, Pages J, Marciano S, Fernández N, Silva J, Anders M, Zerega A, Ridruejo E, Ameigeiras B, D'Amico C, Gaite L, Bermúdez C, Cobos M, Rosales C, Romero G, McCormack L, Reggiardo V, Colombato L, Gadano A, Silva M. Fatty liver disease, an emerging etiology of hepatocellular carcinoma in Argentina. *World J Hepatol* 2018; 10(1): 41-50 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/41.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.41

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer related death worldwide and the main cause of death among patients with cirrhosis[1,2]. The incidence of HCC varies according to geographic location, closely linked to the prevalence of chronic hepatitis C (HCV) or B virus (HBV) infections as well as the prevalence of alcoholic liver disease.

With the advent of new direct antiviral drugs (DAAs) for HCV treatment, epidemiological changes have been reported in the natural history of liver disease[3]. First, the improvement of decompensated cirrhotic patients, the consequence and the increasing rate of delisting from liver transplantation (LT) waitlist after eradication of HCV. On the other hand, a lower wait-listing ratio of patients with HCV decompensated cirrhosis has been observed[3]. However, the proportion of patients listed for LT with HCC has been increased during the era of DAAs[4].

This increasing incidence of HCC has been attributed in developed countries due to a stepwise increase in the incidence of non-alcoholic fatty liver disease (NAFLD), recognized as an emergent cause of end stage liver disease and HCC[4,5]. NAFLD is the expression of a pandemic disease, which is associated with the metabolic syndrome, obesity and type 2 diabetes. Indeed, it is estimated that during the next 20 years, non-alcoholic steatohepatitis (NASH), the progressive form of NAFLD, will constitute the most frequent cause of cirrhosis and HCC in developed countries[6].

However, HCC epidemiological reports from developing areas, including South America, have been heterogeneous[7-14]. Furthermore, no studies have tackled the issue of changing trends in etiologies of HCC over time. Our present aim was to evaluate changes in etiology of HCC in Argentina during the last seven years, focusing on two aspects, the potential changes associated with DAA's era of HCV treatment, as well as changes associated with NAFLD-HCC.

MATERIALS AND METHODS

This longitudinal observational cohort study was conducted between January 1 2009 and January 1 2016 in 14 regional hospitals from Argentina. Sites were instructed to enroll all eligible patients on a sequential basis and data to be recorded from medical charts into a web-based electronic system.

A cohort of consecutive adult patients (> 17 years of age) with newly diagnosed HCC was included. Criteria for inclusion required patients to have newly diagnosed HCC either by pathological criteria or imaging evaluation as recommended by international guidelines[15,16].

Etiologies of HCC considering the primary diagnosis of liver disease included HCV (anti HCV positivity), HBV (hepatitis B surface antigen positivity), alcoholic liver disease (alcohol intake exceeding 30 g), NAFLD, cryptogenic cirrhosis (CC), cholestatic liver diseases (*i.e.*, primary biliary cholangitis, primary and secondary sclerosing cholangitis), autoimmune hepatitis and other causes including metabolic diseases or miscellaneous causes (*e.g.*, hereditary hemochromatosis, Wilson disease, toxic liver disease).

NAFLD diagnosis was achieved on histological ground or clinically according to international guidelines[17]. Patients were classified as presenting clinical NAFLD provided that all other etiologies of liver disease were ruled out, fatty liver was present on abdominal ultrasound (US) and alcohol consumption was excluded (30 g for men and 20 g women). Histological NAFLD was defined by an excessive hepatic fat accumulation associated with insulin resistance and the presence of > 5% of steatosis in liver biopsy. NAFLD included non-alcoholic fatty liver and NASH.

Baseline patient and tumor characteristics were recorded at HCC diagnosis including patients demographics, previous US surveillance, performance status (Eastern Cooperative Oncology Group, ECOG grade 0-4)[18], liver fibrosis stage (I-IV) assessed by liver biopsy elastography, other non-invasive measurements or by clinical data (presence of esophageal varices or ascites or splenomegaly > 120 mm diameter, or features related to portal hypertension), Child Pugh score and laboratory variables. Serum alpha-fetoprotein (AFP) levels at diagnosis were categorized in three cut-off values: $\leqslant$ 100 ng/mL, 101-1000 ng/mL and > 1000 ng/mL[19].

Specific major co-morbidities for each subject were also registered including: diabetes mellitus, severe chronic pulmonary disease, coronary heart disease and congestive heart disease, previous ischemic or hemorrhagic stroke, peripheral vascular disease, chronic kidney failure (glomerular filtration rate < 30 mL/min) and non-HCC cancer.

Tumor characteristics and treatments performed during follow-up were also registered. Computed tomography (CT) or magnetic resonance images (MRI) were included to assess tumor burden, which was classified according to Barcelona Clinic Liver Cancer staging (BCLC criteria)[16].

Study end-points

Our study focused on changing trends of HCC etiologies at different periods from 2009 to 2016. A stratified per-etiology analysis and per Liver Transplant (LT) and non-LT centers was performed. Complete patient follow-up was assessed in all included subjects from HCC diagnosis until death or last medical visit.

All procedures followed were in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[20]. This study was approved by the Austral University School of Medicine and by all 14 centers; complied with the ethical standards (institutional and national) and with Helsinki Declaration of 1975, as revised in 2008. Patient consent was obtained in all subjects included.

Statistical analysis

Institutional clinical research committee reviewed the statistical methods of this study. Categorical data were compared using Fisher's exact test (2-tailed) or χ^2 test. Continuous variables were compared with Student's *t*-test or Mann-Whitney *U* test according to their distribution, respectively. For survival analysis, Cox regression multivariate analysis estimating hazard ratios (HR) and 95%CI for baseline variables related with 5-year mortality was performed. Confounding

Table 1 Patients' baseline characteristics

Variable	*P* values
Age, yr (± SD)	62 ± 10
Male gender, *n* (%)	537 (75.9)
Non-cirrhotic liver, *n* (%)	89 (12.6)
Child Pugh A/B/C, *n* (%)	352 (49.7)/238 (33.6)/118 (16.7)
Comorbidities, *n* (%)	299 (42.2)
Diabetes mellitus, *n* (%)	196 (27.7)
Ascites, *n* (%)	253 (35.7)
Mild	144 (20.3)
Moderate-severe	109 (15.4)
Encephalopathy, *n* (%)	147 (20.8)
Grade Ⅰ-Ⅱ	137 (19.3)
Grade Ⅲ-Ⅳ	10 (1.4)
Esophageal varices, *n* (%)	394 (56.7)
ECOG 0-2/3-4, *n* (%)	637 (89.9)/71 (10.1)
Median HCC number, (IQR)	1.0 (1.0-2.0)
Largest HCC diameter, mm (IQR)	53 ± 37
Within Milan, *n* (%)	334 (46.9)
Bilobar involvement, *n* (%)	159 (22.1)
Diffuse HCC pattern, *n* (%)	28 (3.9)
Median AFP, ng/mL (IQR)	23.0 (5.0-337.0)
≤ 100 ng/mL, *n* (%)	476 (66.3)
101-1000 ng/mL, *n* (%)	128 (17.7)
> 1000 ng/mL, *n* (%)	115 (16.0)
Tumor vascular invasion, *n* (%)	74 (10.4)
Extrahepatic disease, *n* (%)	48 (6.8)

ECOG: Eastern Cooperative Oncology Group; HCC: Hepatocellular carcinoma; AFP: alpha-fetoprotein.

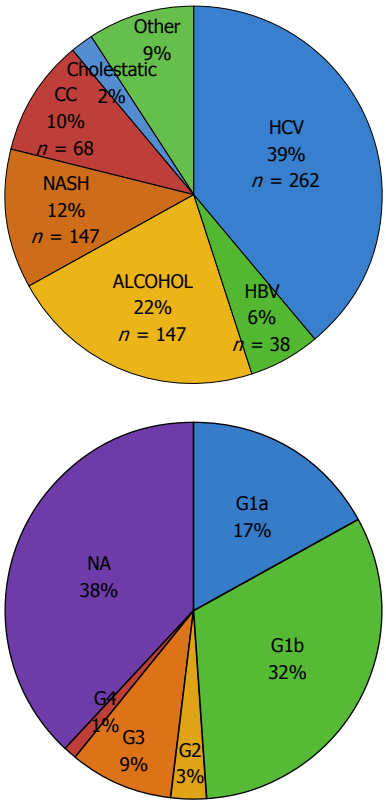

Figure 1 Etiologies oh hepatocellular carcinoma in the overall cohort and hepatitis C virus genotypes. HCV was the main cause of liver disease related with hepatocellular carcinoma including cirrhotic and non-cirrhotic patients. Distribution of known HCV genotypes (G) showed that G1b was the most frequent (*n* = 88), followed by G1a (*n* = 47), G3 (*n* = 27), G2 (*n* = 8) and G4 (*n* = 2). HCV: Hepatitis C virus.

effect was defined when more than 20% of change in the crude HR. Kaplan Meier survival curves were compared using the log-rank test (Mantel-Cox) and adjustment of each final model was evaluated with proportional hazards through graphic and statistical evaluation (Schoenfeld residual test). Calibration was assessed by comparison of observed and predicted curves and evaluation of the goodness of fit of the model by Harrell's c-statistic index. Collected data was analyzed using STATA 10.0.

RESULTS

A total of 708 consecutive adult patients with newly diagnosed HCC from 14 centers were included. Out of 14 hospitals, 6 were LT centers, which contributed with the follow-up of 484 patients (68.4% of the study cohort).

Baseline patients and tumor characteristics

Table 1 describes the baseline patients' characteristics. Non-cirrhotic patients accounted for 12.6% of the included cohort (*n* = 89). Overall prevalence of diabetes mellitus (DBT) was 27.7% (Table 1).

Overall, HCV was the most frequent cause of underlying liver disease. Etiology of HCC in cirrhotic patients was as follows: 37% HCV infection (*n* = 262), 20.8% alcoholic liver disease (*n* = 147), 11.4% NAFLD (*n* = 81), 9.6% cryptogenic cirrhosis/liver disease (CC, *n* = 68), 5.4% HBV infection (*n* = 38), 2.2% of cholestatic disease and autoimmune hepatitis (*n* = 16), and 9.9% other causes (*n* = 60) (Table 2). Most frequent HCV genotypes (G) were G1b (*n* = 88), followed by G1a (*n* = 47), G3 (*n* = 27), G2 (*n* = 8) and G4 (*n* = 2) (Figure 1).

Etiology of HCC among non-cirrhotic patients included cryptogenic liver disease in 52.8% (*n* = 47), chronic HCV infection in 21.3% (*n* = 19), NAFLD in 10.1% (*n* = 9), chronic HBV infection in 9.0% (*n* = 8), altered iron metabolism in 4.5% (*n* = 4) and chronic alcohol consumption in 2 patients.

At HCC diagnosis, 4.2% (*n* = 30), 43.1% (*n* = 305), 21.3% (*n* = 151), 9.5% (*n* = 67) and 21.9% (*n* = 155) of the patients were within BCLC 0, A, B, C and D stages, respectively. Median serum AFP was 23.0 ng/mL (IQR 5.0; 337 ng/mL, Table 1).

Etiologies of HCC stratified by periods of time

Changes over time for each HCC etiology were analyzed in order to observe any epidemiological changes during the entire period. HCV related HCC was the most frequent etiology along the whole observation period. No significant changes were observed in the proportion of HCV-HCC (Table 2). The second most frequent cause of HCC was alcoholic liver disease, remaining stable during the observation period. On the other hand, a striking 6-fold increase in the proportion of HCC-NAFLD cases was observed since 2009 to 2016 (4.3%

Table 2 Underlying etiologies of liver disease per year (frequencies)

	HCV	HBV	Alcohol	NASH	CC	Cholestasis	AI	Other[1]	Total
2009	24 (34.3)	7 (10.0)	18 (25.7)	3 (4.3)	9 (12.9)	0	0	9 (12.9)	70
2010	51 (48.6)	5 (4.8)	16 (15.2)	5 (4.8)	12 (11.4)	3 (2.9)	1 (0.9)	12 (11.5)	105
2011	34 (35.6)	5 (5.3)	26 (27.4)	10 (10.5)	9 (9.5)	1 (1.0)	1 (1.0)	7 (6.4)	95
2012	43 (38.0)	5 (4.4)	21 (18.6)	14 (12.4)	10 (8.8)	3 (2.6)	0	5 (8.0)	113
2013	43 (33.1)	9 (6.9)	24 (18.5)	14 (10.8)	17 (13.1)	2 (1.5)	1 (0.8)	13 (10.0)	130
2014	43 (36.7)	4 (3.4)	22 (18.8)	15 (12.8)	9 (7.7)	3 (2.6)	0	16 (13.7)	117
2015	24 (30.8)	3 (3.8)	20 (25.6)	20 (25.6)	2 (2.6)	1 (1.3)	0	4 (5.2)	78
Total (%)	262 (37.0)	38 (5.4)	147 (20.8)	81 (11.4)	68 (9.6)	13 (1.8)	3 (0.4)	60 (9.9)	708

[1]Other causes of cirrhosis, Hemochromatosis. NASH: Non-alcoholic steatohepatitis; AI: Autoimmune; CC: Cryptogenic cirrhosis; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Table 3 Comparative analysis between non alcoholic fatty liver disease and other than non alcoholic fatty liver disease

Variable	NAFLD *n* = 81 (11.4%)	Non-NAFLD *n* = 627 (88.6%)	*P* value
Age, yr (± SD)	63 ± 8	62 ± 4	0.39
Gender, male, *n* (%)	68 (83.9)	469 (74.9)	0.06
Non-cirrhotic liver, *n* (%)	7 (8.6)	62 (9.9)	0.72
Child Pugh A/B/C, *n* (%)	41 (50.6)/32 (39.5)/8 (9.9)	311 (49.6)/206 (32.8)/110 (17.5)	0.15
Comorbidities, *n* (%)	60 (74.1)	239 (38.1)	< 0.0001
Diabetes mellitus, *n* (%)	50 (61.7)	146 (23.3)	< 0.0001
Median AFP level, ng/mL	6.6 (4-380)	26 (5.3-332)	0.017
AFP > 1000 ng/mL, *n* (%)	9 (12.0)	97 (16.1)	0.34
Ascites, *n* (%)			
Mild	17 (21.0)	127 (20.3)	0.7
Moderate-severe	10 (12.3)	99 (15.8)	0.7
Encephalopathy, *n* (%)			
Grade Ⅰ-Ⅱ	18 (22.2)	119 (19.0)	0.2
Grade Ⅲ-Ⅳ	3 (3.7)	7 (1.1)	0.2
Esophageal varices, *n* (%)	50 (63.3)	344 (55.8)	0.5
ECOG 0-2, *n* (%)	73 (90.1)	564 (89.9)	0.96
Median HCC number, (IQR)	1 (1-2)	1 (1-2)	0.38
Largest HCC diameter, mm (IQR)	55 ± 37	52 ± 37	0.51
Within Milan, *n* (%)	38 (46.9)	295 (47.0)	0.98
Bilobar involvement, *n* (%)	16 (19.7)	142 (22.7)	0.82
Diffuse HCC pattern, *n* (%)	3 (3.7)	20 (3.2)	0.82

AFP: Alpha-fetoprotein; MELD: Model for end stage liver disease; AFLD: Alcoholic fatty liver disease; Non-NAFLD: Other than NAFLD (includes all other etiologies).

vs 25.6%; $P < 0.0001$). Moreover, NAFLD represented the second cause of HCC in 2015 together with alcoholic liver disease (Table 2).

We further considered patients with cryptogenic cirrhosis and DBT ($n = 22/68$) as potentially NAFLD. This new group was merged with NAFLD accounting for 14.5% ($n = 103$) of the entire cohort. Its incidence increased from 8.6% in 2009 to 16.2% in 2014 and 25.6% in 2016, respectively ($P = 0.014$). Prevalence of DBT was similar between HCC cirrhotic and non-cirrhotic patients (23.2% *vs* 28.2%; $P = 0.38$).

Comparative analysis between NAFLD and non-NAFLD HCC patients

A comparative analysis between NAFLD and non-NAFLD-HCC was performed. Mean age was similar in both groups. Only a small proportion of patients were non-cirrhotic (8.6% NAFLD *vs* 9.9% non-NAFLD; $P = 0.72$). There were no significant differences regarding Child Pugh score, MELD score and presence of clinically significant portal hypertension (Table 3).

Comorbidities were most frequently observed in the NAFLD group, particularly there was a higher prevalence of diabetes mellitus (DBT) (61.7% *vs* non-NAFLD 23.3%; $P < 0.0001$). When a stratified analysis was performed comparing the prevalence of DBT, there were no changes observed ranging from 8.9% in 2009 to 17.3% in 2012 ($P = 0.89$). As previously shown, the increasing proportion of NAFLD in the overall cohort was not in parallel with an increasing prevalence of DBT.

Regarding HCC burden, there were no significant differences with previous surveillance (NAFLD 59.5% *vs* non-NAFLD 57.9%; $P = 0.81$) and BCLC staging at HCC diagnosis. Median AFP was lower in the NAFLD-HCC when compared to non-NAFLD group (6.6 ng/mL *vs* 26 ng/mL; $P = 0.02$) (Figure 2).

Etiology of HCC in non-liver transplant vs liver transplant centers

Among LT centers, the main etiology of HCC during 2009

Table 4 Baseline pre-treatment variables associated with 5-year mortality, univariate cox regression

Variable	5-yr mortality rate, (%)	Hazard ratio (95%CI)	*P* value
Age (yr)		1.02 (1.01; 1.04)	< 0.0001
Gender: male (*n* = 537)	42.7	1.08 (0.83; 1.42)	0.58
Female (*n* = 170)	42.3		
Comorbidity			
Yes (*n* = 299)	45.1	1.08 (0.86; 1.37)	0.49
No (*n* = 409)	40.6		
Diabetes mellitus			
Yes (*n* = 196)	38.8	0.83 (0.63; 1.08)	0.17
No (*n* = 512)	44		
NAFLD			
Yes (*n* = 81)	35.9	1.16 (0.81; 1.69)	0.41
No (*n* = 627)	56.9		
ECOG 0-2			
Yes (*n* = 637)	37.9	0.19 (0.14; 0.26)	0.0001
No (*n* = 71)	84.5		
BCLC 0-A			
Yes (*n* = 335)	26	0.29 (0.23; 0.38)	0.0001
No (*n* = 373)	57.4		
Cirrhosis			
Yes (*n* = 639)	42.9		
No (*n* = 69)	39.1	0.86 (0.58; 1.28)	0.45
Child Pugh			
A (*n* = 352)	34.5	-	
B (*n* = 238)	41.6	1.38 (1.06; 1.83)	0.019
C (*n* = 118)	68.6	3.23 (2.41; 4.34)	0.0001
Clinically significant portal hypertension			
Yes (*n* = 484)	40.2	1.22 (0.94; 1.57)	0.13
No (*n* = 224)	43.7		
AFP > 1000 ng/mL			
Yes (*n* = 106)	64.1	3.09 (2.31; 4.15)	0.0001
No (*n* = 569)	39.5		
Tumor vascular invasion			
Yes (*n* = 74)	77	4.74 (3.48; 6.44)	0.0001
No (*n* = 634)	38.5		
Extrahepatic tumor disease			
Yes (*n* = 48)	70.8	3.29 (2.25; 4.81)	0.0001
No (*n* = 660)	40.5		

Normal Values: Alpha-fetoprotein 0.6-4.4 ng/mL. AFP: Alpha-fetoprotein; BCLC: Barcelona clinic liver cancer; ECOG: Eastern cooperative oncology group; HCC: Hepatocellular carcinoma; LT: Liver transplantation; WL: Waiting list; NAFLD: Non-alcoholic fatty liver disease.

was HCV, remaining stable over the years, becoming the second cause of HCC in 2015. Furthermore, the main cause of HCC during 2015 was NAFLD, showing 6-fold increase from 2009 to 2014 and 2015 (5.8% *vs* 13.9% *vs* 36.9%, respectively; $P < 0.0001$). Alcoholic liver disease was the second overall cause of HCC, which was unchanged since 2009 among the different periods.

In non-transplant centers, HCV was the most frequent cause of HCC during all analyzed periods, followed by alcoholic liver disease. No significant changes in the proportion of patients with HCV or alcoholic liver disease were observed in this group. An increasing number of NAFLD-HCC cases were observed, which was lower than that observed in LT centers (LT centers 5.8% to 36.9%; $P < 0.0001$ *vs* non-LT centers 0% to 9%; P = NS).

Etiologies and impact on patient survival

Both uni and multivariate Cox regression analysis were performed as shown on Table 4 evaluating baseline patient and HCC characteristics associated with worse survival. Neither the presence of comorbidities (HR 1.08; CI 0.86; 1.37) nor DBT were related to mortality (HR 0.83; CI 0.63; 1.08). When considering different HCC etiologies, neither NAFLD nor viral etiologies presented higher mortality rates during the follow-up (Figure 3).

On the multivariate model, independent variables associated with death were age HR 1.03 (CI 1.01; 1.04), BCLC 0-A stages *vs* non 0-A stages HR 0.50 (CI 0.37; 0.68), Child Pugh B or C *vs* A HR 1.54 (CI 1.61; 2.05), HR 2.59 (CI 1.84; 3.66), respectively; AFP > 1000 ng/mL at HCC diagnosis HR 2.09 (CI: 1.52; 2.87) and HCC vascular invasion HR 2.83 (CI: 1.75; 3.53) (Table 5).

DISCUSSION

To the best of our knowledge this is the first multicenter cohort study from a South American country evaluating longitudinal changes in etiologies of HCC. This changing etiologic scenario documented in developed countries is replicated in developing ones[4,5]. The documented increase in NAFLD-HCC in our study entails itself a

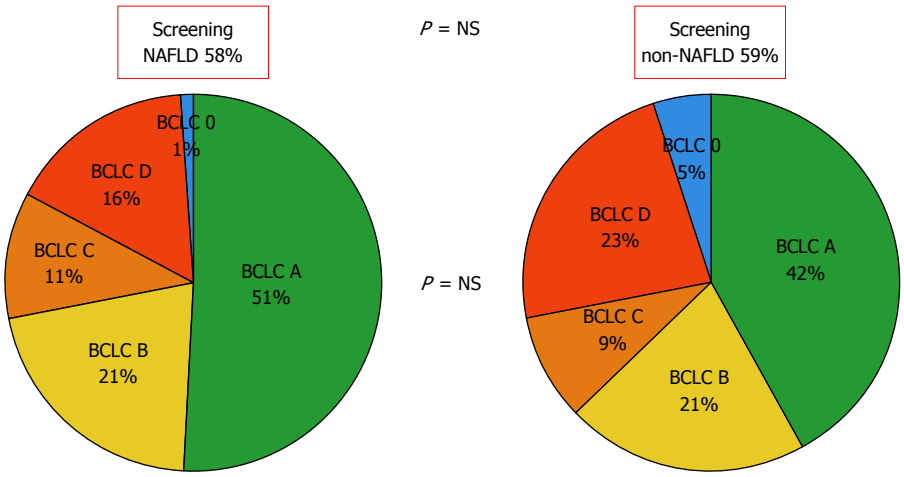

Figure 2 Comparative analysis regarding hepatocellular carcinoma previous surveillance and Barcelona Clinic Liver Cancer staging at diagnosis between non alcoholic fatty liver disease and other etiologies of liver disease. NAFLD: Non-alcoholic fatty liver disease: Non-NAFLD: Other than NAFLD (includes all other etiologies).

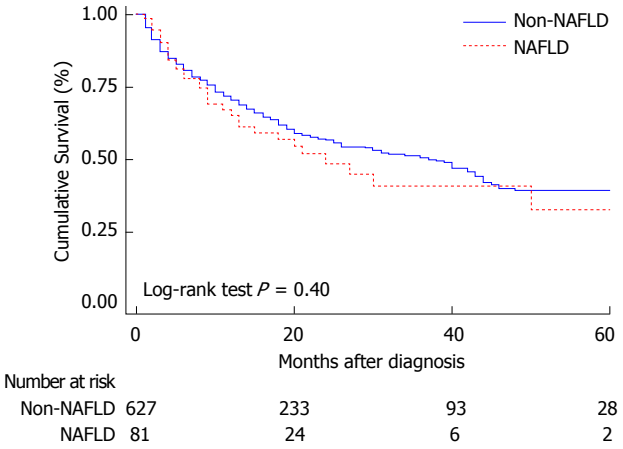

Figure 3 Comparative survival between non alcoholic fatty liver disease and other etiologies of liver disease. NAFLD: Non-alcoholic fatty liver disease; Non-NAFLD: Other than NAFLD (includes all other etiologies).

challenging impact on Public Health worth to be considered in developing countries. HCV infection was the main etiology of HCC throughout the entire observation period, including the last two years, at which time DAA's therapy was available in Argentina (since 2014). Second, as reported in other regions of the world, fatty liver disease was observed as an increasing cause of HCC. The prevalence of DBT was significantly higher in NAFLD-HCC when compared to non-NAFLD patients. Finally, NAFLD-HCC was not associated with a higher mortality rate.

In a recently published multicenter Latin American retrospective cohort study, the main etiologies of HCC were HCV in almost half of the cases, followed by alcoholic liver disease, HBV infection and NAFLD in nine percent[8]. Another multicenter study from this region including HCC LT patients showed that the most frequent cause of HCC was HBV[9]. This discrepancies show that the reported HCC etiology largely reflects country-to-country epidemiological differences. Whereas in Brazil the main etiology has been shown to be HBV, in Argentina the main HCC etiology has been associated with HCV followed by alcoholic liver disease[9,10,21]. However, no previous studies have evaluated etiologic changes longitudinally.

It is already known that in developed countries, a high-fat diet leads to obesity, insulin resistance, metabolic syndrome and type 2 DBT. Unbalanced hypercaloric diets, as well as an increasing consumption of sugar containing beverages might lead to a profound Public Health intervention. These socioeconomically changes have been occurring not only in developed but also in developing regions worldwide.

NAFLD has been related to an increasing rate of overall cardiovascular morbid-mortality[6]. Consequently, NAFLD will become responsible of an increase in medical resource use in the next years that demands specific health prevention programs focusing on diet and exercise in order to avoid not only liver but also cardiovascular disease development. As previously mentioned, a six-fold increase in NAFLD from 2009 to 2015 was observed in our cohort, mainly in LT centers whereas alcoholic liver disease was a leading cause of HCC in non-LT centers. This finding has been observed in Argentina by other authors[10].

DBT leads to fibrosis progression, cirrhosis and

Table 5 Multivariate cox regression analysis of risk factors associated with 5-year mortality

Variable	HR	95%CI	*P* value
Age	1.03	1.01; 1.04	< 0.0001
BCLC 0-A	0.50	0.37; 0.68	< 0.0001
Child Pugh B[1]	1.54	1.61; 2.05	0.003
Child Pugh C[1]	2.59	1.84; 3.66	< 0.0001
AFP > 1000 ng/mL	2.09	1.52; 2.87	< 0.0001
Tumor vascular invasion	2.84	1.75; 3.53	< 0.0001

[1]Compared to Child Pugh A, Harrell's concordance statistic was 0.76.

HCC[6,22,23]. We observed that the increasing prevalence of NAFLD-HCC was not in parallel with the increasing prevalence of DBT over the years. When we included as "potentially NAFLD" those patients with cryptogenic cirrhosis with DBT, a similar 6-fold increase since 2009 to 2015 was observed. Alternatively, we did not considered obese patients with cryptogenic cirrhosis as probable NAFLD as reported by other authors[24]. In our series, body mass index was not recorded. However, we performed a sub analysis considering cryptogenic/DBT patients and NAFLD. Our epidemiological results are in line with that from developed countries, suggesting that NAFLD would have a major Public Health implication in the upcoming years.

Third, from a comparative and specific intergroup variability, there were no differences between non-NAFLD and NAFLD-HCC patients, except from a higher prevalence of diabetes mellitus and lower AFP values in NAFLD-HCC group. Interestingly, higher AFP values were described in other studies in this group of patients in comparison to other etiologies[22]. However, this significant difference did not impact on patient survival, although AFP > 1000 ng/mL at HCC diagnosis was an independent predictor of worse survival in the multivariate Cox regression analysis. There were no major differences in HCC tumor burden between NAFLD and non-NAFLD-HCC. Heterogeneous data has been published regarding this topic. Some studies described tumor differences whereas others did not[25,26].

We acknowledge limitations of cohort studies with no control group, in which several factors might be biased. However, a strict revision of the data was centrally requested, investigators who performed the final analysis did not participate in the data collection to avoid differential outcome assessment on exposure and a complete follow-up and outcome assessment was performed in all patients included. Second, the definition of NAFLD was mainly based on clinical assessment. The lack of histological evaluation of NAFLD and NASH in most of the patients might have biased the results. However, this clinical definition is accepted worldwide by international guidelines[17]. Third, an important information or selection bias regarding the lack of body mass index has been mentioned earlier regarding cryptogenic/obese patients. This might have resulted in a lower number of patients in the group of NAFLD reported in our study. Finally, combination of different etiologies (*e.g.*, alcoholic liver disease plus chronic HCV) was not considered in order to include the main factor of chronic liver disease.

In conclusion, NAFLD related HCC has been recognized as a growing burden in the United States and in some European regions. This changing etiologic scenario has been observed in in high-income countries and might even be happening in developing ones. In Argentina, even though HCV infection is still the main cause of HCC, recent changing trends in etiologies of HCC in Argentina suggests that NAFLD might be the leading cause of HCC in the next years, becoming an important Public Health issue. However, prospective studies will be necessary to confirm our findings.

ARTICLE HIGHLIGHTS

Research background

The incidence of Hepatocellular carcinoma (HCC) has been increasing during the last years and is the second leading cause of cancer related death worldwide. The cause of HCC is closely linked to the prevalence of chronic hepatitis C (HCV) or B virus (HBV) infections as well as the prevalence of alcoholic liver disease. With the advent of the new direct antiviral drugs (DAAs) for hepatitis C (HCV) treatment, epidemiological changes have been already reported in the natural history of liver disease. One of these epidemiological changes in developed countries is due to the stepwise increase in the incidence of non-alcoholic fatty liver disease (NAFLD), an emergent cause of end stage liver disease and HCC.

Research motivation

Indeed, it is estimated that during the next 20 years, non-alcoholic steatohepatitis (NASH), the progressive form of NAFLD, will constitute the most frequent cause of cirrhosis and HCC in developed countries. However, reports regarding epidemiological changes of HCC from developing areas, including South America, have been heterogeneous and none have focused on changing trends in etiologies of HCC over time.

Research objectives

Our aim was to evaluate changes in the etiology of HCC in Argentina during the last seven years, particularly focusing on potential changes associated with NAFLD-HCC.

Research methods

This cohort study was conducted between January 1 2009 and January 1 2016 in 14 regional hospitals from Argentina. Criteria for inclusion required patients with newly diagnosed HCC as recommended by international guidelines. Etiologies of HCC included viral hepatitis, alcoholic liver disease (alcohol intake exceeding 30 g/d), NAFLD, cryptogenic cirrhosis (CC), cholestatic liver diseases (*i.e.*, primary biliary cholangitis, primary and secondary sclerosing cholangitis), autoimmune hepatitis and other causes including metabolic diseases or miscellaneous causes (*e.g.*, hereditary hemochromatosis, Wilson disease, toxic liver disease). NAFLD diagnosis was established on histological ground or clinically according to international guidelines. Baseline patient and tumor characteristics at HCC diagnosis were recorded. Tumor burden was classified according to Barcelona Clinic Liver Cancer staging (BCLC criteria). Complete patient follow-up was assessed in all included subjects from HCC diagnosis until death or last medical visit.

Categorical data were compared using Fisher's exact test (2-tailed) or Chi-Square test and continuous variables were compared with Student's *t*-test or Mann-Whitney *U* test according to their distribution, respectively. For survival analysis, Cox regression multivariate analysis estimating hazard ratios (HR) and 95%CI for baseline variables related with 5-year mortality was performed. Kaplan Meier survival curves were compared using the log-rank test.

Research results

A total of 708 consecutive adult patients with newly diagnosed HCC from 14 centers were included. Out of 14 hospitals, 6 were LT centers, which contributed with the follow-up of 484 patients (68.4% of the study cohort). Non-cirrhotic patients accounted for 12.6% of the included cohort (n = 89). The prevalence of diabetes mellitus (DBT) was 27.7%. Between 2009 and 2016, HCV related HCC was the most frequent etiology along the whole observation period. No significant changes were observed in the proportion of HCV-HCC. The second most frequent cause of HCC was alcoholic liver disease, remaining stable along the observation period. On the other hand, a striking 6-fold increase in the proportion of HCC-NAFLD cases was observed since 2009 to 2016 (4.3% *vs* 25.6%; P < 0.0001). NAFLD was the second cause of HCC in 2015 together with alcoholic liver disease. In addition, when patients with cryptogenic cirrhosis/liver disease and DBT (n = 22/68) were considered together as potentially metabolic syndrome/NAFLD, the NAFLD/Cryptogenic + DBT group represented 14.5% (n = 103) of the entire cohort and increased from 8.6% in 2009 to 16.2% in 2014 and 25.6% in 2016, respectively (P = 0.014). There was a higher prevalence of diabetes mellitus (DBT) (61.7% *vs* non-NAFLD 23.3%; P < 0.0001) in the NAFLD group. The increasing proportion of NAFLD in the overall cohort was not in parallel with an increase of DBT in this population. On the other hand, lower median AFP values at HCC diagnosis were observed between NAFLD-HCC and non-NAFLD groups (6.6 ng/mL *vs* 26 ng/mL; P = 0.02). Neither NAFLD nor other HCC etiologies were associated with higher mortality.

Research conclusions

To the best of our knowledge this is the first multicenter cohort study from a South American country evaluating longitudinal changes in etiologic trends. This changing etiologic scenario documented in developed countries is replicated in developing ones. The documented increase in NAFLD-HCC in our study including a developing country entails in itself a challenging impact on Public Health worth to be considered. HCV infection was the main etiology of HCC throughout the entire observation period, including the last two years, at which time DAA's therapy was available in Argentina. Second, as reported in other regions of the world, fatty liver disease was observed as an increasing cause of HCC. The prevalence of DBT was significantly higher in NAFLD-HCC when compared to non-NAFLD patients. Finally, NAFLD-HCC was not associated with an increasing risk of mortality, adjusted for the presence HCC surveillance and BCLC stage. NAFLD has been related to an increasing rate of overall cardiovascular morbid-mortality. Consequently, NAFLD will become responsible of an increase in medical resource use in the next years that demands specific health prevention programs focusing in diet and exercise in order to avoid not only liver but also cardiovascular disease development. As previously mentioned, a six-fold increase in NAFLD from 2009 to 2015 was observed in our cohort, mainly in LT centers whereas alcoholic liver disease was a leading cause of HCC in non-LT centers.

Research perspectives

NAFLD related HCC has been recognized as a growing burden in the United States and some European regions. This etiologic scenario is not only changing in high-income countries but also it might be happening in developing ones. In Argentina, even though HCV infection is still the main cause of HCC, recent changing trends in etiology of HCC in Argentina suggests that NAFLD might be the leading HCC cause in the next years, becoming an important Public Health issue.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Current state and clinical outcome in Turkish patients with hepatocellular carcinoma

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Informed consent statement: This study is a retrospective cohort study, therefore informed consent to be included in the study is not required according to the regulations of Republic of Turkey. All the treatments were established and indicated treatment modalities, and no experimental tool or medication were used in the study. Nevertheless, all patients were needed to give written permission, before undergoing specific treatments including TACE, TARE, liver transplantation, and surgical resection. In addition, according to the regulations of Istanbul University, at admission all patients give informed consent for their medical information can be used for research purposes anonymously.

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Abstract

AIM

To investigate clinical, etiological, and prognostic features in patients with hepatocellular carcinoma.

METHODS

Patients with hepatocellular carcinoma who were followed-up from 2001 to 2011 were included in the study. The diagnosis was established by histopathological and/or radiological criteria. We retrospectively reviewed clinical and laboratory data, etiology of primary liver disease, imaging characteristics and treatments. Child-Pugh and Barcelona Clinic Liver Cancer stage was determined at initial diagnosis. Kaplan-Meier survival analysis was done to find out treatment effect on survival. Risk factors for vascular invasion and overall survival were investigated by multivariate Cox regression analyses.

RESULTS

Five hundred and forty-five patients with hepatocellular carcinoma were included in the study. Viral hepatitis was prevalent and 68 patients either had normal liver or were non-cirrhotic. Overall median survival was 16 (13-19) mo. Presence of extrahepatic metastasis was associated with larger tumor size (OR = 3.19, 95%CI: 1.14-10.6). Independent predictor variables of vascular invasion were AFP (OR = 2.95, 95%CI: 1.38-6.31), total tumor diameter (OR = 3.14, 95%CI: 1.01-9.77), and hepatitis B infection (OR = 5.37, 95%CI: 1.23-23.39). Liver functional reserve, tumor size/extension, AFP level and primary treatment modality were independent predictors of overall survival. Transarterial chemoembolization (HR = 0.38, 95%CI: 0.28-0.51) and radioembolization (HR = 0.36, 95%CI: 0.18-0.74) provided a comparable survival benefit in the real life setting. Surgical treatments as resection and transplantation were found to be associated with the best survival compared with loco-regional treatments (log-rank, $P < 0.001$).

CONCLUSION

Baseline liver function, oncologic features including AFP level and primary treatment modality determines overall survival in patients with hepatocellular carcinoma.

Key words: Hepatocellular carcinoma; Cirrhosis; Alfa-fetoprotein; Prognosis; Treatment; Survival

Core tip: Hepatocellular carcinoma is a leading cause of cancer-related death with curative treatment options limited to orthotopic liver transplantation, surgical resection and local ablation. Our study confirmed that liver functional reserve, tumor extension and alfa-fetoprotein level are among the most important determinants of patient survival. Survival benefit of non-curative treatments including transarterial chemoembolization and Yttrium-90 radioembolization remains an area of uncertainty. In this study we showed that transarterial chemoembolization and Yttrium-90 radioembolization provided a significant and comparable survival benefit in patients with hepatocellular carcinoma in the real-life setting. We concluded that primary modality of treatment for hepatocellular carcinoma is a major determinant of patient survival that should be incorporated while estimating prognosis.

Ekinci O, Baran B, Ormeci AC, Soyer OM, Gokturk S, Evirgen S, Poyanli A, Gulluoglu M, Akyuz F, Karaca C, Demir K, Besisik F, Kaymakoglu S. Current state and clinical outcome in Turkish patients with hepatocellular carcinoma. *World J Hepatol* 2018; 10(1): 51-61 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/51.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.51

INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most frequently diagnosed cancers worldwide, and it comprises 70%-85% of all primary liver malignancies[1]. HCC is a leading cause of cancer-related death in the world, which is estimated to be more than 600000 deaths per year[2]. A unique characteristic of HCC is that most patients have liver cirrhosis at the time of diagnosis. Even in the absence of liver cirrhosis, HCC almost always develops within the spectrum of a chronic liver disease[3]. A variety of important risk factors for the development of HCC have been identified including but not limited to hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, hereditary hemochromatosis, and cirrhosis of almost any cause[3]. Etiological risk factors associated with the development of HCC are also important due to their relationship with their implications for treatment and prognosis of the disease. In addition to these etiological factors, tumor related factors including histological grade, size and number of nodules, and patient related factors as age, severity of underlying liver cirrhosis and performance status of the patient play a crucial role in determining the outcome of the disease[4]. Therefore, several prognostic scoring systems were developed to predict the prognosis for patients with HCC, and to individualize treatment by matching best therapeutic option with the patient who is most likely to benefit. Barcelona Clinic Liver Cancer (BCLC) classification

which is the most widely used system, comprises four stages that are based on the number and size of nodules, vascular invasion and extrahepatic metastasis, Child-Pugh score (CPS) and performance status of the patient[5]. BCLC system also provides a treatment algorithm to be applied for each stage in patients with HCC. Nevertheless, there are still many problems in determining disease prognosis and selecting patients for appropriate treatment. No classification is completely satisfactory as a result of many other risk factors, including tumor histology, serum alfa-fetoprotein (AFP) level, presence of variant estrogen receptors and diabetes mellitus, which also influence patient survival. Besides, primary treatment modality, which is among the most important determinants of patient outcome, has not been evaluated as a prognostic indicator in relation to other determinants of survival.

Despite the advances in screening, diagnosis and treatment of HCC, a substantial amount of patients are diagnosed at a later stage of the disease which may preclude curative treatment options. Therefore, novel strategies to facilitate early diagnosis and a better estimation of prognosis are needed to improve patient survival. In the present study, we investigated clinical, etiological, and prognostic features in our large, single center cohort of patients with HCC who were diagnosed, treated and followed-up in the last decade. Primary objective of the study was to define potential factors that have influence on prognosis, specifically to determine survival benefit associated with primary treatment modality of HCC in a real life setting. Secondary objective of the study was to find out the relationship between pre-diagnosis screening characteristics, clinical stage of the disease at diagnosis and overall survival.

MATERIALS AND METHODS

Patients

Patients with HCC who were followed-up in the Department of Gastroenterohepatology, Istanbul Faculty of Medicine, Istanbul University between January 2001 and August 2011 were included in this single center, retrospective cohort study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and it was approved by the ethics committee of Istanbul Faculty of Medicine, Istanbul University. The diagnosis of HCC was based on the recommendations reported by the EASL panel of experts in 2001[6]. According to these recommendations; the diagnosis was established by histopathological and/or radiological criteria. CPS was calculated at baseline in cirrhotic patients as previously described[7]. BCLC stage was determined in every patient with HCC at initial diagnosis according to the extent of tumor, performance status, CPS, vascular invasion and extrahepatic spread[5]. We reviewed demographic, clinical and staging characteristics, laboratory data, etiology of primary liver disease, imaging characteristics and treatments of HCC patients. Number and size of nodules, total tumor diameter (TTD), type of tumor (single nodule, multinodular or diffuse-infiltrative), presence of major vascular involvement and extrahepatic metastasis were determined according to baseline imaging records. Survival data of all patients were updated as of November 2011.

Diagnostic evaluation

All patients with a suspicion of primary liver cancer were evaluated by clinical, laboratory and imaging studies including a 4-phase computerized tomography (CT) scan or dynamic contrast enhanced magnetic resonance imaging (MRI). The diagnosis was made if there were radiologic hallmarks of HCC as arterial hypervascularity and venous/late phase wash out. In the absence of radiologic hallmarks of HCC or if findings were inconsistent on contrast enhanced CT or MRI, a biopsy was obtained and assessed by an expert hepatopathologist. Extrahepatic metastasis was screened by a contrast-enhanced chest CT and whole-body bone scan.

All patients with a diagnosis of HCC had baseline physical examination, and results of standard laboratory investigations including complete blood count, renal and liver function tests, screening tests for hepatitis viruses (hepatitis B surface antigen-HBsAg, hepatitis B core antibody-anti-HBc total and hepatitis C antibody-anti-HCV) and AFP. If HBsAg or anti-HCV was detected to be positive further tests [HBeAg, anti-HBe, HBVDNA (PCR), and anti-Delta total (plus HDVRNA when positive) or HCVRNA, respectively] were obtained. Most patients with HCC already had a diagnosis of a chronic liver disease, and remaining patients with unrecognized liver disease had undergone detailed evaluations to assess the presence of other etiologies including alcoholic liver disease, hemochromatosis, Budd-Chiari syndrome, non-alcoholic fatty liver disease, and autoimmune liver diseases. An upper gastrointestinal endoscopy was performed to evaluate the presence of esophageal or gastric varices in each patient.

Treatments

Treatment of HCC was guided by BCLC classification, however most patients were listed for OLT using the expanded criteria after 2001 as previously described[8]. Treatment options included surgical resection, OLT, percutaneous ablation (RFA or ethanol/acetic acid ablation), TACE, Yttrium-90 radioembolization and systemic therapy using sorafenib. Curative partial hepatectomy was performed in patients with tumors confined to one lobe of the liver that shows no radiographic evidence of invasion of the hepatic vasculature, no evidence of portal hypertension and adequate liver functional reserve. All candidates for surgical resection underwent indocyanine green test

Table 1 Treatment modalities and the stage of the disease in patients with hepatocellular carcinoma

Treatment modality	Pts within expanded criteria, *n* (%)	Pts within Milan criteria, *n* (%)	Total, *n*	BCLC stage				Survival, *n* (%)
				0-A	B	C	D	
Surgical resection	24 (89)	19 (70)	27	18	9	0	0	16 (59)
OLT	47 (84)	41 (73)	56	24	13	7	12	56 (100)
Percutaneous ablation	16 (94)	15 (88)	17	11	1	3	2	10 (59)
TACE	118 (69)	99 (58)	172	81	47	34	10	80 (46)
Yttrium-90	11 (58)	10 (53)	19	7	5	7	0	10 (53)
Sorafenib	3 (19)	2 (13)	16	1	2	10	3	8 (50)
No treatment	88 (37)	61 (26)	238	24	28	54	132	39 (16)

HCC: Hepatocellular carcinoma; Pts: Patients; BCLC: Barcelona Clinic Liver Cancer; OLT: Orthotopic liver transplantation; TACE: Transarterial chemoembolization.

to determine operative risk before hepatectomy. Percutaneous ablation was selected in patients who did not meet resectability criteria and had a single tumor ⩽ 3 cm in diameter. Patients without a suitable living-donor were listed for OLT. Listed patients who had an anticipated time to OLT more than 6 mo underwent percutaneous ablation, TACE or Yttrium-90 radioembolization decided by physician's discretion according to tumor characteristics and hepatic reserve. Patients with advanced stage HCC who were not candidates for curative treatments underwent TACE, Yttrium-90 radioembolization or sorafenib therapy. In terminal stage, patients were followed-up under natural course with best supportive care.

Twenty-seven patients were suitable for hepatic resection and underwent surgery. Twelve patients who had 1 or 2 small (⩽ 3 cm) nodules underwent percutaneous ablation with RFA, and 5 patients with a single nodule ⩽ 2 cm underwent percutaneous acetic acid/ethanol injection. Two-hundred and sixty-seven patients who were ineligible for surgical resection or percutaneous ablation, but had tumor characteristics that are compatible with expanded criteria were listed for OLT. A total of 56 patients (47 within expanded criteria) with HCC underwent OLT during the follow-up period, due to the shortage of cadaveric organs or unavailability of a suitable living donor. The number of patients who underwent TACE was 172. Among them 90 patients underwent 1 session, 53 patients underwent 2 sessions, and 29 patients underwent ⩾ 3 sessions of TACE. The distribution of treatment modalities in the remaining patients were as follows: 19 patients underwent Yttrium-90 radioembolization, 16 patients received systemic therapy with sorafenib, and 238 patients received no treatment until the end of the follow-up. Treatment characteristics of patients with HCC were summarized in Table 1.

Statistical analysis

Continuous variables are presented as mean ± standard deviation (SD) or median (range) while categorical variables were expressed as frequencies (%). Differences between frequencies were evaluated using Pearson χ^2 or Fisher's exact test when necessary. Predictor variables of vascular invasion and extrahepatic metastasis were investigated by univariate and multivariate logistic regression analyses. Univariate Cox regression analyses were performed to find out factors associated with overall survival of patients with HCC. Variables which showed a significant influence ($P < 0.05$) were included in a multivariate Cox proportional hazard model to find out independent prognostic factors that affect overall survival. The results of the model were presented as a hazard ratio (HR) with 95% confidence interval (CI). We performed Kaplan-Meier analyses to determine cumulative survival probabilities and treatment effect on overall survival. Log-rank test was used for the statistical comparison of Kaplan-Meier curves. All statistical analyses were performed using IBM SPSS v20 (IBM SPSS Inc., Chicago, IL, United States). A two tailed *P*-value < 0.05 was considered statistically significant.

RESULTS

Patients

A total of 545 patients (449 male, mean age 59.5 ± 10) with HCC who were diagnosed and followed-up between January 2001 and August 2011 were included in the study. The diagnosis of HCC was established by CT or MRI in 459 patients, and the remaining patients underwent liver biopsy due to inconsistent findings in radiological examinations. The number of patients with underlying chronic liver disease was 532, 13 (2.3%) patients had normal liver without any identifiable risk factor for the development of HCC. 350 (66%) of patients with chronic liver disease were already aware of their underlying liver disease at the time of the diagnosis of HCC. However, only 110 patients (31.4%) were under regular follow-up with a combined use of scheduled liver ultrasonography and AFP measurement. The mean estimated duration of chronic liver disease was 69 ± 60 mo (range, 3-420 mo).

The number patients according to underlying etiology for chronic liver disease were as follows: 287 patients with chronic HBV, 120 patients with chronic HCV, 37 patients with chronic delta hepatitis, 10 patients with co-infection of HBV and HCV, 39 patients

Table 2 Baseline demographic, clinical and laboratory characteristics of patients

Characteristics	
Number of patients (%)	545 (100)
Male, *n* (%)	449 (82)
Female, *n* (%)	96 (18)
Age (yr)	
Mean ± SD	59.5 ± 10
Median (range)	60 (19-85)
Chronic liver disease, *n* (%)	532 (97.6)
Chronic viral hepatitis, *n* (%)	454 (83.3)
HBV (monoinfection)	287 (52.6)
HCV (monoinfection)	120 (22)
Hepatitis D	37 (6.7)
HBV + HCV co-infection	10 (1.8)
Cirrhosis, *n* (%)	477 (87.5)
Child A	247 (45.3)
Child B	140 (25.7)
Child C	90 (16.5)
Diagnostic method for HCC, *n* (%)	
CT	26 (4.8)
MRI	433 (79.4)
Liver biopsy	86 (15.8)
Treatment, *n* (%)	
No treatment	238 (43.7)
Hepatic resection	27 (5)
OLT	56 (10.3)
TACE	172 (31.5)
Yttrium-90 radioembolization	19 (3.5)
RFA	12 (2.2)
Ethanol/acetic acid ablation	5 (0.9)
Sorafenib	16 (2.9)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; CT: Computerized tomography; MRI: Magnetic resonance imaging; OLT: Orthotopic liver transplantation; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation.

with cryptogenic liver disease, 21 patients with alcoholic liver disease, 10 patients with non-alcoholic fatty liver disease, 5 patients with autoimmune liver disease, 2 patients with Budd-Chiari syndrome and 1 patient with hemochromatosis. Among patients with chronic viral hepatitis, there were 24 patients who had significant alcohol consumption (> 210 g/wk) which may contribute to the severity of underlying liver disease. The majority of patients with chronic liver disease had cirrhosis at different stages (CPS A, 247 patients; CPS B, 140 patients; CPS C, 90 patients), and 68 patients (12.5%) were pre-cirrhotic based on clinical and/or histopathological examinations. Baseline patient characteristics are summarized in Table 2.

Tumor characteristics and staging

The number of patients with a single tumor was 333 (61%), and the remaining patients had multinodular (191 patients, 35%) or diffuse HCC (21 patients, 3.9%). Median AFP level was 62 ng/mL (range, 1-223169 ng/mL), and 241 (44.2%) patients had an AFP level > 100 ng/mL. At the time of the diagnosis, the number of patients within Milan and expanded criteria were 247 (45%) and 307 (56%), respectively. The distribution of patients according to BCLC classification was as follows: BCLC 0, 14 patients (2.6%); BCLC A, 152 patients (27.9%); BCLC B, 105 patients (19.2%); BCLC C, 115 patients (21.1%); BCLC D, 159 patients (29.2%). Extrahepatic metastasis and macroscopic vascular invasion were diagnosed in 26 (4.8%) and 37 (6.8%) patients, respectively. The only predictor variable for the presence of extrahepatic metastasis at initial diagnosis was TTD (TTD ⩾ 5 cm; OR = 3.19, 95%CI: 1.14-10.6, P = 0.029). Stage of liver disease, tumor type, HBV infection, number of nodules, presence of vascular invasion and AFP level did not predict extrahepatic metastasis (Table 3). Univariate logistic regression analyses showed that HBV infection, multinodular and diffuse-infiltrative HCC, TTD, and AFP level were associated with vascular invasion at initial diagnosis of HCC. At multivariate analysis, independent predictor variables of vascular invasion were found to be AFP > 200 ng/mL (OR = 2.95, 95%CI: 1.38-6.31, P = 0.005), TTD > 5 cm (OR = 3.14, 95%CI: 1.01-9.77, P = 0.047) and HBV infection (OR = 5.37, 95%CI: 1.23-23.39, P = 0.025) (Table 4).

Table 3 Univariate logistic regression analyses of possible predictors of extrahepatic metastasis (n = 545)

	Univariate		
	OR	95%CI	*P* value
Total tumor diameter			
⩾ 5 cm	3.19	1.18-8.59	0.022
Tumor type			
Solitary HCC	Reference	-	-
Multinodular HCC	1.17	0.52-2.66	0.71
Diffuse-infiltrative HCC	1.06	0.13-8.43	0.96
Vascular invasion	1.86	0.53-6.51	0.33
HBV infection	1.76	0.73-4.26	0.21
Number of nodules	1.21	0.92-1.59	0.18
Stage of liver disease			
Normal or precirrhotic liver	Reference	-	-
Child-Pugh A	2.29	0.51-10.2	0.28
Child-Pugh B	1.22	0.23-6.47	0.81
Child-Pugh C	1.14	0.19-7.01	0.89
AFP level			
> 100 ng/mL	1.30	0.59-2.85	0.52
> 200 ng/mL	1.59	0.72-3.50	0.25
> 400 ng/mL	1.48	0.64-3.39	0.36
> 1000 ng/mL	1.91	0.81-4.52	0.14

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; AFP: Alfa-fetoprotein.

Factors associated with overall survival

Cumulative overall median survival was 16 (13-19) mo in the whole cohort (Figure 1A). The best survival outcome was achieved in patients with HCC who underwent surgical treatments as OLT and hepatic resection (HR = 0.07, 95%CI: 0.04-013, P < 0.001, Figure 1B). Treatment modalities including TACE, Yttrium-90 radioembolization, RFA were also found to be associated with improved overall survival (Table 5). Sorafenib therapy demonstrated a survival benefit, yet with a borderline significance. Univariate Cox

Table 4 Univariate and multivariate logistic regression analysis of factors associated with vascular invasion (n = 545, R^2 = 0.15)

Variables	Univariate			Multivariate		
	OR	95%CI	*P* value	OR	95%CI	*P* value
TTD ⩾ 5 cm	6.56	2.29-18.79	< 0.001	3.14	1.01-9.77	0.047
Tumor type						
Solitary HCC	Reference	-	-	Reference	-	-
Multinodular HCC	2.07	1.01-4.25	0.047	1.54	0.72-3.30	0.270
Diffuse-infiltrative HCC	6.63	2.14-20.51	0.001	2.71	0.82-8.94	0.100
Extrahepatic metastasis	1.86	0.53-6.51	0.330			
Etiology of liver disease						
Hepatitis C	Reference	-	-	Reference	-	-
Hepatitis B	6.29	1.48-26.76	0.013	5.37	1.23-23.39	0.025
Other (non-viral)	4.52	0.89-22.91	0.069	3.76	0.72-19.74	0.120
Stage of liver disease						
Normal or precirrhotic liver	Reference	-	-			
Child-Pugh A	0.60	0.24-1.53	0.290			
Child-Pugh B	0.53	0.18-1.52	0.240			
Child-Pugh C	0.62	0.20-1.94	0.420			
AFP level						
> 100 ng/mL	3.77	1.79-7.96	< 0.001			
> 200 ng/mL	4.18	2.05-8.52	< 0.001	2.95	1.38-6.31	0.005
> 400 ng/mL	2.81	1.43-5.52	0.003			
> 1000 ng/mL	3.54	1.78-7.05	< 0.001			

TTD: Total tumor diameter; HCC: Hepatocellular carcinoma; AFP: Alfa-fetoprotein.

Table 5 Univariate and multivariate Cox regression analyses of factors associated with overall survival (n = 545)

	Univariate			Multivariate		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Patient related factors						
Age	0.99	0.98-1.01	0.360			
Gender	0.82	0.60-1.10	0.180			
Etiology of liver disease						
Hepatitis C	Reference	-	-	Reference	-	-
Hepatitis B	1.28	0.98-1.66	0.074	0.98	0.74-1.30	0.900
Other (non-viral)	1.41	1.002-1.99	0.049	1.12	0.78-1.60	0.540
Stage of liver disease						
Normal or precirrhotic liver	Reference	-	-	Reference	-	-
Child-Pugh A	1.52	0.99-2.30	0.051	1.29	0.84-1.99	0.250
Child-Pugh B	3.16	2.04-4.88	< 0.001	1.81	1.13-2.89	0.013
Child-Pugh C	10.46	6.57-16.66	< 0.001	5.35	3.24-8.83	< 0.001
Tumor related factors						
Total tumor diameter ⩾ 5 cm	3.07	2.40-3.93	< 0.001	1.74	1.30-2.33	< 0.001
Multinodular or diffuse-infiltrative	2.02	1.61-2.53	< 0.001	1.23	0.95-1.59	0.120
Extrahepatic metastasis	2.53	1.63-3.93	< 0.001	2.17	1.37-3.45	0.001
Vascular invasion	3.48	2.42-5.01	< 0.001	2.74	1.84-4.06	< 0.001
AFP level > 200 ng/mL	2.59	2.07-3.23	< 0.001	2.19	1.72-2.80	< 0.001
Treatment modalities *vs* no treatment						
Surgical treatments (OLT, hepatic resection)	0.07	0.04-0.13	< 0.001	0.12	0.06-0.24	< 0.001
TACE	0.24	0.19-0.31	< 0.001	0.38	0.28-0.51	< 0.001
Yttrium-90 adioembolization	0.37	0.19-0.72	0.003	0.36	0.18-0.74	0.005
RFA	0.12	0.04-0.38	< 0.001	0.18	0.05-0.57	0.004
Ethanol/acetic acid ablation	0.60	0.22-1.63	0.318	0.79	0.28-2.22	0.660
Sorafenib	0.51	0.25-1.04	0.063	0.52	0.25-1.10	0.088

AFP: Alfa-fetoprotein; OLT: Orthotopic liver transplantation; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation.

regression analyses showed that tumor related factors associated with overall survival of patients with HCC were TTD, tumor-type, vascular invasion, extrahepatic metastasis, and AFP level (Table 5). Patient-related factors including age and gender did not have significant influence on overall survival, yet stage of liver disease significantly predicted overall survival. HBV (HR = 1.28, 95%CI: 0.98-1.66, P = 0.074) and non-viral etiologies (HR = 1.41, 95%CI: 1.002-1.989, P = 0.049) were found to have a borderline influence on patient survival compared to HCV. In multivariate Cox proportional hazard model, stage of liver disease,

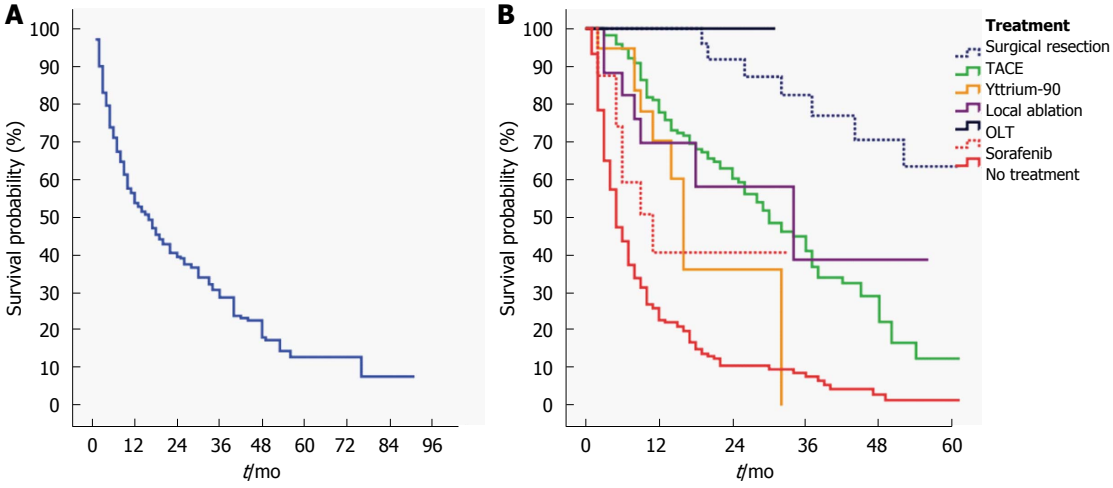

Figure 1 Overall survival in patients with hepatocellular carcinoma. A: Overall median survival in the whole cohort; B: Survival curves was stratified by primary treatment modality.

tumor-related factors including TTD, vascular invasion, extrahepatic metastasis, and AFP level retained significance regarding their influence on overall survival. Treatments including OLT/hepatic resection, RFA, TACE, and Yttrium-90 radioembolization were independently associated with improved overall survival). TACE and Yttrium-90 radioembolization provided a comparable survival benefit (Table 5).

Pre-diagnostic follow-up characteristics and overall survival

Among patients with chronic liver disease (532 patients), regular follow-up screening for HCC was performed in 110 (21%) patients. The remaining patients either were not aware of the underlying liver disease or did not have adequate access to health care services due to social, economic or cultural issues. Patients who had regular follow-up and screening with AFP-ultrasonography were diagnosed at an earlier BCLC stage (stage 0-A; 63/110, 57% *vs* 135/422, 32%, $P < 0.001$). The number of patients within Milan (69/110, 63% *vs* 177/422, 42%, $P < 0.001$) and expanded criteria (85/110, 77% *vs* 220/422, 52%, $P < 0.001$) was significantly higher in patients who underwent regular screening follow-up compared to patients who did not. Patients within Milan or expanded criteria, patients who were diagnosed at earlier BCLC stages and patients who had regular follow-up screening for HCC had a significantly better survival (Figure 2; log-rank, $P < 0.001$).

DISCUSSION

In this retrospective, single center, observational cohort study, we investigated possible risk factors including patient, tumor and treatment-related determinants of overall survival in patients with HCC. Most patients had cirrhosis and viral etiologies, especially HBV infection, were prevalent in our cohort. Two-third of patients with chronic liver disease was aware of their liver condition, and only one-third of them were under regular surveillance for early diagnosis of HCC. Implementation of regular surveillance was associated with diagnosis at earlier stages of HCC, in which curative treatments were amenable. Approximately, a half of the cohort was suitable for OLT according to Milan or expanded criteria, but only a minority of those patients could undergo OLT due to cadaveric organ shortage and absence of a suitable living donor.

In the present study we evaluated patient, tumor and treatment-related prognostic factors associated with overall survival by univariate and multivariate analyses. Among patient-related factors only the stage of liver disease was found to be associated with overall survival. Age, gender and etiology of liver disease did not predict mortality in multivariate analysis. Except, HBV infection was independently associated with vascular invasion in our study, which is consistent with the earlier reports showing a more aggressive and infiltrative behavior[9], and more frequent vascular invasion in HBV-related HCC[10]. However, the role of liver disease etiology in determining prognosis of patients with HCC is controversial. There are a number of studies with conflicting results. In an early study, HBV-related HCC was shown to have a poor prognosis compared with HCV-related tumors, which becomes statistically significant only in patients with advanced HCC[11]. In this study, patients underwent surgery (OLT or resection) and loco-regional (ablation or TACE) treatments or received no therapy. In another study, patients with HCV infection were reported to have a higher cumulated recurrence rate after hepatic resection for small HCC (≤ 3 cm) than in patients with HBV infection. In a subsequent study by Bozorgzadeh *et al*[12], HCV-positive and negative patients who underwent OLT were retrospectively reviewed, and HCV infection was found to have a negative impact on tumor-free and overall survival. Franssen *et al*[13]

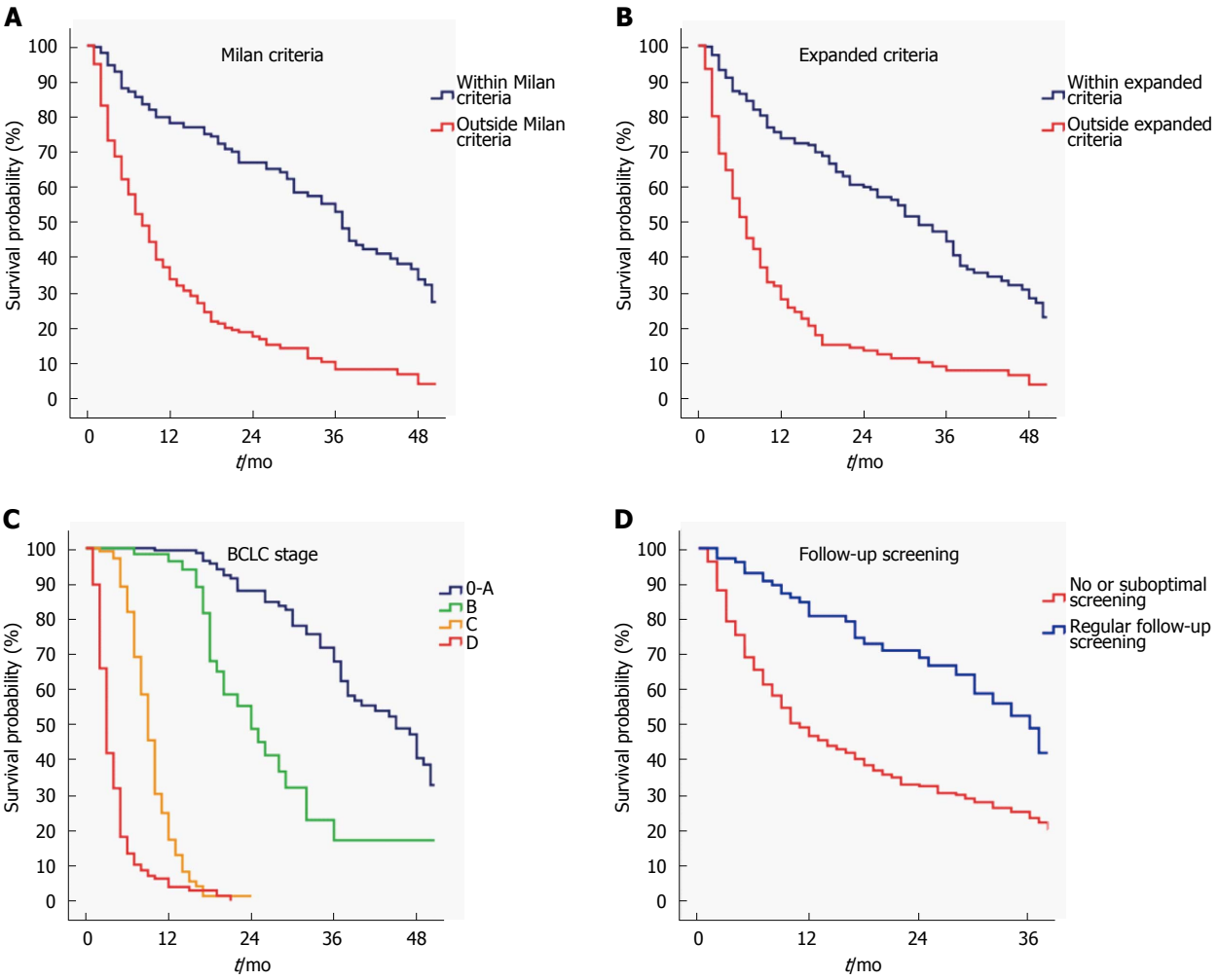

Figure 2 Overall survival according to oncological stage and pre-diagnostic screening characteristics. Patients within Milan (A) or expanded criteria (B), patients who were diagnosed at earlier BCLC stages (C) and patients who had regular follow-up screening for HCC (D) had a significantly better survival (log-rank, $P < 0.001$). HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer.

similarly found that survival and recurrence rates after both OLT and resection were better in HBV than in HCV-related HCC. There are also other studies confirming that HCV infection has a bad influence on overall and disease-free survival if a curative surgical treatment is applied[14-18]. Contrarily, the results of cohort studies which included HCC patients treated with loco-regional (RFA, TACE, *etc.*) modalities or best supportive care, either demonstrated no relationship between etiology and prognosis or showed a slightly negative influence on survival in HBV-related HCC[11,19-21]. In the era of regular surveillance, early diagnosis can suppress the effect of etiology in determining prognosis of HCC.

Our results were consistent with previous reports proving the association between tumor burden/extension and mortality. Total tumor size predicted mortality independent from the number of nodules which may suggest the up to 3 nodules criteria is too strict for selection of OLT candidates. This concept was also highlighted in recent studies showing combination of total tumor volume and AFP are better criteria to increase the number of OLT candidates with an acceptable post-transplant tumor-free survival[22,23]. Other tumor-related factors including extrahepatic metastasis and vascular invasion were also found to be independent prognostic factors.

AFP cannot be considered a sensitive diagnostic marker having a reported sensitivity of 60% when 20 ng/mL is chosen as a cut-off value for the diagnosis of HCC[24]. However, it can provide important prognostic implications even in different patient and treatment settings. Serum AFP level at presentation was clearly shown to correlate with tumor size and extent[25]. There is also substantial relationship between tumor growth and rise in serum AFP level[26]. In the present study, significantly elevated AFP level (> 100 ng/mL) was detected in less than half of the cohort, but it was found to be an independent predictor of both vascular invasion and mortality. Interestingly, we did not find any relationship between serum AFP level and presence of extrahepatic metastasis at diagnosis. Extrahepatic metastasis was only associated with total tumor diameter. To date, a number of studies indicated that AFP level is associated with overall and

recurrence-free survival in HCC patients who received OLT[27] or underwent surgical resection[13,28-30]. With this regard, a model including AFP level in addition to Milan criteria was suggested recently by Duvoux *et al*[31] to improve patient selection for OLT in HCC. AFP can also be used to guide treatment decision in patients with early-stage HCC. In a study by Ebara *et al*[26], risk factors for exceeding the Milan criteria and overall survival after successful RFA in patients with early-stage HCC were investigated. It was reported that an AFP level higher than 100 ng/mL and local recurrence within 1 year of initial successful RFA were associated with earlier recurrence and overall survival. In a subsequent study by Suh *et al*[32], it was shown that the combination of AFP and prothrombin induced by vitamin K absence-II can be a useful marker to select patients with high recurrence risk after RFA for early-stage HCC (< 3 cm).

There are several other studies that investigated predictors of survival in patients undergoing curative and non-curative therapies[11,19,33-35]. The common finding among all studies is that tumor volume/extension, baseline liver function and serum AFP level are independent predictors of prognosis, which are also confirmed by our results. However, primary mode of treatment was not appropriately included in the multivariate analysis in any of those studies. In our study, we showed that liver functional reserve, tumor extension, and baseline AFP level influences overall survival regardless of the primary treatment modality, which is another decisive factor for survival in patients with HCC. Therefore, it should be incorporated into the clinical decision making for the selection of initial treatment option. Our study showed that the choice among initial treatment options has an utmost importance that substantially influence prognosis of patients with HCC. In the present study, surgical treatments and RFA were found to be far better than other loco-regional treatments and systemic therapy with sorafenib. Ethanol/acetic acid ablation was not associated with any survival benefit. TACE and Yttrium-90 radioembolization performed similar efficacy which is demonstrated by comparable hazard ratios after adjustment of confounding factors. Systemic treatment with sorafenib was associated with a survival advantage at a borderline significance compared with no treatment, which can be explained by low number of patients receiving systemic therapy.

In conclusion, surgical treatments and RFA are best options to achieve optimal survival rates in the long-term, and there is still a need for improvement of current surveillance methods for earlier detection of HCC to facilitate those curative treatments for most of the patients. Instead of being a diagnostic marker, baseline AFP level should be considered as a prognostic marker to identify those patients with dismal prognosis. Primary treatment modality of HCC should be considered as a prognostic indicator and should be taken into account while estimating overall survival.

ARTICLE HIGHLIGHTS

Research background

HCC is a leading cause of cancer-related death with curative treatment options limited to orthotopic liver transplantation, surgical resection and local ablation. Several prognostic scoring systems were developed to predict the prognosis for patients with HCC, and to individualize treatment by matching best therapeutic option with the patient who is most likely to benefit. Nevertheless, no classification is completely satisfactory because of many other risk factors which also influence patient survival.

Research motivation

Primary treatment modality, which must be among the most important determinants of patient outcome, has not been evaluated as a prognostic indicator in relation to other determinants of survival until now. Therefore, the authors investigated the association between established prognostic factors of HCC and treatment to show how chosen treatment modality affects the prognosis.

Research objectives

Primary objective of the study was to define potential factors that have influence on prognosis, specifically to determine survival benefit associated with primary treatment modality of HCC in a real-life setting. Secondary objective of the study was to find out the relationship between pre-diagnosis screening characteristics, clinical stage of the disease at diagnosis and overall survival.

Research methods

In the present study, the authors investigated clinical, etiological, and prognostic features in a large, single-center cohort of patients with HCC who were diagnosed, treated and followed-up in the last decade. The diagnosis was established by histopathological and/or radiological criteria that was based on the recommendations reported by the EASL panel of experts in 2001. The authors reviewed demographic, clinical and staging characteristics, laboratory data, etiology of primary liver disease, imaging characteristics and treatments of HCC patients. Number and size of nodules, total tumor diameter (TTD), type of tumor, presence of major vascular involvement and extrahepatic metastasis were determined according to baseline imaging records. Univariate and multivariate Cox regression analyses were performed to find out factors associated with overall survival of patients with HCC.

Research results

A total of 545 patients with HCC who were diagnosed and followed-up between January 2001 and August 2011 were included in the study. Predictor variables of vascular invasion and extrahepatic metastasis were investigated by univariate and multivariate logistic regression analyses. The authors showed that HBV infection, multinodular and diffuse-infiltrative HCC, TTD, and AFP level were associated with vascular invasion at initial diagnosis of HCC. At multivariate analysis, independent predictor variables of vascular invasion were found to be AFP > 200 ng/mL, TTD > 5 cm and HBV. The only predictor variable for the presence of extrahepatic metastasis at initial diagnosis was TTD. Stage of liver disease, tumor type, HBV infection, number of nodules, presence of vascular invasion and AFP level did not predict extrahepatic metastasis. The best survival outcome was achieved in patients with HCC who underwent surgical treatments as OLT and hepatic resection. Treatment modalities including TACE, Yttrium-90 radioembolization, RFA were also found to be associated with improved overall survival. Ethanol/acetic acid ablation was not associated with any survival benefit. Systemic treatment with sorafenib was associated with a survival advantage at a borderline significance compared with no treatment, which can be explained by low number of patients receiving systemic therapy. Patients who had regular follow-up and screening with AFP-ultrasonography were diagnosed at an earlier BCLC stage and had a significantly better survival.

Research conclusions

It has been known that liver functional reserve, tumor extension and alfa-

fetoprotein level are among the most important determinants of patient survival. The authors showed that in addition to patient and tumor related factors, initial choice of treatment is a strong and independent predictor of survival. Survival benefit of non-curative treatments including transarterial chemoembolization and Yttrium-90 radioembolization has been an area of uncertainty. Transarterial chemoembolization and Yttrium-90 radioembolization provided a significant and comparable survival benefit in patients with hepatocellular carcinoma in the real-life setting.

Research perspectives

Primary modality of treatment for hepatocellular carcinoma is a major determinant of patient survival that should be incorporated while estimating prognosis in the future trials evaluating benefits of investigational new drugs.

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ORIGINAL ARTICLE

Retrospective Study

Predicting early outcomes of liver transplantation in young children: The EARLY study

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Informed consent statement: The need for written informed consent was waived for this retrospective chart review study by the Research Ethics Board.

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

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Abstract

AIM

To determine potentially modifiable predictors of early outcomes after liver transplantation in children of age < 3 years.

METHODS

This study was a retrospective chart review including all consecutive children of age less than 3-years-old having had a liver transplant done at the Western Canadian referral center from June 2005 to June 2015.

Pre-specified potential predictor variables and primary and secondary outcomes were recorded using standard definitions and a case report form. Associations between potential predictor variables and outcomes were determined using univariate and multiple logistic [odds ratio (OR); 95%CI] or linear (effect size, ES; 95%CI) regressions.

RESULTS

There were 65 children, of mean age 11.9 (SD 7.1) mo and weight 8.5 (2.1) kg, with biliary-atresia in 40 (62%), who had a living related donor [LRD; 29 (45%)], split/reduced [21 (32%)] or whole liver graft [15 (23%)]. Outcomes after liver transplant included: ventilator-days of 12.5 (14.1); pediatric intensive care unit mortality of 5 (8%); re-operation in 33 (51%), hepatic artery thrombosis (HAT) in 12 (19%), portal vein thrombosis (PVT) in 11 (17%), and any severe complication (HAT, PVT, bile leak, bowel perforation, intraabdominal infection, retransplant, or death) in 32 (49%) patients. Predictors of the prespecified primary outcomes on multiple regression were: (1) HAT: split/reduced (OR 0.06; 0.01, 0.76; P = 0.030) or LRD (OR 0.16; 0.03, 0.95; P = 0.044) *vs* whole liver graft; and (2) ventilator-days: surgeon (P < 0.05), lowest antithrombin (AT) postoperative day 2-5 (ES -0.24; -0.47, -0.02; P = 0.034), and split/reduced (ES -12.5; -21.8, -3.2; P = 0.009) *vs* whole-liver graft. Predictors of the pre-specified secondary outcomes on multiple regression were: (1) any thrombosis: LRD (OR 0.10; 0.01, 0.71; P = 0.021) or split/reduced (OR 0.10; 0.01, 0.85; P = 0.034) *vs* whole liver graft, and lowest AT postoperative day 2-5 (OR 0.93; 0.87, 0.99; P = 0.038); and (2) any severe complication: surgeon (P < 0.05), lowest AT postoperative day 2-5 (OR 0.92; 0.86-0.98; P = 0.016), and split/reduced (OR 0.06; 0.01, 0.78; P = 0.032) *vs* whole-liver graft.

CONCLUSION

In young children, whole liver graft and surgeon was associated with more complications, and higher AT postoperative day 2-5 was associated with fewer complications early after liver transplantation.

Key words: Liver transplantation; Pediatric; Complications; Thrombosis; Antithrombin

Core tip: In a retrospective review of 65 consecutive children having had liver transplant at age less than 3-year-old, done at a single referral institution, earlier post-operative complications were independently statistically associated with whole liver graft (compared to split/reduced or living related graft), surgeon, and lower antithrombin levels day 2-5 postoperatively. The finding that lower antithrombin levels were associated with any thrombosis, any severe complication, and ventilator days is a novel finding that should be confirmed by others.

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INTRODUCTION

One-year graft and survival rates after pediatric liver transplantation (LT) approach 80%-90% and 90% respectively[1,2]. We found that long-term neurocognitive outcome in patients under 3-years-old at time of LT, assessed in 89% of survivors at 4.5 years of age, was shifted to the left of population norms [full scale intelligence quotient mean 93.9, standard deviation (SD) 17.1], with intelligence scores < 70 (below two SDs from the population mean, expected in 2.27% of the normative population) in 6%[3]. These patients often had significant postoperative complications in the intensive care unit, and these acute posttransplant illnesses (*e.g.*, use of inotropes, infection, higher creatinine) were associated with adverse neurocognitive outcomes[3]. These findings are important because "the early years" are increasingly recognized as the period of greatest vulnerability to, and greatest return on investment from, preventing adverse events[4-7]. Adverse long-term outcomes can have lasting and profound impacts on future quality of life, education, earning potential, and healthcare utilization[4-7]. In addition, these complications are life-threatening, involve repeat surgeries, prolong intensive care unit stay, and are stressful for patients, families, and the medical team.

There have been previous studies reporting the incidence of acute complications in the pediatric intensive care unit (PICU) postLT. The main complications include the following: hepatic artery thrombosis (HAT; < 10%)[8], portal vein thrombosis (PVT; < 10%)[9], biliary leak (< 15%)[10], bowel perforation (< 10%)[11], infection, and resulting retransplantation (in < 15%) and reoperations (in up to 50%)[12]. These postLT complications are predictors of 6-mo graft and patient survival[13]. Some risk factors for these complications have been suggested, including graft type, and transplant era (year of surgery); however, these are variable between studies[10,13-15]. Recipient age and weight are often not predictors[8,16-18].

In this study, we aimed to determine potentially modifiable prespecified acute care variables that may be associated with prespecified primary and secondary acute intensive care postoperative outcomes in young LT recipients at our center over the past 10 years. In addition, we aimed to explore novel potential predictors of adverse outcomes, including: written comments made about abnormal liver vessels or biliary anatomy in the dictated operating report, measures of postoperative fluid balance (*i.e.,* highest hemoglobin, first day of negative fluid balance, first day of using furosemide, lowest central venous pressure); and

measures of postoperative coagulation status (*i.e*. time to start of heparin and achieving a therapeutic heparin level, lowest antithrombin levels, and use of other anticoagulants).

MATERIALS AND METHODS

Ethics statement

This study was approved by the University of Alberta Health Research Ethics Board (Pro00031805).

Study design

This was a retrospective observational cohort study. The charts of all patients meeting the eligibility criteria were reviewed. Inclusion criteria were having a LT done at age < 3 years at the Stollery Children's Hospital between June 2005 to June 2015. Patients having a multivisceral transplant were excluded. Potential predictor variables collected included descriptive pretransplant demographics, transplant surgery details, and early postoperative variables (see Tables E1 to E3 in Additional File 1). PICU outcomes recorded included length of stay, mortality, graft survival, retransplant, reoperations, and complications (HAT, PVT, bile leak, bowel perforation, and infection) (see Table E4 in Additional File 1). A severe complication was defined as any one of: HAT, PVT, bile leak, bowel perforation, intraabdominal infection, death, or retransplant.

Variables and outcomes were determined by review of the patient chart by one of the authors (RA), including: written notes, laboratory results, radiology reports, operating room surgical dictations, and anesthesia records. To verify accurate recording of severe complication outcomes, all patient charts with a severe complication were reviewed by a second author (ARJ) to ensure agreement, and severe complications were cross-checked in the independent LT database at our institution. A case report form, including strict conservative definitions of variables and outcomes, was agreed upon by all authors prior to chart review (Additional File 2).

Prior to any data analysis, we prespecified the primary and secondary outcomes. The primary outcomes were HAT and ventilator days; the secondary outcomes were any severe complication and any thrombosis (HAT or PVT). After local presentation of results, we were asked to add posthoc secondary outcomes of 6-mo graft survival, and to compare outcomes by year category and weight category; we include these results, acknowledging them to be posthoc, exploratory, and to be interpreted with caution.

Also prior to any data analysis, the following variables were prespecified to be used in the univariate analyses: preoperative (biliary atresia; growth failure - < 5th percentile for weight or height; albumin; graft type - whole liver, split/reduced, or living donor), operative (surgery duration; cold ischemia time; warm ischemia time; artery vascularity end-to-end anastomosis or graft; fascia closed; comment about hepatic artery, portal vein, biliary, or any one of these anatomical concerns in the dictated operative report; packed red blood cell volume transfused), and postoperative (heparin started - hour; therapeutic heparin level by day 3; highest hemoglobin day 1 and day 2-5; lowest anti-thrombin day 1 and day 2-5; other anticoagulant used - dipyridamole, dextran, or aspirin; first day of negative fluid balance; first day of furosemide use; lowest central venous pressure day 1 and day 2-5) variables.

Statistical analysis

The statistical methods of this study were reviewed by Elham Khodayari Moez, MSc, PhD candidate, from School of Public Health, University of Alberta, Edmonton, Alberta, Canada. Data was entered into a REDCap database, and transferred to SPSS version 19 for analysis[19]. For binary outcomes, univariate followed by multiple logistic regression was used to identify potential predictors. For continuous outcomes, univariate followed by multiple linear regression was used to identify potential predictors. The possibility of presence of any correlation structure in the data caused by surgeon clusters was assessed using intraclass correlations (ICCs). For all the outcomes, ICCs were small and indicated no correlation structure among the observations within surgeon clusters. Therefore, the assumption of independent observations, required for regression modelling, was met.

In all multiple regressions, the following prespecified, as likely clinically significant, variables were included: Weight, pediatric end-stage liver disease (PELD) score, year of surgery, and surgeon. All three surgeons were Fellows of the Royal Society of Surgeons of Canada and performed all the adult and pediatric LTs during the entire 10-year period; transplant cases were done by whichever surgeon was on service at that time. Variables significant at $P < 0.10$ on univariate analysis were also used in the multiple regressions. In patients having a retransplant during their PICU stay, only variables from the first LT surgery, and the first 5 days' time after the first LT were used. Dummy variables were created for analysis of graft type (in three categories) and surgeon (in three categories). Multiple regressions were performed if missingness of a variable was < 5%, with those patients excluded from the analysis (*i.e.,* no imputation of missing variables). Results are presented as odds ratios (ORs) with 95%CIs for logistic regressions, and effect sizes (ESs) with 95%CIs for linear regressions. For the multiple regressions, a *P*-value $\leqslant 0.05$ was accepted as statistically significant.

RESULTS

Description of the cohort

There were 65 patients meeting the eligibility criteria over the 10 years. The patients were 11.9 (SD 7.1)

Table 1 Univariate and multiple logistic regressions for the primary outcome of hepatic artery thrombosis after liver transplantation

Variable	Univariate logistic regression, n = 65		Multiple logistic regression, n = 65	
	Odds ratio (95%CI)	*P*-value	Odds ratio (95%CI)	*P*-value
Yr	1.10 (0.89, 1.35)	0.371		
Weight	0.65 (0.41, 1.04)	0.073		
PELD	0.98 (0.93, 1.04)	0.514		
Surgeon 2 *vs* 1	2.04 (0.41, 10.27)	0.388		
Surgeon 3 *vs* 1	4.33 (0.87, 21.60)	0.074		
Surgeon 2 *vs* 3	0.47 (0.10, 2.17)	0.334		
Fascia closed on admission	3.9 (1.1, 14.3)	0.04		
Hepatic artery any comment	3.9 (1.06, 14.31)	0.04		
Any operating note comment[1]	9.82 (1.18, 81.58)	0.034		
First day use of furosemide, n = 54[2]	1.21 (1.05, 1.41)	0.011		
Graft type R/SL *vs* WL	0.04 (0.01, 0.42)	0.006	0.06 (0.01, 0.76)	0.03
Graft type LR *vs* WL	0.10 (0.02, 0.48)	0.004	0.16 (0.03, 0.95)	0.044

[1]No meaningful difference if we use "any operating note comment" instead of "hepatic artery any comment" in the multiple regression; [2]If multiple regression is done with first day of furosemide (data available for n = 54), furosemide is significant with OR 1.67 (95%CI: 1.03, 2.73), P = 0.039, meaning the later furosemide is started the higher is the risk of HAT. CI: Confidence interval; HAT: Hepatic artery thrombosis; LR: Living related liver graft; PELD: Pediatric end-stage liver disease score; R/SL: Reduced or split liver graft; WL: Whole liver graft.

mo of age, of 8.5 (SD 2.1) kg weight (23% ⩽ 7 kg; 52% with growth failure), with biliary atresia in 40 (62%), and with 34 (52%) having had a previous Kasai procedure. Graft type was whole liver in 15 (23%), reduced size/split graft in 21 (32%), and living related graft in 29 (45%) patients. A comment about concerning anatomy of the vessels or biliary tract was recorded for 39 (60%) patients. A therapeutic heparin level by day 3 was obtained for 20 (31%) patients. PICU mortality was 5 (8%), and in survivors the ventilation days and PICU days were 12.5 (SD 14.1) and 21 (SD 21) d respectively. Complications were common, with HAT in 12 (19%), PVT in 11 (17%), biliary leak in 15 (23%), bowel perforation in 5 (8%), intraabdominal infection in 18 (28%), retransplant in 9 (14%), and any severe complication in 32 (49%) patients. First graft survival was 52 (80%) in the PICU, and 51 (78%) at 6 mo. More details of the preoperative, operative, postoperative, and outcome variables are given in Tables S1-4 (Additional File 1).

Primary outcomes

The univariate and multiple logistic regression results for HAT are shown in Table 1. Reduced/split liver (OR 0.06; 95%CI: 1, 0.76; P = 0.03), and living donor liver (OR 0.16; 95%CI: 0.03, 0.95; P = 0.044) transplant had lower risk of HAT than whole liver transplant. The later the first use of furosemide (OR 1.67; 95%CI: 1.03, 2.73; P = 0.039) was also associated with higher risk of HAT.

The univariate and multiple linear regression results for ventilator days in the 60 survivors are shown in Table 2. Ventilator days were shorter for reduced/split liver (ES -12.5; 95%CI: -21.8, -3.2; P = 0.009) than for whole liver transplants. The lowest antithrombin day 2-5 was associated with ventilator days: The higher the antithrombin, the shorter the ventilator days (ES -0.23; 95%CI: -0.47, -0.02; P = 0.034). This is shown graphically in Figure S1 (Additional File 1).

Secondary outcomes

The univariate and multiple logistic regression results for any severe complication are shown in Table 3. Reduced/split liver (OR 0.06; 95%CI: 0.01, 0.78; P = 0.032) transplant had a lower severe complication risk than whole liver transplant. The lowest antithrombin day 2-5 was associated with severe complication risk: the higher the antithrombin, the lower the risk (OR 0.92; 95%CI: 0.86, 0.98; P = 0.016). This is shown graphically in Figure S2 (Additional File 1).

The univariate and multiple logistic regression results for any thrombosis are shown in Table 4. Reduced/split liver (OR 0.10; 95%CI: 0.01, 0.85; P = 0.034) and living donor liver (OR 0.10; 95%CI: 0.01, 0.71; P = 0.021) transplants had a lower risk of thrombosis than whole liver transplants. The lowest antithrombin day 2-5 was associated with thrombosis: the higher the antithrombin, the lower the risk (OR 0.93; 95%CI: 0.87, 0.99; P = 0.038). This is shown graphically in Figure S3 (Additional File 1).

Posthoc outcomes

The univariate and multiple logistic regression results for 6-mo graft survival are shown in Table 5. Reduced/split liver (OR 15.4; 95%CI: 1.01, 234.9; P = 0.049) had better graft survival than whole liver transplant. The lowest antithrombin day 2-5 was associated with graft survival: the higher the anti-thrombin, the higher the graft survival (OR 1.08; 95%CI: 1.00, 1.16; P = 0/049). This is shown graphically in Figure S4 (Additional File 1).

Year (2005-2010 *vs* 2011-2015) and weight (> 7 kg *vs* ⩽ 7 kg) were analyzed as categorical variables for their association with the primary and secondary outcomes and mortality. On univariate analysis (independent sample t-test or Fisher's exact test,

Table 2 Univariate and multiple linear regressions for the primary outcome of postoperative ventilator days after liver transplantation in *n* = 60 survivors

Variable	Univariate linear regression, *n* = 60		Multiple linear regression, *n* = 58	
	Effect size (95%CI)	*P*-value	Effect size (95%CI)	*P*-value
Yr	-0.34 (-1.51, 0.83)	0.568		
Weight	-1.36 (-3.06, 0.34)	0.114		
PELD	0.03 (-0.30, 0.35)	0.866		
Surgeon 2 *vs* 1	-0.77 (-8.69, 7.14)	0.845		
Surgeon 3 *vs* 1	12.09 (3.15, 21.03)	0.009	10.67 (1.34, 20.01)	0.026
Surgeon 2 *vs* 3	-12.86 (-22.45, -3.28)	0.009	-9.69 (-19.24, -0.15)	0.047
Surgery duration, *n* = 54[1]	-0.037 (-0.069, -0.005)	0.026		
Fascia closed on admission	8.10 (0.52, 15.68)	0.037		
Heparin started hour, *n* = 58[2]	0.31 (0.17, 0.45)	0.001		
Highest hemoglobin day 2-5	0.27 (0.001, 0.54)	0.049	0.22 (-0.04, 0.48)	0.096
Lowest anti-thrombin day 1, *n* = 44	-0.38 (-0.65, -0.11)	0.007		
Lowest anti-thrombin day 2-5, *n* = 58	-0.35 (-0.57, -0.13)	0.003	-0.24 (-0.47, -0.02)	0.034
First day furosemide used, *n* = 521	1.01 (0.31, 1.70)	0.005		
Graft type R/SL *vs* WL	-11.61 (-21.48, -1.74)	0.022	-12.53 (-21.82, -3.23)	0.009
Graft type LR *vs* WL	-9.27 (-18.73, 0.19)	0.055	-7.29 (-15.75, 1.17)	0.096

[1]If we add "surgery duration" and "first day furosemide used" (*n* = 49) neither variable is significant, and if add only "first day furosemide used" also not significant; [2]"Heparin started h" was not added to the multiple regression because it may be a marker of how worried the medical team are about bleeding *vs* thrombosis risk, and how long the INR is elevated [the correlation of "heparin started (h)" and "time for INR to be ⩽ 2" is *r* = 0.66] (*i.e.*, it may be an outcome, and not a determinant of outcome), and if added the effect size is 0.21 (0.07, 0.36); *P* = 0.005. INR: International normalized ratio; LR: Living related liver graft; PELD: Pediatric end-stage liver disease score; R/SL: Reduced or split liver graft; WL: Whole liver graft.

Table 3 Univariate and multiple logistic regression for the secondary outcome of any severe complication after liver transplantation

Variable	Univariate logistic regression, *n* = 65		Multiple logistic regression, *n* = 62	
	Odds ratio (95%CI)	*P*-value	Odds ratio (95%CI)	*P*-value
Yr	0.97 (0.83, 1.14)	0.721		
Weight	1.02 (0.81, 1.29)	0.857	1.44 (0.98, 2.12)	0.064
PELD	0.96 (0.92, 1.00)	0.067		
Surgeon 2 *vs* 1	2.96 (0.92, 9.53)	0.069	10.07 (1.49, 67.87)	0.018
Surgeon 3 *vs* 1	6.11 (1.52, 24.50)	0.011	17.29 (1.85, 161.4)	0.012
Surgeon 2 *vs* 3	0.49 (0.12, 2.03)	0.322		
Surgery duration, *n* = 59	0.995 (0.99, 1.00)	0.043		
Artery vascularity	8.96 (1.03, 77.66)	0.047		
Biliary anatomy comment	8.96 (1.03, 77.66)	0.047		
Highest hemoglobin day 2-5	1.04 (1.00, 1.09)	0.052		
Lowest anti-thrombin day 2-5, *n* = 62	0.95 (0.91, 0.99)	0.019	0.92 (0.86, 0.98)	0.016
First day of furosemide, *n* = 54[1]	1.23 (0.99, 1.54)	0.067		
Graft type LR *vs* WL	0.30 (0.08, 1.15)	0.079		
Graft type R/SL *vs* WL	0.22 (0.05, 0.95)	0.042	0.06 (0.01, 0.78)	0.032

[1]If multiple regression is done with first day of furosemide (*n* = 54), then furosemide is not significant. Surgery duration is collinear with surgeon, so only surgeon was used in the multiple regression. LR: Living related liver graft; PELD: Pediatric end-stage liver disease score; R/SL: Reduced or split liver graft; WL: Whole liver graft.

as appropriate), year category was not statistically significantly associated with any outcome, and this was confirmed when year was used as a categorical variable in the multiple logistic and linear regressions (Table S5, Additional File 1). On univariate analysis, weight category was associated with ventilator days (10.5, SD 13.2 *vs* 20.4, SD 15.2, *P* = 0.03), with a trend toward an association with graft survival (43/50, 86% *vs* 9/15, 60%; *P* = 0.06). However, these associations were not confirmed when weight was used as a categorical variable in the multiple logistic and linear regressions (Table S6, Additional File 1).

DISCUSSION

Liver transplant in young children is life-saving for end-stage liver disease, yet patients are known to have a high risk of post-operative complications[20,21]. We aimed to determine potentially modifiable perioperative variables that are independently associated with complications post LT. There are several important findings from this study of 65 young patients having LT over the past 10 years. First, although PICU patient (92%) and graft (80%) survival were high, patients experienced a combination of significant postoperative

Table 4 Univariate and multiple logistic regression for the secondary outcome of any thrombosis after liver transplantation

Variable	Univariate logistic regression, *n* = 65		Multiple logistic regression, *n* = 62	
	Odds ratio (95%CI)	*P*-value	Odds ratio (95%CI)	*P*-value
Yr	0.96 (0.81, 1.14)	0.656		
Weight	0.74 (0.53, 1.03)	0.075		
PELD	0.97 (0.93, 1.02)	0.218		
Surgeon 2 *vs* 1	1.50 (0.37, 6.03)	0.568		
Surgeon 3 *vs* 1	7.20 (1.75, 29.57)	0.006	8.66 (0.99, 75.63)	0.051
Surgeon 2 *vs* 3	0.21 (0.05, 0.88)	0.033		
Surgery duration, *n* = 59[1]	0.99 (0.98, 1.00)	0.017		
Fascia closed on admission	2.55 (0.84, 7.78)	0.1		
Hepatic artery any comment[2]	2.55 (0.84, 7.78)	0.1		
Biliary anatomy comment[2]	5.12 (1.08, 24.20)	0.039		
Any operating note comment	3.44 (0.99, 11.94)	0.052		
Lowest anti-thrombin d2-5, *n* = 62	0.95 (0.91, 1.00)	0.03	0.93 (0.87, 0.99)	0.038
First day use of furosemide, *n* = 54[1]	1.24 (1.04, 1.47)	0.018		
Graft type R/SL *vs* WL	0.12 (0.03, 0.54)	0.006	0.10 (0.01, 0.85)	0.034
Graft type LR *vs* WL	0.10 (0.03, 0.44)	0.002	0.10 (0.01, 0.71)	0.021

[1]If multiple regression is done with first day furosemide (*n* = 54) a trend for first day of furosemide [OR 1.37 (0.99, 1.90), *P* = 0.062] is found, meaning the later the furosemide is started, the higher the risk of thrombosis; [2]"Any operating note comment" was used as it is collinear with "hepatic artery any comment" and "biliary anatomy comment". If instead, we remove "any operating note comment" and add "hepatic artery any comment" and "biliary anatomy comment", there is no meaningful change to the regression results. LR: Living related liver graft; OR: Odds ratio; PELD: Pediatric end-stage liver diseases score; R/SL: Reduced or split liver graft; WL: Whole liver graft.

Table 5 Univariate and multiple logistic regression for the posthoc secondary outcome of 6-mo first graft survival after liver transplantation

Variable	Univariate logistic regression, *n* = 65		Multiple logistic regression, *n* = 62	
	Odds ratio (95%CI)	*P*-value	Odds ratio (95%CI)	*P*-value
Yr	1.08 (0.88, 1.32)	0.46		
Weight	1.65 (1.01, 2.68)	0.046		
PELD	1.02 (0.97, 1.07)	0.508		
Surgeon 2 *vs* 1	0.49 (0.10, 2.47)	0.388		
Surgeon 3 *vs* 1	0.17 (0.04, 0.84)	0.03		
Surgeon 2 *vs* 3	2.83 (0.63, 12.71)	0.174		
Fascia closed on admission	0.21 (0.06, 0.75)	0.016		
Biliary atresia	0.23 (0.05, 1.14)	0.072		
Surgery duration, *n* = 59[1]	1.01 (1.00, 1.01)	0.097		
Heparin started (h), *n* = 62[2]	0.97 (0.94, 0.99)	0.019		
Lowest anti-thrombin d2-5, *n* = 62	1.07 (1.01, 1.13)	0.018	1.08 (1.00, 1.16)	0.049
First day furosemide used, *n* = 54[1]	0.88 (0.78, 0.99)	0.035		
Graft type R/SL *vs* WL	8.31 (1.41, 49.06)	0.019	15.39 (1.01, 234.9)	0.049
Graft type LR *vs* WL	5.47 (1.27, 23.64)	0.023		

Surgery duration and surgeon are collinear, so we could not enter both in the regression. [1]If we added "first day furosemide used" (*n* = 54), furosemide is not significant; [2]If we added "heparin started h", it was not significant. LR: Living related liver graft; PELD: Pediatric end-stage liver disease score; R/SL: Reduced or split liver graft; WL: Whole liver graft.

complications, including HAT (19%), PVT (17%), bile leak (23%), bowel perforation (8%), intraabdominal bleeding (11%), abdominal compartment syndrome (12%), and intraabdominal infection (28%). These complications necessitated reoperation of the abdomen for 51% (median 2 episodes, interquartile range 1-4), retransplantation for 14%, and renal replacement therapy for 14% of all patients. Second, there were few independent predictors of our primary and secondary outcomes. When adjusted for prespecified clinically important variables (weight, year, PELD score, and surgeon) and those variables significant at $P \leqslant 0.10$ on univariate analysis, whole liver graft had higher risk of complications (of HAT, ventilator days, any severe complication, any thrombosis, and graft loss), and a novel predictor, the lower the antithrombin level on day 2-5 postoperative, had higher risk of complications (ventilator days, any severe complication, any thrombosis, and graft loss). Third, we found no statistically significant change in outcomes over time on multiple regressions. Fourth, we found no statistically significant association of recipient weight with adverse outcomes on multiple regressions. Fifth, some of the novel predictors we examined were not associated with complication rates on multiple regressions. This included growth

failure (below 5th percentile on weight or height), surgical comments about concerning anatomy of the transplant vessels (hepatic artery or portal vein) or biliary tract, whether fascia was closed on admission to PICU, measures of postoperative fluid status (*e.g.*, use of furosemide, lowest central venous pressure, and highest hemoglobin), and measures of anticoagulation (*e.g.*, achieving a therapeutic heparin level).

This study cohort was similar to those reported in the literature, allowing cautious generalization of the findings to other centers. For example, the United States Scientific Registry of Transplant Recipients 2014 annual data report found 50.6% of recipients had previous abdominal surgery, and 4.8% had previous PVT, compatible with our rate of previous (Kasai procedure) surgery for 52%, and previous PVT in 9%[1]. The SPLIT database found that in LT for biliary atresia, retransplant rates were 11%, and reoperation was required in 48%, comparable to our rates of 14% and 51% respectively[12]. The SPLIT group also found that reoperation within 30 d was more common in split, reduced and living donor grafts (which accounted for 77% of our grafts)[14], and that 30-d survival was 93%, again comparable to our findings[14]. A recent meta-analysis found that 1-year pediatric patient and graft survival for whole liver grafts was 91% and 84.9%, and for technical variant grafts 87.7% and 77.2% respectively, comparable to our 6-mo patient and graft survivals of 89% and 78%[15].

Nevertheless, there are some differences from other reported cohorts that should be acknowledged. The rates of HAT, PVT, and bile leak were higher than in most reports. For example, HAT is often in the range of 5%-10% (compared to our rate of 19%)[2,8,11,14,15,17,18,22-27], PVT in the range of 5%-15% (compared to our rate of 17%)[2,11,15-18,24-26,28,29], and bile leak in the range of 2%-15% (compared to our rate of 23%)[10,15,16,26,29]. A report from the SPLIT database found high rates of vascular complications (25%), PVT (16%), and bile leak (21%) in the 6.7% of recipients with complex vascular anomalies[30]; however, we did not find an association of abnormal anatomy comments and thrombosis or severe complications. Although some reports have found complication rates to have improved over time, these are usually reports from the 1990s, with the improvements seen by the early-to-mid 2000s[10,11,14,17,18,24,28,31]. This is similar to reports that have found that young age and smaller weight is no longer a risk factor for complications[2,8,13,14,16-18,22,26,27,29,31]. Indeed, we did not find that year or weight was an independent predictor of complication rates. Finally, there are few reports of the incidence of bowel perforation or intraabdominal infection rates for comparison[11,13,21,26]. Quality improvement initiatives at all centers, including our own, may be needed to reduce these complication rates[32,33].

There are some novel findings from this study that warrant further investigation. First, the independent association of low antithrombin levels postLT with any thrombosis, any severe complication, graft loss, and ventilation days has not, to our knowledge, previously been examined or reported. Antithrombin is an anticoagulant produced by the liver, with its effect mediated by irreversibly inhibiting plasma serine proteases (including activated factors X and thrombin); this effect is greatly accelerated by heparin[34]. In addition, antithrombin has antiinflammatory properties[34]. Although antithrombin does not have beneficial effects in critically ill patients in general, it has not been studied in the setting of LT patients who are high risk for thrombosis[34]. Anticoagulation management after liver transplant is not standardized and often not reported in publications[35,36]. The hemostatic system during liver transplant is in a complex and precarious rebalance, with thrombotic complications often higher than bleeding complications, and is an area in need of extensive study[37-41]. The SPLIT research agenda specifically suggests a randomized trial of different anticoagulation profiles measuring the combined endpoints of PVT, HAT, and reexploration for intraabdominal bleeding[42]. Treatment with antithrombin concentrate intravenously should be considered for low antithrombin levels in such protocols.

Second, the independent association of surgeon with outcomes has not, to our knowledge, previously been reported. The literature suggests that this is an expected finding, for several reasons. The outcomes among centers are highly variable, with most large centers reporting excellent outcomes[11,17,18,24-26,28], and some smaller centers reporting poor outcomes[43-47]. Some authors have reported a decrease in complication rates over time associated with what they call technical experience[10,17,18,27,29,48]. The SPLIT group has reported that the center where transplant is done is a predictor of patient and graft survivals (which in turn are predicted by complication rates), and that after adjusting for center there is no effect of age on transplant outcomes[13]. The Kid's Inpatient Database also found mortality varied by region[48]. The SPLIT Clinical Care and Quality Improvement Committee recently reported that they considered HAT and biliary complications as "essentially surgical complications"[32]. These data suggest that surgical technique is a potentially modifiable variable affecting outcome, and more study is needed to determine what accounts for these differences in outcomes among surgeons, something that is beyond the scope of our study.

Third, whole liver grafts were associated with higher complication rates in our study. This is contrary to the findings from a recent meta-analysis comparing outcomes between whole liver *vs* technical variant grafts in pediatrics, where the ORs for 1-year patient and graft survival were 1.62 and 1.78, and for PVT and biliary complications were 0.45 and 0.42 for whole liver grafts compared to technical variant grafts[15]. The SPLIT group also reported that whole liver grafts have lower rates of biliary complications, PVT, and reoperation compared to split, reduced or

living donor grafts[14]. Nevertheless, there is conflicting literature. Some groups have reported that graft type is not related to biliary complications[10,16,29]. Most groups have reported that graft type is not related to HAT[15], and UNOS data and some single center data suggests graft survival is better after living donor grafts than deceased donor grafts, particularly in younger patients[11,27,49,50]. It is possible that our findings of higher complication rates with whole liver grafts is due to the small patient size in which whole grafts contributed to intraabdominal hypertension and vascular compression[9,23]. In support of our findings, an analysis of the UNOS database examining liver transplants for biliary atresia from 2002-2014 found that in recipients ⩽ 7 kg the 1-, 5- and 10-year graft survival was lowest for whole liver grafts, and the vascular thrombosis and liver retransplantation rates highest for whole liver grafts, unlike in recipients weighing 7-14 kg and > 14 kg[51]. Future studies should determine whether graft type affects outcome differently in the youngest patients.

There are limitations to this study. First, this was a single-center, retrospective, observational study of 65 patients having had LT at age < 3 years over a time-period of 10 years. Some variables were missing from the medical records for several patients (*e.g*., blood products given during the transplant surgery; size of hepatic artery, hepatic veins, or portal vein; and graft-to-recipient body size ratio). The subjective evaluations of the vessels and biliary anatomy in the surgical notes were not standardized, and thus difficult to interpret. As such, the findings cannot show cause and effect, and are only hypothesis generating; whether treatment to increase antithrombin levels can improve outcomes is unknown and requires prospective study, ideally in a randomized trial as suggested in the SPLIT research agenda[42]. In addition, other centers should confirm the findings to determine the generalizability of the results. Second, the "any severe complication" outcome was not based on the Clavien-Dindo classification of surgical complications; however, the definition we used would include only complications of Grade Ⅲ-Ⅴ, and mostly of Grade Ⅳ (life-threatening requiring ICU management)[52]. In addition, the main outcomes overlapped; for example, HAT was a component of "any thrombosis" and "any severe complication", and graft survival was often determined by thrombosis and other severe complications. Third, the novel predictor, lowest antithrombin on day 2-5 postoperative, could have been a finding during the development of the adverse outcome (*i.e.,* thrombosis), and thus may not be a modifiable predictor. Finally, the posthoc outcomes should be interpreted with caution given the multiple statistical testing and small cohort.

This study has several strengths. This was a modest sample size of young patients having a LT. Although retrospective, the outcomes and potential predictor variables collected were objective, and were all clearly defined prior to data collection. The predictors and primary and secondary outcomes were pre-specified prior to analysis, to prevent "data dredging". Some novel variables were examined, and found not associated with outcomes (*e.g.*, central venous pressure, heparin therapeutic level by day 3, surgical comments about concerning anatomy of the transplant vessels or biliary tract). Some other novel predictors were associated with outcomes (*e.g.*, antithrombin level day 2-5 and surgeon) which generate novel hypotheses for future research.

Patients under 3-years-old having LT had high patient (92%) and graft (80%) survivals. These patients not infrequently experienced a combination of significant postoperative complications, including HAT, PVT, bile leak, bowel perforation, intraabdominal bleeding, abdominal compartment syndrome, and intraabdominal infection, sometimes necessitating reoperation of the abdomen, retransplantation, and kidney dialysis. Whole liver graft was independently associated with a higher risk of complications. A novel predictor, the lower the antithrombin level on days 2-5 postoperative, was independently associated with a higher risk of complications. Other centers should determine whether antithrombin levels are associated with outcomes after LT in young children, and a prospective trial comparing anticoagulation strategies that incorporate antithrombin treatment should be considered.

ARTICLE HIGHLIGHTS

Research background

Postoperative intensive care unit complications after liver transplantation in young children are common, and associated with significant morbidity and mortality. Risk factors for these early complications are poorly studied.

Research motivation

Postoperative intensive care unit complications in young children can require reoperation, threaten liver graft viability, and prolong length of stay. These complications include thrombosis of vessels necessary for blood flow to the liver graft (*i.e.* hepatic artery thrombosis and portal vein thrombosis), other life-threatening events requiring intensive care management (*i.e.* bile leak, bowel perforation, intraabdominal infection, or retransplant), or death. Identifying risk factors for these complications can generate hypotheses for future research testing, leading to improved outcomes after liver transplantation.

Research objectives

The authors aimed to determine potentially modifiable prespecified acute care variables that may be associated with prespecified primary and secondary acute intensive care postoperative outcomes in young liver transplant recipients at our center over the past 10 years. In addition, the authors aimed to explore novel potential predictors of adverse outcomes, including written comments made about abnormal liver vessels or biliary anatomy in the dictated operating report, measures of postoperative fluid balance, and measures of post-operative coagulation status. The authors identified risk factors that are potentially modifiable and that should be confirmed by future research.

Research methods

This study was a retrospective chart review including all consecutive children of age less than 3-years-old having had a liver transplant done at the Western Canadian referral center from June 2005 to June 2015. Prespecified potential predictor variables and primary and secondary outcomes were recorded using

standard definitions and a case report form. Associations between potential predictor variables and outcomes were determined using univariate and multiple logistic (odds ratio, OR; 95% confidence interval, CI) or linear (effect size, ES; 95%CI) regressions.

Research results

There were several important results from this study. First, although pediatric intensive care unit (PICU) patient (92%) and graft (80%) survivals were high, patients experienced a combination of significant postoperative complications, including hepatic artery thrombosis (19%), portal vein thrombosis (17%), bile leak (23%), bowel perforation (8%), intraabdominal bleeding (11%), abdominal compartment syndrome (12%), and intraabdominal infection (28%). These complications necessitated reoperation of the abdomen for 51% (median 2 episodes, interquartile range 1-4), retransplantation for 14%, and renal replacement therapy for 14% of all patients. Second, there were few independent predictors of our primary and secondary outcomes. When adjusted for prespecified clinically important variables (weight, year, pediatric end-stage liver disease score, and surgeon) and those variables significant at $P \leq 0.10$ on univariate analysis, whole liver graft had higher risk of complications (of hepatic artery thrombosis, ventilator days, any severe complication, any thrombosis, and graft loss). A novel predictor, the lower the antithrombin level on day 2-5 post-operative, had higher risk of complications (ventilator days, any severe complication, any thrombosis, and graft loss). The independent association of surgeon with outcomes (of any thrombosis, any severe complication, and ventilator days) has not, to our knowledge, previously been reported. Third, the authors found no statistically significant change in outcomes over time on multiple regressions. Fourth, the authors found no statistically significant association of recipient weight with adverse outcomes on multiple regressions. Fifth, some of the novel predictors the authors examined were not associated with complication rates on multiple regressions. This included: growth failure (below 5th percentile on weight or height), surgical comments about concerning anatomy of the transplant vessels (hepatic artery or portal vein) or biliary tract, whether fascia was closed on admission to PICU, measures of postoperative fluid status (*e.g.*, use of furosemide, lowest central venous pressure, and highest hemoglobin), and measures of anticoagulation (*e.g.*, achieving a therapeutic heparin level). Future study is required to confirm our findings. Treatment with antithrombin concentrate intravenously should be considered for low antithrombin levels in studies of anticoagulation protocols.

Research conclusions

Patients under 3-years-old having liver transplant had high patient (92%) and graft (80%) survival. These patients not infrequently experienced a combination of significant postoperative complications, including hepatic artery thrombosis, portal vein thrombosis, bile leak, bowel perforation, intraabdominal bleeding, abdominal compartment syndrome, and intraabdominal infection, sometimes necessitating reoperation of the abdomen, retransplantation, and kidney dialysis. Whole liver graft was independently associated with a higher risk of complications. Surgeon was independently associated with a higher risk of complications. A novel predictor, the lower the antithrombin level on day 2-5 postoperative, was independently associated with a higher risk of complications. Antithrombin is an anticoagulant produced by the liver, with its effect mediated by irreversibly inhibiting plasma serine proteases (including activated factors X and thrombin); this effect is greatly accelerated by heparin. In addition, antithrombin has antiinflammatory properties. Although antithrombin does not have beneficial effects in critically ill patients in general, it has not been studied in the setting of liver transplant patients who are high risk for thrombosis. Other centers should determine whether antithrombin levels are associated with outcomes after liver transplant in young children, and a prospective trial comparing anticoagulation strategies that incorporate antithrombin treatment should be considered. In addition, more study is needed to determine what accounts for differences in outcomes among surgeons.

Research perspectives

The findings are hypothesis generating and require confirmation by other centers, ideally in prospective studies. Future prospective observational research is needed to confirm the findings that whole liver graft, surgeon, and low antithrombin postoperatively are risk factors for complications. If confirmed, future randomized controlled trials of anticoagulation strategies after liver transplant in young children are needed, and these should include monitoring and treatment of antithrombin levels.

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ORIGINAL ARTICLE

Retrospective Study

Collagen proportionate area correlates to hepatic venous pressure gradient in non-abstinent cirrhotic patients with alcoholic liver disease

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Abstract

AIM

To explore the relationship between collagen proportionate area (CPA) and portal hypertension-related clinical manifestations in alcoholic liver disease (ALD).

METHODS
Retrospective study with chart review of patients with ALD adressed to our center between January 2012 and December 2013 for a transjugular liver biopsy (TJLB) and hepatic hemodynamic study. Patients were included if they met the following criteria: (1) Medical indication for a liver biopsy in the setting of ALD; (2) recent (< 15 d) clinical, radiological, endoscopic and biological data available; and (3) estimated follow-up of at least 6 mo. Liver tissue from cirrhotic subjects obtained from transjugular liver biopsies was stained with PicroSirius red and computer-assisted digital image analysis to determine fibrosis density using CPA was performed.

RESULTS
We included 61 patients with alcoholic ALD, subdivided in 41 active alcohol drinkers and 20 durably abstinent patients. Nine healthy liver donors served as controls. Mean CPA in patients with ALD was 7.1%, with no difference between active drinkers and abstinent patients (P = 0.17). Using a fibrosis density cutoff of 5%, we observed a positive correlation between high fibrosis density and the hepatic venous pressure gradient (HVPG) only in active drinkers (P = 0.02). At 12-mo of follow-up, in the group of active alcohol drinkers, patients reaching a composite outcome showed a higher HVPG value as compared to those who did not (18.5 mmHg *vs* 14.5 mmHg P < 0.04) whereas CPA values were similar (6.9% *vs* 11%, P = 0.23).

CONCLUSION
In active alcoholic ALD, CPA correlates to portal pressure but only HVPG predicts clinical events, pointing to the role of alcohol as a modulator of portal hypertension.

Key words: Fibrosis; Hepatic venous pressure gradient; Cirrhosis; Chronic advanced liver disease; Collagen proportionate area

Core tip: This is the first study exploring the relationships between fibrosis density assessed by collagen proportionate area (CPA) in liver biopsy, hepatic venous pressure gradient (HVPG) and development of clinical manifestations of portal hypertension in patients with chronic advanced alcoholic liver disease addressed for liver investigations. The results suggest a positive correlation between high fibrosis density and the HVPG only in active drinkers. HVPG, but not CPA, predicts clinical events in active alcohol drinkers pointing to the role of alcohol as a modulator of portal hypertension.

Restellini S, Goossens N, Clément S, Lanthier N, Negro F, Rubbia-Brandt L, Spahr L. Collagen proportionate area correlates to hepatic venous pressure gradient in non-abstinent cirrhotic patients with alcoholic liver disease. *World J Hepatol* 2018; 10(1): 73-81 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/73.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.73

INTRODUCTION

Determination of liver fibrosis is crucial in the management of patients with chronic liver disease[1]. Fibrosis is associated with the development of portal hypertension (PHT) which has a strong negative impact on patients' outcome and survival[2]. The diagnosis of liver fibrosis is often made at an advanced stage in the population of excessive drinkers, when they come to medical attention for portal-hypertensive complications[3,4]. The development of fibrosis is of particular interest in alcoholic liver disease (ALD) as compared to other etiologies, with regards to the pattern of distribution (pericellular, centrilobular, periportal)[5,6], the large amount of fibrosis[7] and the typical histological lesions (including steatohepatitis) reported to accelerate disease progression[8].

Traditionally, liver biopsy is the gold standard for the detection and staging of liver fibrosis. However, recent advances in non-invasive strategies, including serum markers, transient elastography and other elasticity-based radiological techniques are now able to provide reliable information on liver fibrosis (no significant fibrosis *vs* advanced fibrosis or cirrhosis) and may avoid unnecessary liver biopsy in a subset of patients with viral[9] or alcohol related liver disease[10]. However, when a liver biopsy is indicated, the transjugular route allows to obtain both a liver tissue specimen and the measurement of the hepatic venous pressure gradient (HVPG) which is the reference method to measure portal pressure in the clinical setting[11]. This parameter is a predictor of clinical decompensation and PHT-related complications when equal or superior to 10 mmHg[11], and may be influenced by factors including histological lesions[12] and alcohol intake[13]. Thus, both architectural distortion of liver lobule by fibrosis and dynamic components participate to intrahepatic resistance to blood flow[14] and are associated with increased HVPG value in patients with ALD.

Fibrosis in a liver biopsy can be assessed by histological staging systems that are based on a semi-quantitative evaluation and provide information on the importance of architectural changes. The optimal histological staging system for ALD is not universally accepted. Common tools such as Ishak and METAVIR have been mostly developed in chronic viral hepatitis in which fibrosis predominates around the portal tracts. Thus, application of a score used in non-alcoholic fatty liver disease might be more appropriate in patients with ALD, as both diseases share several similarities in terms of pathogenic mechanisms and morphological alterations.

The quantitative measurement of liver fibrosis by a computer-assisted digital image analysis of a liver tissue specimen collagen proportionate area (CPA)

overcomes the limitations of semiquantitative scores, demonstrated a good correlation with HVPG and noninvasive markers of fibrosis[15], and is of prognostic significance. These results, however, have been mostly obtained in HCV-related liver disease[16,17], and very few data are available in ALD. Therefore, we undertook the present study to explore the relationships between fibrosis density in liver biopsy, HVPG and development of clinical manifestations of PHT in patients with chronic advanced alcoholic liver disease (cALD) addressed for liver investigations. We aimed to better understand the relative prognostic contribution of HVPG and liver fibrosis quantification in subjects with advanced ALD.

MATERIALS AND METHODS

Patients

Consecutive patients with ALD adressed to the Gastroenterology and Hepatology Division of Geneva University Hospitals between January 2012 and December 2013 for a transjugular liver biopsy (TJLB) and hepatic hemodynamic study were eligible for this study. Both active alcoholic patients and abstinent patients were eligible for inclusion. Abstinent patients were defined as patients who did no drink any glass of alcohol for the last 6 mo before the inclusion. Abstinence or relapse status was self reported. Patients were included if they met the following criteria: (1) Medical indication for a liver biopsy in the setting of ALD; (2) recent (< 15 d) clinical, radiological, endoscopic and biological data available; and (3) estimated follow-up of at least 6 mo. Complete portal vein thrombosis, multifocal hepatocellular carcinoma and coexistent sepsis were considered as exclusion criteria. A group of healthy candidates for living donation, who underwent a protocol TJLB, served as controls. It is our policy to perform a liver biopsy early in patients eligible for living donation in agreement with our institutional ethical committee.

Liver function tests and clinical symptoms used for the determination of both Child-Pugh and model for end-stage liver disease (MELD) scores were recorded at baseline. During a 12-mo follow-up, liver related death or liver transplantation, as well as clinically relevant episodes including ascitic decompensation, overt episodes of hepatic encephalopathy (HE) and PHT-related bleeding were carefully documented and considered composite events. Information regarding alcohol consumption or abstinence (given by family or relatives, or extrapolated from biological samples (blood or urine alcohol, liver tests) was extracted from patients file.

Transjugular liver biopsy

Two trained operators (LS and NG) experts in TJLB performed all the procedures using a standard procedure and material that included both a TJL-101-ET needle set (Cook Europe, Bjaeverskov, Denmark) and an 8F curved catheter (Cordis Europa, Amsterdam, The Netherlands). Under light sedation, the right jugular vein was punctured and the catheter introduced in the right hepatic vein under radiological guidance. The catheter was wedged into a small hepatic venule in order to block blood flow, followed by injection of iodinated contrast media to verify the proper position of the catheter in a wedge position. A stable tracing on the monitor was required to accept the measure as valid, while taking the mid-chest as the external zero reference. The free hepatic venous pressure was measured while the catheter was floating in the hepatic vein close to the ostium of the vena cava. The mean value of at least two measurements of wedge and free pressures recorded in different vascular territories in the right liver lobe were kept for analysis. The difference between wedge- and free hepatic venous pressure, named the HVPG, is the parameter used to assess PHT in a clinical setting[11].

Liver stiffness

Liver stiffness, expressed in kilopascals (kPa), was measured by transient elastometry using the Fibroscan (Echosens®, Paris, France) in patients without ascites, following the recommended criteria for valid measurements[9]. The value measured in advanced fibrosis or cirrhosis are in the range of 15 kPa or higher, with both the M and XL probes providing values of similar performance[18]. The examinators performing liver stiffness measurements (SR, NG, LS) were not aware of HVPG values.

Liver histology

Liver biopsy samples were formalin fixed, paraffin embedded, and serial sections were stained with hematoxylin and eosin, Masson Trichrome and PicroSirius red. The histopathological specimens were thoroughly examined by an expert in liver pathology (LRB) using standard high-power field views, as previously described[19]. We analyzed the presence and severity of the following features, using a scoring system derived from a recent publication on non alcoholic fatty liver[20]: Steatosis (above 33% of hepatocytes: marked steatosis), ballooning (> 2 enlarged hepatocytes with clear reticular cytoplasm on high power field (× 49): Marked ballooning degeneration), and inflammation (< 2 foci of inflammatory cells in the lobule: mild inflammation; > 2 foci: marked inflammation). A detailed fibrosis evaluation was not performed as all patients presented at an advanced stage of ALD that reached the stage of cirrhosis. The examiner was blinded to patients' hemodynamic and liver stiffness data.

The PicroSirius red tissue section was used for CPA using digital image analysis, as reported[16]. Images of the entire specimen were acquired with a digital camera (Leica DC 300 F) connected to a high resolution DMRBE Leica microscope, using a × 10 objective and a × 10 magnification lens. Collagen proportionate area was subsequently quantified using the Qwin Leica Q550IW software (Meyer Instr. TX, United

Table 1 Patients characteristics

Variable	Controls (n = 9)	Alcohol abstinents (n = 20)	Active alcohol drinkers (n = 41)	P value (abstinent *vs* active alcohol drinkers)
Age (yr)	38.2 (35-49)	59.5 (55-65)	56.9 (52-63)	0.40
Male sex	6 (67%)	16 (80%)	33 (80%)	1.0
Ascites	0	11 (61%)	23 (64%)	1.0
HE	0	3 (16%)	7 (21%)	1.0
Esophageal varices	0	15 (83%)	25 (69%)	0.30
HVPG (mmHg)	2 (2-3)	18 (16-19)	18 (14-20)	0.90
Liver stiffness (kPa)	3.8 (3-3.8)	Not available	38.6 (14.6-68.2)	-
Platelet count (G/L)	286 (170-340)	44.5 (20-55)	33.5 (20-53)	0.40
Child-Pugh score	-	9.5 (8-10)	9 (8-11)	0.94
MELD score	-	14 (12-19)	19 (11-22)	0.50

HVPG: Hepatic venous pressure gradient; MELD: Model for end-stage liver disease.

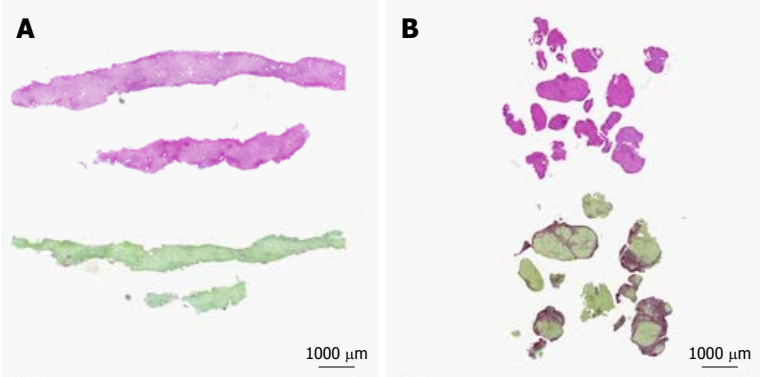

Figure 1 Variability of histological specimens in HE (top) and PicroSirius red (bottom) stained transjugular biopsy specimens. A: Non-cirrhotic sample with 0.01% fibrosis proportion; B: Cirrhotic sample with 21% of fibrosis proportion.

States) which is particularly suited for quantification of microscopic alterations. This fine quantification method allowed us to determine the percentage of PicroSirius red positive area in the biopsy specimen, expressed as 0%-5%, 5%-10%, 10%-20% and > 20%, and represented the fibrosis density (Figure 1). Significant fibrosis was defined as CPA > 5%. This procedure was performed by two trained investigators (SR and NG) under the supervision of the pathologist (LRB).

Statistical analysis

Variables are reported as median and interquartile range (IQR). Comparisons between groups were performed using the Wilcoxon signed rank, chi-square or Fischer's exact tests as appropriate. Correlations between variables were evaluated by Spearman correlation. The results are presented as odds ratios (OR) with 95%CI. A P value < 0.05 was considered statistically significant. Statistical analyses were performed with the program R (R Foundation for Statistical Computing, version 3.0).

RESULTS

Clinical data

The study population consisted of 9 healthy subjects and 61 patients with cALD, divided in 20 patients with longstanding abstinence from alcohol (> 6 mo), and 41 patients who consumed regularly alcohol. The patients' clinical characteristics are described in Table 1. They were predominantly male, with Child-Pugh B cirrhosis, the majority of patients presenting with clinically significant PHT manifest as ascites and esophageal varices, and a history of HE. The mean HVPG value was 18 mmHg in both groups. Only few patients benefited from a transient elastography (n = 12, 12/41 patients in the group of active alcohol drinkers, and 0/20 in the abstinent group) precluding any comparison. This was mostly due to technical limitations related to the presence of ascites or poor quality of measures. Overall, in this population with cALD at a cirrhotic stage, abstinent and active alcohol drinkers presented similar clinical characteristics with regard to the degree of liver failure, HVPG and clinical manifestations of PHT. Only serum albumin was decreased in active alcohol consumers *vs* abstinent patients (23 ± 2 gr/L *vs* 29.5 ± 1.8 gr/L, P = 0.01).

At 12 mo, survival without liver transplantation was 84%. During follow-up, 7 patients died of liver-related causes (4/20 in active alcohol drinkers and 3/41 in abstinent patients), and 3 patients from the abstinent group underwent liver transplantation. A return to regular, moderate alcohol consumption (20-30 gr/d) was reported in the group of patients who qualified as abstinent at baseline. All but 3 patients from the active alcohol drinkers group persisted in a regular alcohol

Table 2 Histological features on transjugular liver biopsy

Variable	Controls (n = 9)	Alcohol abstinents (n = 20)	Active alcohol drinkers (n = 41)	P value (abstinent *vs* active alcohol drinkers)
Cirrhosis	0 (9%)	20 (100%)	41 (100%)	1.0
Marked steatosis	0 (0%)	1 (5%)	21 (51%)	0.001
Marked ballooned hepatocytes	0 (0%)	5 (26%)	25 (64%)	0.01
Marked inflammation	0 (0%)	0 (0%)	1 (2%)	0.9
Fibrosis density (%)	0.7 (0.2-1.5)	3.8 (1.1-11.8)	8.2 (3.8-14.1)	0.17
Fibrosis category				0.48
0%-5%	9	11	15	
5%-10%	0	3	9	
10%-20%	0	3	12	
> 20%	0	3	5	

Table 3 Characteristics of patients with alcoholic liver disease according to fibrosis density

Variable	Fibrosis density ⩽ 5% (n = 26)	Fibrosis density > 5% (n = 35)	P value
Active alcohol	15 (58%)	26 (74%)	0.27
Age (yr)	58 (55-64)	57 (52-63)	0.4
Male sex	24 (92%)	25 (71%)	0.06
Ascites	12 (55%)	22 (69%)	0.4
HE	7 (32%)	3 (10%)	0.07
Esophageal varices	15 (65%)	25 (81%)	0.2
HVPG (mmHg)	16 (11-18)	19 (17-20)	0.01
Liver stiffness (kPa)	19 (12-42)	57 (34-72)	0.5
Platelet count (G/L)	37 (20-52)	38 (20-55)	0.7
MELD score	14 (11-19)	18 (13-22)	0.2
Histology			
Marked steatosis	11 (42%)	11 (31%)	0.4
Marked ballooned hepatocytes	9 (36%)	21 (64%)	0.06
Marked inflammation	0 (0%)	1 (3%)	0.9

HVPG: Hepatic venous pressure gradient; MELD: Model for end-stage liver disease.

consumption (30-50 g/d) during follow-up.

At 12 mo, a composite clinical outcome including ascitic decompensation, portal hypertensive bleeding, or episode of overt HE was reported in 32 patients, with ascites requiring large volume paracentesis being the most prevalent complication. A transjugular intrahepatic shunt (TIPS) procedure was performed in 5 patients, all from the active alcohol group to treat manifestations of PHT.

Histology

Liver biopsy was successful in all patients. It yielded material that measured 8.1 mm (6.8-14.2) in length that was sufficient for an accurate histological diagnosis and additional liver tissue studies, as previously reported[19,21]. In all patients, fragmented material showing diffuse architectural changes with nodular formation and extensive fibrosis were consistent with the diagnosis of cirrhosis. Results of histological evaluation are presented in Table 2. Marked steatosis and ballooned hepatocytes were more prominent in the subgroup of active alcohol drinkers as compared to abstinent patients, an observation which is consistent with published data[22]. Mild lobular inflammation composed in a majority of mononuclear cells was present in all patients with cALD. One patient from the active alcohol group demonstrated marked inflammation without reaching the histological criteria for alcoholic hepatitis.

Determination of fibrosis density by CPA could be performed in all patients and controls, demonstrating, as expected, higher values in patients as compared to controls (Figure 1B). The inter-rater agreement (SR and NG) was good with a kappa index of 0.9. In patients with ALD, the fibrosis density tended to be higher in active alcohol drinkers as compared to alcohol abstinent patients, but without reaching a statistically significant level [8.2% (3.4-14.1) *vs* 3.8% (1.-11.8), P = 0.17, Table 2]. In the subgroup of active alcohol users, using a cut-off value of 5% of fibrosis density (derived from the median value in the whole patient group), patients with low fibrosis (< 5%) had a lower value of HVPG as compared to those with high fibrosis (> 5%) (16 ± 1.9 mmHg *vs* 19 ± 2 mmHg, P < 0.01, Table 3 and Figure 1B). Within subjects with active or abstinent alcohol abuse, in multivariate analysis including HVPG, drinking status and sex, only HVPG was independently associated with fibrosis density [OR 1.2 per unit increase in HVPG, 95%CI (1.1-1.4), P = 0.01].

Except for a trend towards more ballooned hepatocytes in the high fibrosis group, other histological features were similar with regards to fibrosis density in

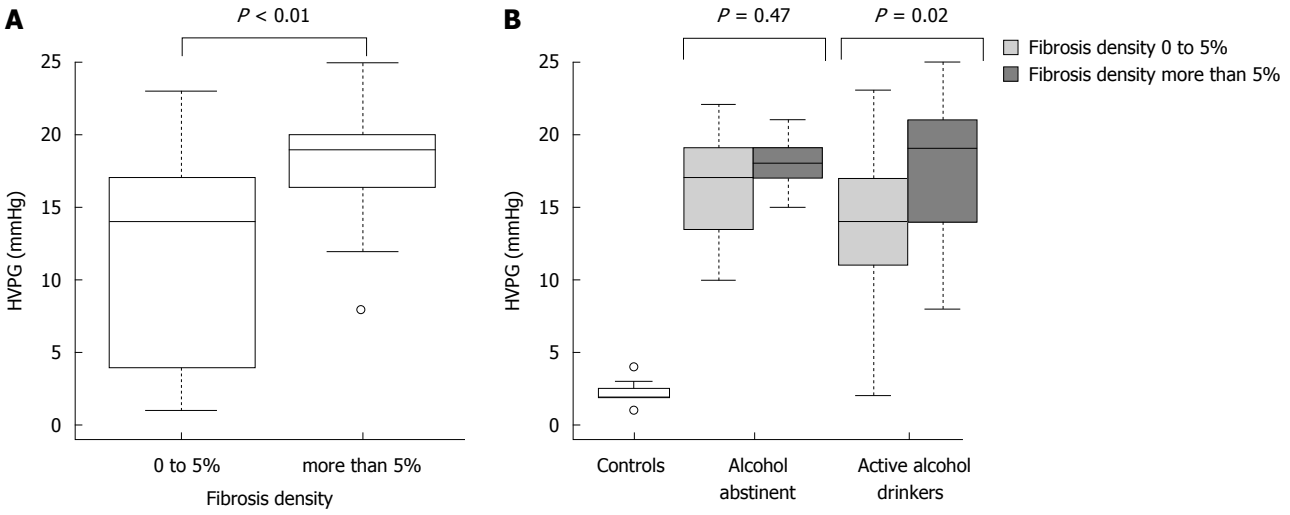

Figure 2 Correlation between hepatic venous pressure gradient and fibrosis density measured by collagen proportionate area. A: Correlation between HVPG and fibrosis density measured by CPA in the whole group of patients; B: Subgroup analysis showing a positive correlation between CPA and HVPG restricted to active alcohol drinkers. HVPG: Hepatic venous pressure gradient; CPA: Collagen proportionate area.

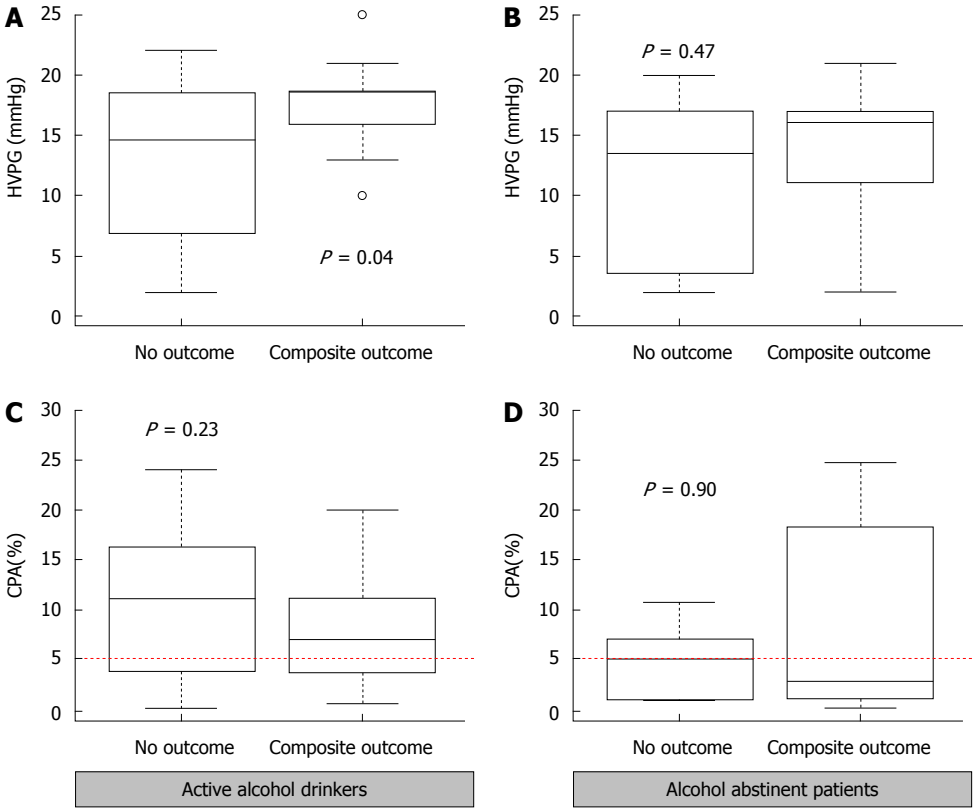

Figure 3 Relationship between hepatic venous pressure gradient (A), collagen proportionate area (B) and development of clinical complications related to portal hypertension during follow-up both in active alcohol drinkers (C) and abstinent patients (D).

the liver biopsy.

Clinical correlations

In the whole group, fibrosis density measured by CPA correlated with HVPG using a 5% cut-off value (Figure 2A). In a subgroup analysis, this correlation was conserved only in active drinkers (see Figure 2B). Figure 3 and Supplementary Table 1 illustrate the relationship between CPA, HVPG and clinical outcome in the subgroups of abstinent and active alcohol drinkers. In the group of active drinkers, patients who reached a composite outcome showed a higher HVPG value as compared to those who did not [19 (16-19) mmHg *vs* 15 (7.5-18) mmHg $P = 0.04$]. Such a difference could not be observed using the CPA [6.9 (3.9-11) % *vs* 11 (4.0-16) %, $P = 0.23$]. In the group of patients

abstinent from alcohol, neither HVPG (P = 0.47) nor CPA (P = 0.90) were associated with the development of a composite clinical outcome during follow-up.

The severity of histological features was not related to CPA or HVPG values, and were not associated with the development of a complication of PHT.

DISCUSSION

In the group of patients with chronic advanced alcoholic liver disease presenting with moderate to severe liver insufficiency and clinical manifestations of PHT, we demonstrate a clear relationship between portal pressure and the precise quantification of liver fibrosis by CPA in transjugular liver biopsies. In multivariate analysis, only HVPG was independently associated with fibrosis density (OR 1.2 per unit increase in HVPG, 95% CI [1.1-1.4], P = 0.01). In this apparently uniform group of alcoholics, we could objectivate differences in the level of HVPG between active drinkers and abstinent patients according to the density of liver fibrosis. Finally, high HVPG in active alcohol drinkers (but not in abstinent patients) was associated with future complications of PHT during follow-up.

The superiority of quantification of liver collagen over semi-quantitative methods to predict clinical outcome has been mostly demonstrated in chronic liver diseases of various etiologies and degrees of liver failure[16]. This approach is of interest to assess fibrosis in liver tissue obtained by needle biopsy, a situation where the number and size of nodules and fibrous septa may be difficult to evaluate with regards to the small size of the specimen. Although biopsies obtained by transjugular route are smaller in size and more fragmented as compared to percutaneous sampling, this method allows an accurate histological diagnosis[21] and a simultaneous measurement of the HVPG. We believe that our results are valid, as we also provide data in healthy subjects submitted to the same CPA and HVPG measures demonstrating striking differences, as expected.

To the best of our knowledge, this is the first study to explore the relationship between CPA and HVPG in a well characterized and homogeneous group of patients with ALD at an advanced stage, and to provide a separate analysis between abstinent and actively drinking subjects. The specificity of ALD as compared to chronic liver diseases of other etiologies includes an elevated density of liver fibrosis[7] and the strong influence of alcohol on PHT complications[13,23]. The former relates to the important collagen deposition and dense perivenular pattern of fibrosis typical of ALD[7], and the latter is associated with the increased intrahepatic resistance promoted by cofactors such as endothelial dysfunction, inflammation and marked steatosis associated with acute alcohol intake[24]. CPA positively correlates with HVPG only in active drinkers. Our findings suggest that active alcohol consumption may influence portal hemodynamic in addition to existing architectural changes due to cirrhosis. Accordingly, an oral administration of 0.5 gr/kg of ethanol increases both HVPG and azygos blood flow in patients with alcoholic cirrhosis and may precipitate variceal bleeding[13]. This observation is consistent with the higher value of HVPG in active alcohol drinkers who developed a clinical complication of PHT during follow-up as compared to those without clinical decompensation[25]. We were not able to identify an influence of histological lesions on parameters such as CPA and HVPG. We speculate that major architectural changes of cirrhosis present in all patients may have blunted the possible role of lesions such as marked steatosis or inflammation on the HVPG.

In view of these results, what would be the use of CPA as a fine quantitative method of measuring fibrosis density in liver biopsy of ALD patients? The diagnosis of cirrhosis is best defined on histological criteria, and subclassification of fibrosis by semi-quantitative scores allows to grade the severity of the disease[26]. Direct quantification of collagen by CPA is another way to provide a detailed analysis of fibrosis and to predict clinical outcomes in patients with cirrhosis of mixed etiologies[16]. In our patients with cirrhotic ALD, high CPA values did not correlate with clinical events. Thus, in this situation, measurement of fibrosis density adds no clinically relevant information. Determination of the amount of fibrosis at an earlier stage of perisinusoidal collagen deposition could: (1) bring valuable prognostic information alone or in association with existing scores; (2) be closely correlated to liver stiffness as a non invasive monitoring method[15]; and (3) provide a promising research tool to monitor the possible regression of fibrosis.

The strength of this study includes a well characterized population of patients with chronic advanced ALD submitted to both hemodynamic and histological baseline evaluation and close follow-up during 12 mo. Nevertheless, we acknowledge that our study suffers from several limitations. First, we limited our study to patients with advanced ALD all at a cirrhotic stage, without providing data on the entire clinical spectrum of ALD. However, we decided to focus on cirrhotic subjects as they are the highest risk of clinical events and HVPG measurement have mostly been validated as prognostic factors in this population Secondly, only a minority of patients had liver stiffness measurement precluding any comparisons with CPA and HVPG. However, the usefulness of liver stiffness is limited in this population as ascites, a frequent complication of cirrhosis, limits the performance of liver stiffness measurement. Third, information on abstinence was self-reported leading to a risk of information bias that could possibly preclude association between HVPG/CPA and clinical outcomes in abstinent patients.

In conclusion, quantification of fibrosis on transjugular liver biopsy in advanced ALD correlated to portal pressure in the subgroup of active drinkers. The HVPG, but not CPA, predicts clinical events in active

alcohol drinkers pointing to the role of alcohol as an important modulator of PHT.

ARTICLE HIGHLIGHTS

Research background

Fibrosis staging in a liver biopsy is based on a semi-quantitative evaluation by the pathologist however inter-observer concordance may be a limiting factor. The quantitative measurement of liver fibrosis by a computer-assisted digital image analysis of a liver tissue specimen collagen proportionate area (CPA) overcomes some limitations of semiquantitative scores.

Research motivation

Previous studies have shown a good correlation between CPA and hepatic venous pressure gradient (HVPG) and association of CPA with prognosis. However, these results have been mostly obtained in HCV-related liver disease, and very few data are available in alcoholic liver disease (ALD).

Research objectives

The objective of the study was to explore the relationships between fibrosis density in liver biopsy, HVPG and development of clinical manifestations of portal hypertension (PHT) in patients with chronic advanced alcoholic liver disease (cALD) addressed for liver investigations. We aimed to better understand the relative prognostic contribution of HVPG and CPA in subjects with advanced ALD.

Research methods

We conduced a retrospective study with chart review of patients with ALD adressed to our center between January 2012 and December 2013 for a transjugular liver biopsy (TJLB) and hepatic hemodynamic study. Patients were included if they met the following criteria: (1) Medical indication for a liver biopsy in the setting of ALD; (2) recent (< 15 days) clinical, radiological, endoscopic and biological data available; (3) estimated follow-up of at least 6 mo. Liver tissue from cirrhotic subjects obtained from transjugular liver biopsies was stained with PicroSirius red and computer-assisted digital image analysis to determine fibrosis density using CPA was performed.

Research results

We included 61 patients with alcoholic ALD, subdivided in 41 active alcohol drinkers and 20 durably abstinent patients. Nine healthy liver donors served as controls. Mean CPA in patients with ALD was 7.1%, with no difference between active drinkers and abstinent patients (P = 0.17). Using a fibrosis density cutoff of 5%, we observed a positive correlation between high fibrosis density and the hepatic venous pressure gradient (HVPG) only in active drinkers (P = 0.02). At 12-month of follow-up, in the group of active alcohol drinkers, patients reaching a composite outcome showed a higher HVPG value as compared to those who did not (18.5 mmHg *vs* 14.5 mmHg P < 0.04) whereas CPA values were similar (6.9% *vs* 11%, P = 0.23).

Research conclusions

This is the first study exploring the relationships between fibrosis density assessed by CPA in liver biopsy, HVPG and development of clinical manifestations of portal hypertension in patients with ALD addressed for liver investigations. The results of this study suggest a positive correlation between high fibrosis density (using a fibrosis density cutoff of 5%), and HVPG only in active drinkers. At 12-mo of follow-up, in the group of active alcohol drinkers, patients reaching a composite outcome showed a higher HVPG value as compared to those who did not. Therefore, HVPG, but not CPA, predicts clinical events in active alcohol drinkers pointing to the role of alcohol as a modulator of portal hypertension.

Research perspectives

The validation of CPA as a quantitative method of measuring fibrosis density in liver biopsy of ALD patients requires further investigations in order to determine a correlation with clinical events, in particular overall and liver-related mortality in various subgroup of patients with difference etiologies of liver disease.

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ORIGINAL ARTICLE

Retrospective Study

Ratio of mean platelet volume to platelet count is a potential surrogate marker predicting liver cirrhosis

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Abstract

AIM

To provide a simple surrogate marker predictive of liver cirrhosis (LC).

METHODS

Specimens from 302 patients who underwent resection for hepatocellular carcinoma between January 2006 and December 2012 were retrospectively analyzed. Based on pathologic findings, patients were divided into groups based on whether or not they had LC. Parameters associated with hepatic functional reserve were compared in these two groups using Mann-Whitney *U*-test for univariate analysis. Factors differing significantly in univariate analyses were entered into multivariate logistic regression analysis.

RESULTS

There were significant differences between the LC group (n = 100) and non-LC group (n = 202) in prothrombin activity, concentrations of alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, cholinesterase, type Ⅳ collagen, hyaluronic acid, indocyanine green retention rate at 15 min, maximal removal rate of technitium-99m diethylene triamine penta-acetic acid-galactosyl human serum albumin and ratio of mean platelet volume to platelet count (MPV/PLT). Multivariate analysis showed that prothrombin activity, concentrations of alanine aminotransferase, aspartate aminotransferase, total bilirubin and hyaluronic acid, and MPV/PLT ratio were factors independently predictive of LC. The area under the curve value for MPV/PLT was 0.78,

with a 0.8 cutoff value having a sensitivity of 65% and a specificity of 78%.

CONCLUSION
The MPV/PLT ratio, which can be determined simply from the complete blood count, may be a simple surrogate marker predicting LC.

Key words: Mean platelet volume; Platelet count; Liver cirrhosis; Hepatic functional reserve; Liver fibrosis

Core tip: Although liver biopsy is considered the gold standard in the diagnosis of liver fibrosis and cirrhosis, liver biopsy is an invasive procedure, with attendant morbidity. Less invasive procedures are needed in the diagnosis of liver cirrhosis. Multivariate analysis showed that the mean platelet volume to platelet count ratio was independently predictive of liver cirrhosis. This ratio, which can be determined from a routine complete blood count, may be a simple surrogate marker predicting liver cirrhosis.

Iida H, Kaibori M, Matsui K, Ishizaki M, Kon M. Ratio of mean platelet volume to platelet count is a potential surrogate marker predicting liver cirrhosis. *World J Hepatol* 2018; 10(1): 82-87 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/82.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.82

INTRODUCTION

Mean platelet volume (MPV) is a machine-calculated measurement of average platelet size, usually included in complete blood count testing. Normal MPV ranges from 7.5 fL to 11.5 fL. Because average platelet size is directly proportional to the numbers of platelets produced, MPV is indicative of platelet production in bone marrow. Moreover, MPV is higher when there is destruction of platelets, as observed in patients with inflammatory bowel disease, immune thrombocytopenic purpura, myeloproliferative diseases and Bernard-Soulier syndrome[1]. MPV may also be higher in patients with pre-eclampsia and those recovering from transient bone marrow hypoplasia[2]. In contrast, abnormally low MPV values are indicative of thrombocytopenia because of impaired platelet production, as observed in patients with aplastic anemia.

Several studies have reported that liver cirrhosis (LC) and fibrosis are related to MPV[3-6]. Increased MPV, as well as decreased platelet count (PLT), were found to reflect a greater degree of fibrosis. These findings suggested that the ratio of MPV to PLT may correlate strongly with the degree of liver fibrosis. This study was, therefore, designed to determine whether liver fibrosis and LC are associated with the MPV/PLT ratio or not.

MATERIALS AND METHODS

This retrospective study assessed samples obtained from 302 patients who underwent liver resection for hepatocellular carcinoma (HCC) between January 2006 and December 2012. All patients were assessed pathologically by stage of fibrosis in nontumor liver tissue using the new Inuyama classification[7]. F0 was defined as no fibrosis (n = 22), F1 as chronic hepatitis with fibrous portal expansion (n = 67), F2 as chronic hepatitis with bridging fibrosis (n = 62), F3 as chronic hepatitis with bridging fibrosis and architectural distortion (n = 51), and F4 as LC with tendency toward nodular formation throughout the whole area. Patients classified as F0-F3 were assigned to the non-LC group (n = 202), and those classified as F4 to the LC group (n = 100).

Parameters associated with hepatic functional reserve were assessed in all patients; these included: MPV, PLT, and the MPV/PLT ratio; prothrombin activity (PT); concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin, cholinesterase, type Ⅳ collagen, and hyaluronic acid; indocyanine green retention rate at 15 min (ICGR15); and, the maximal removal rate of technitium-99m diethylene triamine penta-acetic acid (99mTc-DTPA)-galactosyl human serum albumin (GSA-Rmax), a marker of hepatic functional reserve, as determined by scintigraphy[8,9]. These factors were compared between the LC and non-LC groups. Multivariate regression analysis was performed to identify factors independently predictive of LC, and cutoff values were calculated. In addition, patients were divided by fibrosis stage (F0-F4), and these parameters were compared among the five subgroups.

Comorbidities that could be associated with an increase or decrease in the MPV/PLT ratio, such as inflammatory bowel disease, immune thrombocytopenic purpura, myeloproliferative disease or Bernard-Soulier syndrome, were not observed in any of the patients.

Statistical analysis

Parameters predictive of hepatic functional reserve in the LC and non-LC groups were compared using the Mann-Whitney *U*-test. Factors differing significantly in univariate analyses were entered into multivariate logistic regression analysis. Receiver operating characteristic (ROC) curves were used to calculate areas under the curve (AUC) and cutoff values. All analyses were performed using JMP 9 statistical analysis software (SAS Institute Inc., Cary, NC, United States), with a P value of < 0.05 defined as statistically significant.

RESULTS

There were 161 patients with hepatitis C and 53 patients with hepatitis B. The remaining 88 patients were negative for hepatitis B and C. The average age was 69.6 ± 9.7 years in the non-LC group and 68.2

Table 1 Univariate analysis of factors in non-liver cirrhosis and liver cirrhosis groups

	Non-LC group, *n* = 202	LC group, *n* = 100	*P*-value
Etiology			
HBV	43 (21.2%)	10 (10.0%)	
HCV	89 (44.1%)	72 (72.0%)	
NBNC	70 (34.7%)	18 (18.0%)	< 0.001
Edmonson-Steiner grade			
Ⅰ	30 (30.0%)	36 (17.8%)	0.07
Ⅱ	64 (64.0%)	151 (74.8%)	
Ⅲ	4 (4.0%)	13 (6.4%)	
Ⅳ	2 (2.0%)	2 (1.0%)	
PLT, × 10^4/μL	18.9 ± 8.1	11.6 ± 4.6	< 0.0001
MPV, fL	10.2 ± 0.9	10.8 ± 0.9	< 0.0001
MPV/PLT ratio	0.64 ± 0.30	1.10 ± 0.51	< 0.0001
PT, %	92.9 ± 11.7	82.5 ± 11.1	< 0.0001
AST, IU/L	42 ± 26	51 ± 23	< 0.0001
ALT, IU/L	40 ± 30	47 ± 33	0.02
Total-bilirubin, mg/dL	0.67 ± 0.23	0.90 ± 0.34	< 0.0001
Albumin, g/dL	3.8 ± 0.5	3.6 ± 0.4	0.01
Cholinesterase, IU/L	235 ± 80	193 ± 60	< 0.0001
Type 4 collagen, ng/mL	6.3 ± 2.4	9.1 ± 4.4	0.04
Hyaluronic acid, ng/mL	147 ± 179	312 ± 318	< 0.0001
ICGR15, %	14.1 ± 8.3	22.1 ± 12.1	< 0.0001
GSA Rmax, mg/min	0.62 ± 0.21	0.44 ± 0.17	< 0.0001

All results reported as mean ± standard deviation. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GSA-Rmax: Maximal removal rate of 99mTc-DTPA-galactosyl human serum albumin; HBV: Positive for hepatitis B antigen; HCV: Positive for hepatitis C antibody; ICGR15: Indocyanine green retention rate at 15 min; LC: Liver cirrhosis; MPV: Mean platelet volume; PLT: Platelet count; PT: Prothrombin activity.

± 7.6 years in the LC group (P = 0.21). The ratio of males to females was larger in the non-LC group, with 164 (81.2%) male and 38 (18.8%) female patients; there were 69 (69.0%) male and 31 (31.0%) female patients in the LC group (P = 0.02).

Table 1 compares parameters (univariate analysis) between the LC and non-LC groups. The rate of hepatitis C was greater in the LC group than in the non-LC group (P < 0.001). The Edmondson-Steiner grade[10] for HCC grade Ⅰ was a little smaller and for grade Ⅱ a little larger in the LC group; however, the difference was not significant (P = 0.07). The average PLT was 11.6 ± 4.6 × 10^4/μL and 18.9 ± 8.1 × 10^4/μL, respectively, and the average MPV was 10.8 ± 0.9 fL and 10.2 ± 0.9 fL, respectively (P < 0.05 for each). The MPV/PLT ratio was significantly higher in the LC group than in the non-LC group (1.10 ± 0.51 *vs* 0.64 ± 0.30, P < 0.05). Other factors associated with hepatic functional reserve also differed significantly between the two groups, including PT, the concentrations of AST, ALT, total bilirubin, albumin, cholinesterase, type Ⅳ collagen and hyaluronic acid, ICGR15 and GSA-Rmax (P < 0.05 for each).

Table 2 shows multivariate analysis of factors predictive of LC in these patients. MPV/PLT ratio, PT, and concentrations of AST, ALT, total bilirubin and hyaluronic acid were independent predictors of LC. The highest odds ratio was 3.71 for the MPV/PLT ratio. Although albumin, cholinesterase and type Ⅳ collagen concentrations, as well as ICGR15 and GSA-Rmax, were also predictors of LC on univariate analysis, they were not independently predictive on multivariate analysis.

The ROC curves of all six independently predictive factors (MPV/PLT ratio, PT, and concentrations of AST, ALT, total bilirubin and hyaluronic acid) are shown in Figure 1. Calculation of AUC for all six factors showed that the MPV/PLT ratio had the highest AUC (0.78). A cutoff value of 0.8 had a sensitivity of 65% and a specificity of 78% in predicting LC. This ratio was a better predictor of LC than other parameters of hepatic functional reserve.

Patients were also divided by individual fibrosis stage and MPV/PLT ratio determined for each stage. The average MPV/PLT ratios for patients classified as F0-1, F2, F3 and F4 were 0.54 ± 0.24, 0.65 ± 0.29, 0.79 ± 0.35 and 1.10 ± 0.51, respectively, with each pairwise difference being statistically significant (Figure 2).

Additionally, we examined the correlation between the MPV/PLT ratio and the pathological inflammation level according to the new Inuyama classification. The average MPV/PLT ratios for patients classified as A0, A1, A2 and A3 were 0.68 ± 0.21, 0.70 ± 0.45, 0.82 ± 0.39 and 0.73 ± 0.10, respectively. There was no significant correlation between MPV/PLT and pathological inflammation level (P = 0.214).

DISCUSSION

LC is a result of advanced liver disease, in which normal liver tissue is replaced by fibrotic tissue. These changes lead to loss of liver function. LC is most frequently caused by alcoholism, infection with hepatitis B and hepatitis C viruses, and fatty liver

Table 2 Multivariate analysis of factors predicting liver cirrhosis

		Odds ratio	*P*-value	95%CI
MPV/PLT ratio	⩾ 0.71, *n* = 151 < 0.71, *n* = 151	3.71	< 0.0001	1.94-7.28
PT, %	⩾ 89.0, *n* = 151 < 89.0, *n* = 151	2.68	0.0018	1.44-5.06
AST, IU/L	⩾ 39, *n* = 155 < 39, *n* = 147	3.30	0.01	1.30-9.09
ALT, IU/L	⩾ 34, *n* = 153 < 34, *n* = 149	2.57	0.04	1.02-7.09
Total-bilirubin, mg/dL	⩾ 0.7, *n* = 177 < 0.7, *n* = 125	1.89	0.04	1.00-3.61
Albumin, g/dL	⩾ 3.8, *n* = 168 < 3.8, *n* = 134	0.95	0.89	0.47-1.89
Cholinesterase, IU/L	⩾ 211, *n* = 152 < 211, *n* = 150	0.98	0.96	0.48-1.98
Type 4 collagen, ng/mL	⩾ 6.5, *n* = 166 < 6.5, *n* = 136	0.95	0.86	0.51-1.72
Hyaluronic acid, ng/mL	⩾ 124, *n* = 153 < 124, *n* = 149	2.28	0.008	1.23-4.26
ICGR15, %	⩾ 14.3, *n* = 151 < 14.3, *n* = 151	1.40	0.30	0.72-2.68
GSA Rmax, mg/min	⩾ 0.555, *n* = 151 < 0.555, *n* = 151	1.51	0.24	0.75-3.04

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; GSA-Rmax: Maximal removal rate of 99mTc-DTPA-galactosyl human serum albumin; ICGR15: Indocyanine green retention rate at 15 min; MPV: Mean platelet volume; PLT: Platelet count; PT: Prothrombin activity.

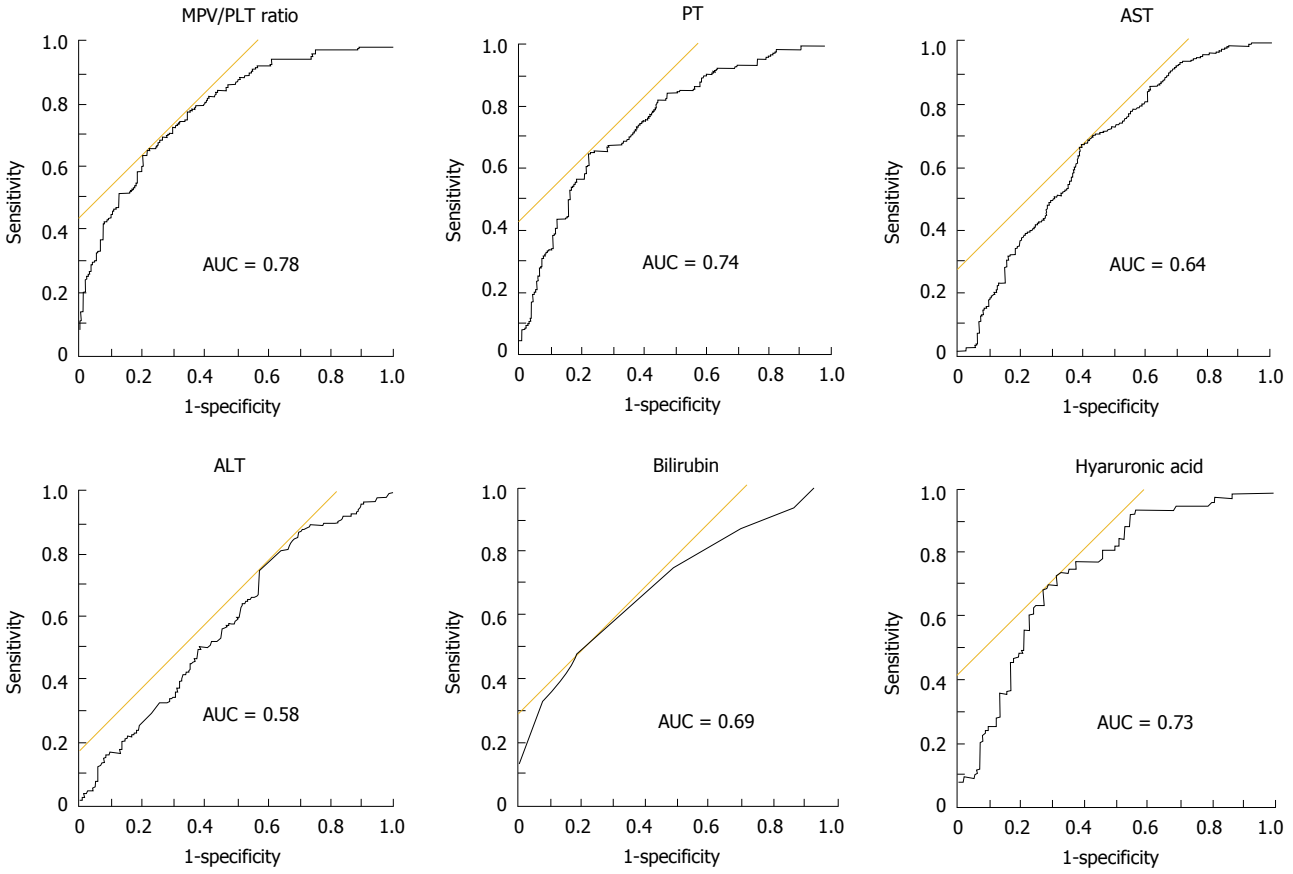

Figure 1 Receiver operating characteristic curve analysis of parameters differing significantly in the liver cirrhosis and non-liver cirrhosis groups on multivariate analysis. The area under the curve of MPV/PLT was the highest. A MPV/PLT ratio of 0.8 had a sensitivity of 65% and a specificity of 78%. MPV/PLT: Mean platelet volume to platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin activity.

disease, but it may have many other causes. LC arising from nonalcoholic steatohepatitis (NASH) was recently

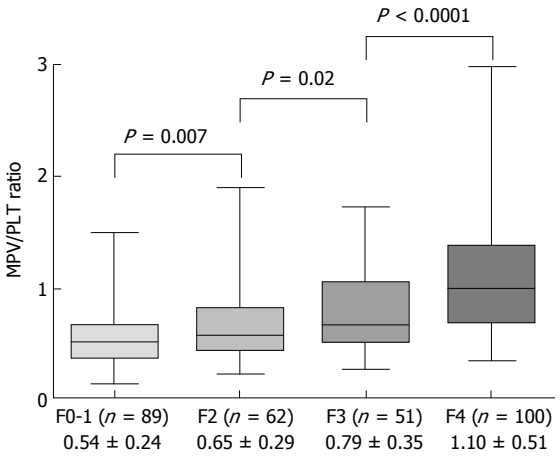

Figure 2 Relationship between MPV/PLT ratio and fibrosis stage. There were significant differences between each pair of stages. MPV/PLT: Mean platelet volume to platelet count.

shown to be a worldwide problem.

The standard method of diagnosing LC is liver biopsy. However, liver biopsy is an invasive procedure and cannot be performed on patients with severe ascites or severe coagulation disorders. Several studies have, therefore, assessed surrogate markers in blood predictive of LC; these include FibroTest (FibroSure) and the AST platelet ratio index[11]. In addition, elastographic methods, such as FibroScan, have been reported to be noninvasive methods of predicting LC[12,13]. We could not compare these methods with the MPV/PLT ratio because we do not have these examination devices.

MPV may be another predictor of LC. Higher MPV has been reported in patients with hepatitis B[14], and LC, fibrosis level and MPV have been reported to correlate in patients with chronic hepatitis B[3-5]. MPV may also be predictive of LC in patients with chronic hepatitis C[6].

MPV has been associated not only with fibrosis stage but with degree of liver inflammation[15]. For example, higher MPV has been observed in patients with NASH[16], and MPV has been found to correlate with the presence of nonalcoholic fatty liver disease (NAFLD)[17], although another study reported no correlation[18]. In addition, MPV may or may not correlate with insulin resistance, which is closely related to NAFLD[19,20]. To date, however, the relationship between NAFLD and MPV has not been determined.

MPV has been reported to strongly correlate with the prognosis of patients with non-small cell lung cancer[21]. In addition, high MPV and MPV/PLT have been found to be associated with a high risk for HCC[22,23]. An examination of the correlation between MPV/PLT ratio and postoperative prognosis of patients undergoing hepatic resection for HCC found that the MPV/PLT ratio was unrelated to overall or recurrence-free survival rate after resection.

This study had several limitations, including its retrospective design, performance at a single center and small sample size. Moreover, all patients included had undergone resection for HCC. Therefore, the results should not be generalized to patients without HCC before verification. Prospective, multicenter studies with large numbers of patients are needed to confirm these findings.

In conclusion, we found that the MPV/PLT ratio was predictive of LC, suggesting that this ratio may be a simple surrogate marker predictive of LC.

ARTICLE HIGHLIGHTS

Background

Several noninvasive methods for predicting cirrhosis have been reported, but liver biopsy is the only method for obtaining a definitive diagnosis. However, liver biopsy is invasive, and a noninvasive diagnostic method is desirable. Mean platelet volume (MPV), the size of platelets, can be determined from routine complete blood count data of blood samples. Generally, if bone marrow hematopoietic function decreases, MPV decreases. In contrast, if spleen function increases, new platelets are made rapidly and MPV increases. In recent years, the relationship between MPV and liver disease has attracted attention.

Research frontiers

There are reports that MPV correlates with liver function, and there are reports that MPV is related to the incidence of HCC. However, there is no report to evaluate the correlation between MPV/platelet count (PLT) and liver function, so we undertook this study.

Innovations and breakthroughs

The authors studied only patients who were diagnosed with cirrhosis histopathologically after liver resection. The MVP/PLT ratio could predict cirrhosis more sensitively than other general liver function tests.

Applications

The MPV/PLT ratio also correlated with the degree of hepatic fibrosis according to the Inuyama classification. The authors examined the relationship between prognosis after hepatic resection of hepatocellular carcinoma and the value of the MPV/PLT ration, but unfortunately no correlation was found.

Terminology

MPV is the size of platelets and can be determined from routine complete blood count data of blood samples. Liver cirrhosis and fibrosis are related to MPV.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy of direct-acting antiviral treatment for chronic hepatitis C: A single hospital experience

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Abstract

AIM

To evaluate the efficacy of direct-acting antivirals (DAAs) in Kanto Rosai Hospital.

METHODS

All patients with hepatitis C virus (HCV) who underwent DAA prescription were enrolled in this study. The present study was a single center retrospective analysis using patients infected with HCV genotype 1 or 2. Resistance analysis was performed by using direct sequencing and cycleave PCR in genotype 1 patients treated with interferon (IFN)-free DAA. The primary endpoint was sustained virologic response at 12 wk after therapy (SVR12).

RESULTS

A total of 117 patients participated in the study, including 135 with genotype 1 and 42 with genotype 2. Of the 135 patients with genotype 1, 16 received protease inhibitor + IFN + ribavirin and all achieved

SVR. Of the 119 patients who received IFN-free DAA (in different combinations), 102 achieved SVR and 9 failed (7/9 were on daclatasvir/asunaprevir and 2/9 on ledipasvir/sofosbuvir). Efficacy analysis was done only for 43 patients who received daclatasvir/asunaprevir. From this analysis, Y93 resistance-associated substitutions were significantly correlated with SVR.

CONCLUSION

The SVR rate was 98% for genotype 1 and 100% for genotype 2. However, caution is needed for HCV NS5A resistance-associated substitutions that are selected by HCV NS5A inhibitors because cerebrovascular adverse events are induced by some DAA drugs.

Key words: Resistance-associated substitutions; Direct-acting antivirals; Sustained viral response; Hepatitis C

Core tip: Direct-acting antivirals have been approved for the treatment of hepatitis C virus (HCV) genotype 1 and 2 infections in Japan since 2011. In the new era of DAA therapy, predictors who fail to respond to DAA might be compromised by resistance-associated substitutions. There have been few reports of daclatasvir/asunaprevir failure because daclatasvir/asunaprevir is limited in Japan. Therefore, it might be important to report these cases for future research and treatment of HCV.

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INTRODUCTION

Hepatitis C is a worldwide health problem with 170 million carriers globally and 4 million new cases appearing per year[1]. Approximately 70% of hepatocellular carcinoma cases in Japan are attributable to hepatitis C virus (HCV) infection[2,3]. Since the late 1990s in Japan, the management of HCV infection has improved and there has been a decrease in the widespread use of non-sterile needles and blood transfusions[4-7]. Protease inhibitors such as simeprevir or telaprevir resulting in highly sustained virologic responses (SVRs) in HCV patients were introduced in 2011[8-10]. More recently, interferon (IFN)-free DAAs inhibiting key viral functions have become the mainstay of anti-HCV treatment[11-13]. Prior to the introduction of these therapeutic agents, IFN-based treatments were the standard therapy against HCV infection[14], despite the suboptimal SVR induced by this treatment (40%-50%). However, patients responding to IFN therapy and sustaining a loss of HCV RNA are generally regarded as being at low risk of developing liver cirrhosis or hepatocellular carcinoma (HCC)[4]. However, these continuous efforts and advances in anti-HCV therapy may influence improvements in the long-term outcome of patients with HCV.

In the new era of DAA therapy, the reason for patients' failure in responding to DAAs might be related to the presence or development of resistance-associated substitutions (RASs)[15,16]. The aim of this study was to characterize the treatment response of new DAAs in patients infected with HCV.

MATERIALS AND METHODS

Patients

Japanese patients aged 30-87 years with chronic HCV genotype 1 and genotype 2 infections and without decompensated cirrhosis were commenced with DAA treatment. Overall, 177 participants treated with telaprevir or simeprevir with pegylated (PEG)-IFN and ribavirin (RBV) or IFN-free DAA, and in whom SVR12 was judged between November 2012 and March 2017 at Kanto Rosai Hospital were included. Treatment-naïve and treatment-experienced patients were included.

Assessments

Parameters were defined by standard laboratory techniques in Kanto Rosai Hospital. HCV NS5A RASs at Y93 and L31 were detected by commercial direct sequencing and cycleave PCR (SRL Laboratory, Tokyo, Japan) as well as PCR-invader methods (BML Laboratory, Tokyo, Japan). HCV RNA was measured by COBAS *Taq*Man PCR assay version 2.0 (Roche, Tokyo, Japan), with a lower limit of quantification of 25 IU/mL. For 10 patients who received either telaprevir or simeprevir with PEG-IFN treatment, the IL28B genotype was defined by PCR amplification and sequencing of the rs8099917, rs1188122 and rs88103142 nucleotide polymorphisms (SRL Laboratory). HCV core amino acids 70 and 99 were defined by PCR direct sequencing (LSI Laboratory, Tokyo, Japan). Liver cirrhosis was diagnosed by ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI) or a liver biopsy.

The primary efficacy end point was the proportion of patients with undetectable HCV RNA at 12 wk post-treatment (SVR12).

Statistical analysis

Analyses were performed using STATA/MP14.0 software (Stata-Corp LP, College Station, TX, United States).

Ethical statement

Before any study procedures were undertaken, informed consent was obtained from all patients. This study conformed to the ethical guidelines of the Declaration of Helsinki, and was approved by the ethics committee of

Table 1 Baseline demographics and patient characteristics

Parameter	Overall, *n* = 177	Genotype 1					Genotype 2
		IFN/TVR/RBV *n* = 5	IFN/SMV/RBV *n* = 11	DCV/ASV *n* = 43	LDV/SOF *n* = 66	OBV/PTV/r *n* = 10	SOF/RBV *n* = 42
Age, median[1]	67.8 (11.0)	62.9 (8.7)	60.2 (8.9)	72.7 (8.3)	66.0 (11.2)	70.9 (6.5)	67.5 (12.6)
> 65, *n* (%)	118 (66.7)	3 (60)	4 (36.4)	37 (88.1)	39 (59.0)	7 (70)	29 (67.4)
Sex, *n* (%)							
Male	79 (44.6)	3 (60)	7 (63.6)	14 (32.6)	31 (47.0)	4 (40)	20 (47.6)
Female	98 (55.4)	2 (40)	4 (36.4)	29 (67.4)	35 (53.0)	6 (60)	22 (52.4)
HCV RNA, median Log_{10} LGE[1]	6.1 (0.8)	6.5 (0.56)	6.2 (1.1)	6.30 (0.5)	6.16 (0.6)	5.4 (0.9)	5.8 (0.9)
> 100000 IU/mL, *n* (%)	109 (61.6)	4 (80)	9 (81.8)	32 (76.2)	43 (0.7)	2 (20)	19 (45.2)
Cirrhosis present, *n* (%)							
Yes	74 (41.8)	0 (0)	0 (0)	34 (79.0)	29 (44.0)	3 (30)	8 (18.6)
No	103 (58.2)	5 (100)	11 (100)	9 (20.1)	37 (56.0)	7 (70)	34 (81.4)
HCV treatment history, *n* (%)							
Naïve	132 (74.6)	1 (20)	2 (18.2)	25 (58.1)	63 (95.5)	9 (90)	32 (76.2)
Prior IFN-based treatment	45 (25.4)	4 (80)	9 (81.8)	18 (41.8)	3 (4.5)	1 (1)	10 (23.8)
History of HCC, *n* (%)							
Yes	26 (14.7)	1 (20)	0 (0)	19 (44.1)	3 (4.5)	0 (0)	3 (9)
No	151 (85.3)	4 (80)	11 (100)	24 (55.8)	63 (95.5)	10 (100)	39 (90.7)
Laboratory values							
Baseline platelet count, mean ($\times 10^4/\mu L$)[1]	15.1 (6.5)	15.4 (3.4)	15.1 (6.2)	11.5 (5.8)	15.5 (6.5)	18.0 (5.96)	17.6 (6.0)
Baseline ALT level, mean (IU/L)[1]	51.2 (37.3)	41.8 (9.7)	50.1 (50.5)	53.1 (27.8)	60.3 (45.2)	39.9 (26.8)	38.9 (28.6)
Baseline AFP level, mean (ng/mL)[1]	12.1 (17.6)	5.6 (1.6)	7.18 (9.1)	23.4 (27.2)	8.99 (11.6)	9.9 (11.6)	6.8 (6.9)

[1]The standard deviation is given in parentheses. AFP: Alpha fetoprotein; ALT: Alanine aminotransferase; DCV/ASV: Daclatasvir/asunaprevir; HCV: Hepatitis C virus; IFN: Interferon; LDV/SOF: Ledipasvir/sofosbuvir; OBV/PTV/r: Ombitasvir/paritaprevir/ritonavir; RBV: Ribavirin; SMV: Simeprevir; TVR: Telaprevir.

Japan Organization of Occupational Health and Safety Kanto Rosai Hospital (2015-2017).

RESULTS

Baseline demographics and characteristics

Among 177 cases, 16 patients with genotype 1 were assigned to telaprevir or simeprevir with PEG-IFN and RBV, and 119 were assigned to IFN-free DAA [daclatasvir/asunaprevir (DCV/ASV), ledipasvir/sofosbuvir (LDV/SOF), ombitasvir/paritaprevir/ritonavir (OBV/PTV/r)]. Forty-two patients were treated with SOF and RBV for genotype 2. The average age ± standard deviation of the patients was 67.8 ± 11.0 years. Of these, the group with the highest average age of 72.7 ± 8.3 years was prescribed DCV/ASV. The number and proportion of males and females were 79 (44.6%) and 98 (55.4%), respectively. There were 74 cases (46.2%) with cirrhosis, including 21 cases diagnosed pathologically and 45 (25.4%) patients who had experienced IFN-based treatment previously. Twenty-six (14.7%) patients had a history of curative HCC (Table 1).

Among 16 patients with IFN-based protease inhibitor treatment, 10 were tested for the polymorphism NS5A region of IL28B, and HCV core amino acids 70 and 91. In both treatment groups, patients with the mutation who were predicted to have a low treatment response were included (Table 2).

Treatment response and efficacy of all DAA therapy

SVR12 was achieved in 167 of 177 (94.4%) patients. All 16 who received protease inhibitor with PEG-IFN and RBV (5 with teraprevir, 11 with simeprevir) achieved SVR12. All 42 patients with genotype 2 who received the treatment with SOF with RBV achieved SVR12. There was no case of relapse to the date of this paper. The response rate of the IFN-free DAA regimen (DCV/ASV, LDV/SOF, OBV/PTV/r) is shown in Table 3. Of the 43 patients who were treated with DCV/ASV, 1 patient broke through and 6 relapsed. Of the 66 patients on LDV/SOF, 2 relapsed and 2 had severe adverse events, including subarachnoid hemorrhage and cerebral hemorrhage. Although medication was stopped at 8 wk and 6 wk after prescription, SVR was achieved. Two patients also relapsed with LDV/SOF treatment. Of the 10 patients who have been on OBV/PTV/r, 1 was lost to follow-up.

Analysis of RASs

NS5A RASs were analyzed in 82 patients with IFN-free DAA treatment (Figure 1). Of these, 2 relapsed patients with wild-type Y93 and 1 with Y93 hetero were treated with DCV/ASV. Three relapsed patients with wild-type L31 were also treated with DCA/ASV. Another 6 patients that failed to achieve SVR with DAA treatment had not obtained NS5A RASs prior to treatment. Of the 9 failure patients, 7 were diagnosed as cirrhosis before DAA treatment, and 4 had a history of curative HCC (Table 4).

Patients who failed to respond to the initial IFN-free DAA regimen were given second-line therapies. Four patients were enrolled to LDV/SOF with RBV therapy in another hepatitis core hospital in Kanagawa prefecture and SVR was achieved in 3 of these patients, with 1 relapsing. One patient treated with LDV/SOF achieved

Table 2 Baseline characteristics of IL28B and NS5A polymorphisms

	IFN/TVR/RBV *n* = 5	IFN/SMV/RBV *n* = 5
IL28B SNP (*n*)		
rs8099917		
T/T	4	1
T/G	1	2
G/G	0	1
rs11881222		
A/A	4	1
A/G	0	2
G/G	1	1
rs88103142		
T/T	4	1
T/C	1	2
C/C	0	1
NS5A aa70[1]		
Wild-type	3	0
Mutant	2	4
Competitive	0	1
NS5A aa91[1]		
Wild-type	3	0
Mutant	2	2
Competitive	0	3

[1]aa HCV core amino acid. IFN: Interferon; RBV: Ribavirin; SMV: Simeprevil; TVR: Telaprevir.

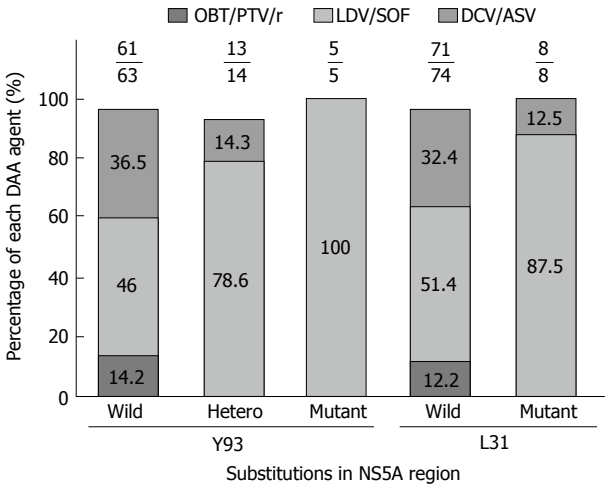

Figure 1 SVR rates for NS5A resistance-associated substitutions and each interferon-free agent. The number above each column is the number of cases with SVR (numerator) and total cases (denominator). Two relapsed patients with wild-type Y93, 1 with Y93 hetero and 3 relapsed patients with wild-type L31 were treated with DCV/ASV. Another 6 patients that failed to achieve SVR with DAA treatment had not obtained NS5A RASs prior to treatment. Another patient had no relapse regardless of the presence or absence of RASs. DAA: Direct-acting antivirals; DCV/ASV: Daclatasvir/asunaprevir; RAS: Resistance-associated substitution; SVR: Sustained virologic response.

SVR. One patient is now undergoing daclatasvir/asunaprevir/beclabuvir (known as DCV-TRIO) treatment (Table 4).

Of the 25 patients having HCC history and treated with IFN-free DAA, 4 had recurrence to date. Of these, 2 came back with extremely rapid growth of HCC.

Multivariable logistic regression for SVR factors using patients with DCV/ASV treatment was performed using two models. Regression using all baseline variables as covariates (model 1) showed HCV RNA levels were independently associated with SVR. Model 2 was built with suspected variables from DAA failure patients (Table 5) and showed that only Y93 RAS was associated with SVR (Table 5).

DISCUSSION

This study of patients with HCV infection demonstrated that high SVR rates can be achieved with DAA regimens including IFN-based protease inhibitor and IFN-free DAAs. DAAs conferred good effectiveness and safety for both treatment-naïve patients and previously treated cases.

Until recently, PEG-IFN combined with RBV therapy was the only antiviral drug regimen capable of terminating HCV infection[8]. However, SVR was only achieved in about 50% of treated patients[17-19]. Many DAAs have been designed to improve this situation[20]. To activate the IFN pathway, telaprevir, boceprevir and simeprevir were introduced as 1st and 2nd generation HCV protease inhibitors[8-10,20]. However, these agents increase the risk of adverse events, such as anemia, renal failure and severe drug rash. In the initial IFN-free regimen, DCV/ASV eliminated IFN-related toxicity and achieved a SVR24 rate of 84% in chronic hepatitis C patients and 90.9% in liver cirrhosis cases in Japan[21]. The SVR12 rate of LDV/SOF was 100%[12] and for OBV/PTV/r it was 98%[22] in genotype 1 HCV. SOF/RBV and OBV/PTV/r have been approved for genotype 2 HCV, which accounts for up to 30% of chronic HCV infection and which is increasing in prevalence in Japan[23]. Although OBV/PTV/r was limited to use for genotype 2b, the SVR rate was 95-98% when RBV was used[23-25]. The use of IFN-free DAA enables the treatment of IFN ineligible/intolerant individuals with HCV infection.

A low rate of virological failure in genotype 1 was observed in patients with baseline Y93 or L31 variants in NS5A receiving DCV/ASV or OBV/PTV/r treatment[13,22]. It has been reported that pretreatment with NS5A RASs did not impact LDV/SOF therapy[26].

Moreover, there have been few reports of DCV/ASV failure because DCV/ASV is limited in Japan. Therefore, it might be important to report these cases for future research and treatment of HCV.

In the present report, the SVR rate of each therapy in Kanto Rosai Hospital was similar to previous reports[12,13,21,23,27]. In genotype 1 patients, 7 failures with DCV/ASV and 2 with LDV/SOF were reported. Among these, 7 patients were diagnosed cirrhosis and 4 patients who had a history of HCC were also reported. Y93 RAS was correlated to SVR failure in DCV/ASV cases. In 2 relapsers with LDV/SOF, DAA RAS could not detected. Subsequently, it was revealed that the core genotype of HCV was 1a and 2a in these patients.

We experienced 2 patients with subarachnoid

Table 3 Response during and after treatment with direct-acting antivirals

Response	Overall, *n* = 119	Genotype 1			Genotype 2
		DCV/ASV *n* = 43	LDV/SOF *n* = 66	OBV/PTV/r *n* = 10	SOF + RBV *n* = 42
HCV RNA < LLOQ during treatment[1], *n* (%)	119 (100)	41 (100)	66 (100)	9 (90)[3]	42 (100)
HCV RNA < LLOQ after end of treatment[1], *n* (%)	118 (98.3)	42 (97.6)	66 (100)	9 (90)[3]	42 (100)
SVR12[2], *n* (%)	109 (91.6)	35 (83.3)	64 (97)	9 (90)[3]	42 (100)
On-treatment failure, *n* (%)	1 (0.8)	1 (2.3)	0 (0)	0 (0)	0 (0)
Relapse, *n* (%)	8 (6.7)	6 (16.7)	2 (3)	0 (0)	0 (0)

[1]LLOQ (lower limit of quantification) = 25 IU/ML; [2]SVR: Sustained virologic response; [3]One case lost to follow-up. DCV/ASV: Daclatasvir/asunaprevir; LDV/SOF: Ledipasvir/sofosbuvir; OBV/PTV/r: Ombitasvir/paritaprevir/ritonavir; RBV: Ribavirin.

Table 4 NS5A RASs and clinical course in patients with failure of DAAs

Patient No.	Sex	Age in yr	LC[1]	HCC[2]	Initial DAA	NS5A RASs			Second DAA	Second result
						Before DAA	After DAA (invader)	After DAA (cycleave)		
1	Female	73	No	No	DCV/ASV	NA	Y93H L31F Q54H A92V	Y93 mutant L31 mutant	LDV/SOF/RBV	SVR
2	Female	77	Yes	Yes	DCV/ASV	NA	Y93H L31M Q24Q/R	Y93 mutant L31 mutant	LDV/SOF/RBV	Relapse
3	Female	71	Yes	No	DCV/ASV	NA	NA	Y93 wild-type L31mutant	LDV/SOF/RBV	SVR
4	Female	78	Yes	No	DCV/ASV	NA	NA	Y93 mutant L31 mutant	LDV/SOF/RBV	SVR
5	Male	74	Yes	Yes	DCV/ASV	NA	Y93H L31V Q54y Q62D	Y93 mutant L31 mutant	LDV/SOF	SVR
6	Female	83	Yes	Yes	DCV/ASV	Y93Y/H L31L	Y93H L31M L31V	Y93 mutant L31 wild-type	No	NA
7	Male	71	Yes	No	DCV/ASV	Y93Y L31L	NA	Y93 wild-type L31 mutant	DCV-TRIO	Undergoing
8	Female	66	No	No	LDV/SOF	Y93Y L31L	NA	Failure	Waiting	NA
9	Male	78	Yes	Yes	LDV/SOF	NA	NA	Failure	Waiting	NA

[1]Diagnosed as cirrhosis; [2]A history of curative treatment for hepatocellular carcinoma. DAA: Direct-acting antivirals; DCV/ASV: Daclatasvir/asunaprevir; DCV-TRIO: Daclatasvir/asunaprevir/beclabuvir; LDV/SOF: Ledipasvir/sofosbuvir; NA: Data not available; RAS: Resistance-associated substitution; RBV: Ribavirin; SVR: Sustained virologic response. Failure: Could not be detected.

Table 5 Multivariable logistic regression models for SVR in patients with DCV/ASV

	Odds ratio	95%CI	*P*-value
Model 1: All variables			
Platelet count	0.00	-0.01-0.27	0.71
AFP level	0.00	-0.00-0.01	0.44
ALT level	0.00	-0.00-0.01	0.31
HCV RNA level	0.26	0.02-0.45	0.04[a]
Age	0.02	-0.01-0.04	0.14
Sex	-0.13	-0.39-0.12	0.28
Y93	0.23	-0.31-0.77	0.38
L31	-0.17	-1.05-0.70	0.68
History of HCC	-0.29	-0.68-0.92	0.13
Cirrhosis	-0.30	-0.38-0.26	0.67
Prior IFN	-0.15	-0.41-0.99	0.21
Model 2: Limited suspicious covariates			
Age	0.00	-0.13-0.14	0.93
Y93	0.48	0.08-0.87	0.02[a]
L31	-0.42	-1.09-0.24	0.2
Cirrhosis	-0.15	-0.37-0.08	0.19

Model 1: The baseline model considered with all covariates obtained. Model 2: Limited to covariates suspected from Table 4. [a]$P < 0.05$ were considered statistically significant. AFP: Alpha fetoprotein; ALT: Alanine aminotransferase; HCC: Hepatocellular carcinoma; IFN: Interferon.

hemorrhage and cerebral hemorrhage, and these discontinued LDV/SOF therapy. They were 51- and 68-year-old females without cirrhosis and other medical history. In 2016, postmarketing surveillance data were reported in Japan, and 31 cases of severe cerebrovascular disease were reported[28]. As far as we know, there is no detailed report about cerebrovascular adverse reaction. Therefore, the physiological mechanism underlying the cerebrovascular adverse events is unclear. Caution is needed when prescribing LDV/SOF therapy.

Two patients had aggressive and rapid HCC recurrence after treatment with DAA. The assumption that the use of DAAs may induce HCC relapse had been reported[29]. The surveillance of HCC must be taken strictly after DAA treatment in patients with prior HCC.

Recent reports demonstrated that the SVR rate was only 69% for salvage therapy for patients who failed to respond to NS5A inhibitors[30]. Prior DCV/ASV treatment is associated with a failure of LDV/SOF for multiple HCV NAS5A RASs[30,31].

We could not treat patients with LDV/SOF and RBV simultaneously because this treatment regimen has not been approved for general insurance. However, the ratio of SVR increased to 75% in initial DAA failure patients, even though multiple NS5A RASs were observed.

The achievement of an SVR of 100% for overall patients with HCV infection may be accomplished in the future.

This study had some limitations. First, data for RASs were not available for all cases. Due to the small sample size, the power of the multiple regression analysis remains low. Second, because this was a study from one hospital, the total number of treatment

cases was small. Third, because DCV/ASV has only been approved in Japan, there are some limitations regarding the generalizability of the results. However, this study provides some important knowledge about HCV treatment.

In conclusion, DAA treatment for HCV infection is highly effective in Kanto Rosai Hospital. However, caution is needed for HCV NS5A RASs that are selected by HCV NS5A inhibitors because cerebrovascular adverse events are induced by some DAA drugs.

ARTICLE HIGHLIGHTS

Research background

In a previous study, it was shown that resistance-associated substitutions (RASs) were predictors of direct-acting antiviral (DAA) failure. No significant adverse effect was reported in the DAA treatment in clinical trials. In this study, the prestudy hypothesis was that another predictor might exist concerning about DAA failure. Another hypothesis was that more severe adverse effects must occur in the real world because patients conditions were more severe than those of clinical trials.

Research motivation

DAAs have been approved for the treatment of hepatitis C virus (HCV) genotype 1 and 2 infections in Japan since 2011. In the new era of DAA therapy, predictors who fail to respond to DAA might be compromised by RASs. There have been few reports of daclatasvir/asunaprevir (DCV/ASV) failure because DCV/ASV is limited in Japan. Therefore, it might be important to report these cases for future research and treatment of HCV.

Research objectives

All patients with HCV infection who underwent DAA prescription were enrolled in this study. Overall, 177 participants treated with DAAs and in whom sustained virologic response at 12 wk after therapy (SVR12) was judged between November 2012 and March 2017 at Kanto Rosai Hospital were included.

Research methods

HCV patients who underwent DAA prescription were enrolled in this study. Resistance analysis was performed by using direct sequencing and cycleave PCR. Multiple regression analysis was performed to evaluate factors related to loss of HCV RNA.

Research results

In total, 117 patients participated in the study, including 135 with genotype 1 and 42 with genotype 2. Of the 135 patients with genotype 1, 16 received protease inhibitor + interferon + ribavirin and all achieved SVR. Of the 119 patients who received interferon-free DAA (in different combinations), 102 achieved SVR while 9 failed, including 7/9 who were on DCV/ASV and 2/9 who were on ledipasvir/sofosbuvir. Efficacy analysis was done only for 42 patients who received DCV/ASV. From this analysis, Y93 RASs were significantly correlated with SVR.

Research conclusions

The SVR rate was 98% for genotype 1 and 100% for genotype 2. NS5A RASs are most likely to affect the outcomes of DAA therapy in our facility.

Research perspectives

The SVR rate was 98% for genotype 1 and 100% for genotype 2. However, caution is needed for HCV NS5A RASs that are selected by HCV NS5A inhibitors because cerebrovascular adverse events are induced by some DAA drugs.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy of intra-arterial contrast-enhanced ultrasonography during transarterial chemoembolization with drug-eluting beads for hepatocellular carcinoma

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Author contributions: Shiozawa K, Watanabe M and Igarashi Y designed the study; Yamamoto S, Matsui T, Saigusa Y, Shiozawa K, Ikehara T and Watanabe M performed transarterial chemoembolization with drug-eluting beads for hepatocellular carcinoma; Watanabe M performed intra-arterial contrast-enhanced ultrasonography with Sonazoid®; Shiozawa K and Watanabe M analyzed the data and wrote the manuscript; Maetani I supervised the study; all authors have read and approved the final version to be published.

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Abstract

AIM

To assess the usefulness of intra-arterial contrast-enhanced ultrasonography (IAUS) during transarterial chemoembolization (TACE) with drug-eluting beads (DEB) for hepatocellular carcinoma (HCC).

METHODS

Thirty two patients with 39 HCC underwent DEB-TACE guided with IAUS, and examined by contrast-enhanced

ultrasonography (CEUS) or dynamic CT after DEB-TACE were enrolled in this study. CEUS findings before DEB-TACE and IAUS findings were compared. Treatments judged to be complete and incomplete for lesions were appropriate and insufficient, respectively. Findings on CEUS and/or dynamic CT performed 1, 3 and 6 mo after DEB-TACE were evaluated using mRECIST (CR/PR/SD/PD).

RESULTS

The treatments were complete and incomplete in 26 and 13 lesions, respectively. On imaging evaluation using CEUS and/or dynamic CT one month after treatment, 25 and 1 lesions were judged to be CR and PR, respectively, and at 6 mo after treatment, the results were CR, PR, SD and PD for 24, 1, 0 and 1 of these lesions, respectively, in the 26 completely treated lesions. Of the 13 lesions in which treatment was incomplete, the results on imaging at one month after treatment were CR, PR, SD and PD for 0, 6, 4 and 3 lesions, respectively. The overall CR rate at 6 mo after treatment was 61.5% (24/39).

CONCLUSION

A combination of DEB-TACE with IAUS can improve the therapeutic effects in patients with HCC.

Key words: Hepatocellular carcinoma; Contrast-enhanced ultrasonography; Drug-eluting beads; Transarterial chemoembolization; Intra-arterial contrast-enhanced ultrasonography

Core tip: To assess the usefulness of intra-arterial contrast-enhanced ultrasonography (IAUS) during transarterial chemoembolization (TACE) with drug-eluting beads (DEB) for 39 hepatocellular carcinoma (HCC). Complete and incomplete treatments were 26 and 13, respectively. One month after treatment, 25 and 1 lesions were judged to be CR and PR, respectively, and at 6 mo after treatment, the results were CR, PR, SD and PD for 24, 1, 0 and 1 of these lesions, respectively, in the 26 completely treated lesions. The overall CR rate at 6 mo after treatment was 61.5% (24/39). A combination of DEB-TACE with IAUS can improve the therapeutic effects in HCC patients.

Shiozawa K, Watanabe M, Ikehara T, Yamamoto S, Matsui T, Saigusa Y, Igarashi Y, Maetani I. Efficacy of intra-arterial contrast-enhanced ultrasonography during transarterial chemoembolization with drug-eluting beads for hepatocellular carcinoma. *World J Hepatol* 2018; 10(1): 95-104 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/95.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.95

INTRODUCTION

Transarterial chemoembolization (TACE) improves survival in patients with intermediate-stage (Barcelona Clinic Liver Cancer[1]; BCLC classification B) hepatocellular carcinoma (HCC)[2]. Drug-eluting beads (DEB) are polyvinyl alcohol-based microspheres that can be loaded with anthracycline drugs, such as doxorubicin[3]. DEB-TACE is now used in catheter-based locoregional therapy that takes advantage of the arterial supply to HCC and spares the surrounding hepatic parenchyma, which receives most of its blood supply from the portal vein[4,5]. When injected through a catheter or microcatheter at the tumor site, DEB act as embolic material, causing tumor ischemia, and also release drugs in a sustained and controlled manner[6].

TACE is commonly guided by digital subtraction angiography (DSA). In DEB-TACE, it is particularly important to prevent inflow of DEB into normal hepatic parenchyma and to administer the beads into the tumor artery because of their effect as a permanent embolic material[7]. However, some HCCs are difficult to visualize on DSA for DEB-TACE due to the complex blood supply.

Contrast-enhanced ultrasonography (CEUS) enables tumor visualization without use of ionizing radiation or the risk of nephrotoxicity associated with contrast-enhanced computed tomography (CT) and catheter-based studies[8,9]. In addition, CEUS is useful for evaluation of the hemodynamics of hepatic tumors and surrounding hepatic parenchyma in real time. We have evaluated the efficacy of HCC treatment with sorafenib (Nexavar; Bayer Healthcare, Leverkusen, Germany) and CyberKnife® (Accuray Incorporated, Sunnyvale, CA, United States) by CEUS using Sonazoid® (Daiichi Sankyo, Tokyo, Japan)[10,11].

Transcatheter (intra-arterial) CEUS (IAUS) has recently been used in DEB-TACE for HCC and metastatic hepatic tumors, and its safety and efficacy in identifying the feeding artery have been evaluated[12-16]. However, the therapeutic effect using IAUS as support for DEB-TACE in HCC has not been examined. In this study, we evaluated the usefulness of IAUS using Sonazoid® in DEB-TACE for HCC.

MATERIALS AND METHODS

Patient characteristics

Of patients who received TACE for HCC at our hospital between June 2015 and September 2016, 32 patients (39 lesions) with lesions visualizable by ultrasonography (US) who gave consent to DEB-TACE and IAUS and could be examined by CEUS or dynamic CT at 1, 3 and 6 mo after DEB-TACE and every 3-4 mo thereafter were included in the study. The patients were 28 males and 4 females; the median age was 73 years old; the underlying liver disease was hepatitis B in 4

Table 1 Baseline patient and tumor characteristics

Characteristics	All (n = 32)
Age (yr) (median)	72 (range 44-89)
Gender: Male/female	28/4
Etiology	
Alcohol/HBV/HCV/NASH	14/4/12/2
Child-Pugh classification A/B	23/9
Previous treatment (y/n)	18/14
Tumor number	39
Tumor size (mm) (median)	21 (range 8-50)
Tumor location	
Peripheral/central	26/13
DCBead (100-300 μm, mL) (median)	0.6 (range 0.2-1.5)
Vascular lake (y/n)	7/32 (22%)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis.

patients, hepatitis C in 12, alcoholic liver disease in 14, and non-alcoholic steatohepatitis (NASH) in 2; and the median tumor diameter was 21 mm (Table 1). All patients were diagnosed with HCC using gray-scale US, dynamic CT and Gd-EOB-DTPA-enhanced magnetic resonance imaging, based on the new guidelines of the American Association for the Study of Liver Diseases[17]. Serum α-fetoprotein (AFP), AFP-L3 fraction, and des-γ-carboxyprothrombin (DCP) levels were referred to for diagnosis, as needed.

We chose all patients based on the Evidence-based Clinical Practice Guidelines for HCC developed by the Japan Society of Hepatology[18]. All 32 patients had hepatic cirrhosis with Child-Pugh classification A or B and an Eastern Cooperative Oncology Group performance status < 2[19]. The all lesions were single HCC ≤ 50 mm in diameter or up to 3 HCCs ≤ 30 mm in diameter. Tumors were selected that were difficult to treat with radiofrequency ablation, such as those with the presence of ascites, close to vessels, or on the liver surface in patients who did not want surgical resection. Exclusion criteria were non-visualizable lesions on US in the supine position, advanced-stage HCC in the BCLC classification, serum total bilirubin > 3 mg/dL, history of heart or renal impairment, iodine allergy, and egg allergy.

The study was performed in accordance with the Declaration of Helsinki, the International Conference on Harmonization Guidelines for Good Clinical Practice, and local laws and regulations. The study was performed after approval by the Ethics Committee of Toho University Medical Center Omori Hospital. All patients provided written informed consent for DEB-TACE and IAUS.

Pretreatment CEUS procedure

Gray-scale US and CEUS using Sonazoid® were performed within one month before DEB-TACE in all patients. All CEUS was performed by two sonographers with 25 and 18 years of experience, respectively, using an Aplio XG (Toshiba Medical Systems, Tokyo, Japan) with a convex probe (PVT-375BT, 3.75-MHz center frequency). The mechanical index (MI) for the acoustic output was set to 0.2 and the dynamic range was set to 60-65 dB. In patients in whom lesions were detected by gray-scale US, the single focus point was set at the lower margin of the lesion. An intravenous bolus injection of Sonazoid® (0.5 mL) was administered *via* a left cubital venous line, followed by flushing with 10 mL of normal saline. The dynamics of enhancement of the lesion were observed in the vascular phase (arterial phase, 0-40 s; portal venous phase, 40-120 s; late phase, > 120 s), and in the postvascular phase 10 min after injection. Subsequently, the feeding blood vessel was identified by re-injection of Sonazoid®[20] in as many lesions as possible.

DEB-TACE procedure

Dynamic CT was performed within one month before DEB-TACE in all patients, a 3D-CT angiogram was prepared, and abdominal angiography was performed with reference to the CT findings. DEB-TACE was performed by an expert interventional radiologist and hepatologist with 31 years of experience and a hepatologist with 19 years of experience. The right groin and upper abdomen were cleansed with iodine and the patient was draped under sterile cloths with exposure of the right groin and upper abdomen. Through a right femoral artery access and after placement of a 3 Fr shepherd hook catheter (Terumo, Tokyo, Japan), the celiac trunk was examined through the arterial and venous phases to define the hepatic artery anatomy and to assess portal vein patency. Then, the hepatic artery was selectively catheterized (segment/subsegment) to study the arterial supply of the target lesions. A coaxial microcatheter [Haruka® (JMS Co., Ltd. Tokyo, Japan) or Wonder III® (UTM Co., Ltd. Aichi, Japan)] was advanced in every feeding artery to allow embolization of the lesion with drug-eluting microspheres [DC beads® (Eisai Co., Ltd. Tokyo, Japan), 100-300 μm in diameter, 1 vial loaded with 50 mg of epirubicin® (Nippon Kayaku Co., Ltd. Tokyo, Japan)] until contrast medium disappeared from the blood vessel within 5-6 heart beats. At this time, DSA and IAUS were performed and tumor enhancement was evaluated. If residual tumor enhancement was observed in any images, DEB-TACE was repeated and disappearance of as much tumor enhancement as possible was confirmed using IAUS, after which the treatment was considered complete.

IAUS procedure

The lesion location was identified with gray-scale US, immediately prior to an echo-contrast study. IAUS was performed by administration of Sonazoid® through the microcatheter and imaging of the area of the target lesion with a dedicated, contrast-specific technique. IAUS was performed by the same expert interventional radiologist and hepatologist with 31 years of experi-

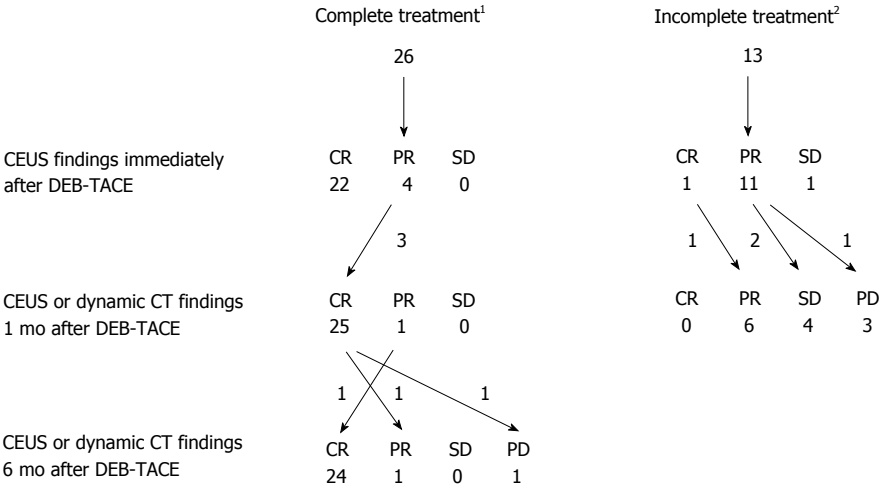

Figure 1 Patient flow diagram. In this diagram, treatment response evaluated by contrast-enhanced ultrasonography (CEUS) and/or dynamic computed tomography (CT) immediately, one month and six months after DEB-TACE for hepatocellular carcinoma (HCC) using mRECIST. Findings on CEUS or dynamic CT were evaluated using mRECIST criteria. [1]Complete treatment: Disappearance of the tumor enhancement after DEB-TACE by IAUS findings; [2]Incomplete treatment: Presence of residual tumor enhancement after DEB-TACE by IAUS findings. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

ence using an Aplio 400 (Toshiba Medical Systems, Tokyo, Japan) with a convex probe (PVT-375BT, 3.75-MHz center frequency). The MI for the acoustic output was 0.2-0.3 and the dynamic range was 60-65 dB. A single focal point was set at the deep site of the lesion. Sonazoid® (0.5 mL diluted with 19.5 mL of distilled water) was used as the contrast medium in IAUS. The diluted Sonazoid® was introduced into the feeding artery by intermittent injection of 0.3-0.5 mL through a microcatheter placed in the artery and flushing with saline at the same flow rate.

The following items were performed in DEB-TACE using IAUS: (1) Before DEB-TACE, the feeding arteries were identified and tumor enhancement was confirmed by IAUS. When a hypoenhanced area was partially observed in the tumor on IAUS, another feeding artery was identified when possible; (2) during DEB-TACE, DSA and IAUS were performed when contrast medium disappeared from the blood vessel within 5-6 heartbeats on fluoroscopy, and the presence or absence of tumor enhancement was evaluated. If tumor enhancement disappeared on DSA, but was detected by IAUS, DEB-TACE was repeated until the tumor enhancement disappeared on IAUS. Treatment was considered complete after disappearance of as much of the contrast image as possible; and (3) video images of all IAUS were stored on the hard disk of the scanner and transferred to a high-performance personal computer.

Image analyses

The items below were investigated after completion of all procedures: (1) In all lesions, CEUS findings before treatment and stored IAUS video images were compared, and disappearance of the tumor enhancement and untreated regions were evaluated. Treatments to be complete and incomplete for lesions were appropriate and insufficient, respectively; and (2) findings on CEUS and/or dynamic CT performed 1, 3, 6 and 12 mo after DEB-TACE and every 3 mo thereafter were evaluated using modified Response Evaluation Criteria in Solid Tumors (mRECIST) criteria [Complete response (CR), Partial response (PR), Stable disease (SD), Progressive disease (PD)][21]. Normally, mRECIST is used to evaluate treatment using dynamic CT, but we applied this method to CEUS findings. In cases judged as SD or PD on imaging after treatment, TACE (DEB-TACE or conventional TACE) was repeated within one month.

Adverse events

Following each procedure, patients were hospitalized for about 6 d and reevaluated with physical examinations and blood tests on days 1, 5 and 30. Safety was monitored by recording postprocedure clinical complications and liver and renal function. The severity of complications was retrospectively graded according to the Common Terminology Criteria for Adverse Events (CTCAE) ver. 4.0 (v4.03; June 14, 2010)[22]. An adverse event was considered to be treatment-related if it occurred within 30 d after DEB-TACE.

RESULTS

The median duration of observation in all patients was 363 d. Since an enhanced area was noted on IAUS when the contrast medium was retained in the feeding vessel on fluoroscopy in all patients, *i.e.*, when contrast medium disappeared from the blood vessel within 5-6 heart beats, additional DEB-TACE was applied. A comparison of CEUS findings before treatment and stored IAUS video images showed that the treatment was complete and incomplete in 26 and 13 lesions, respectively (Figure 1). Of 32 lesions judged to have received complete treatment at the end of treatment, 6 were judged to have received incomplete treatment

Table 2 Adverse events in 32 patients after 39 transarterial chemoembolization with drug-eluting beads procedures

Adverse event	Grade 1-2[4]	Grade 3-4[4]
Fever ≥ 37.5 ℃	19 (48.7)	0
Abdominal pain	3 (7.7)	0
Nausea and/or vomiting	0	0
Catheterization-site bleeding[1]	0	0
Transient renal insufficiency	0	0
Groin hematoma[2]	0	0
Liver decompensation[3]	0	0
Hepatic abscess	0	0
Intratumor bleeding	0	0

Data are given as *n* (%). [1]Controlled with local compressive medication; [2]Both self-limiting and resolved within 2 wk of the procedure; [3]Mild ascites treated successfully with oral aldosterone antagonists; [4]According to the CTCAE, version 4.0 (v4.03). CTCAE: Common Terminology Criteria for Adverse Events.

based on evaluation of stored video images; therefore, combining these two evaluations, complete treatment was achieved in 26 lesions (Figure 2). On imaging evaluation using CEUS and/or dynamic CT one month after treatment, 25 and 1 lesions were judged to be CR and PR, respectively, in the 26 completely treated lesions. At 6 mo after treatment, the results were CR, PR, SD and PD for 24, 1, 0 and 1 of these lesions, respectively, reflecting changes to PR and PD in one lesion each of the 25 lesions judged as CR one month after treatment, and a change to CR in the lesion judged as PR at one month. Of the 24 CR lesions at 6 mo after treatment, imaging evaluation was possible in 21 lesions at 12 mo, and 18 and 3 were judged to be CR and PD, respectively. Of the 13 lesions in which treatment was incomplete (Figure 3), the results on imaging at one month after treatment were CR, PR, SD and PD for 0, 6, 4 and 3 lesions, respectively. No lesion achieved CR at 6 mo after treatment, and additional TACE was applied to all 13 lesions. The overall CR rate at 6 mo after treatment was 61.5% (24/39) (Figure 4).

There was no DEB-TACE-related mortality and no major adverse events were recorded. Within 7 d after DEB-TACE, 3/39 lesions (7.7%) showed a mild transient increase (an increase of more than double compared to before treatment) in liver enzymes, and 19/39 (48.7%) had fever (≥ 37.5 ℃) and 3/39 (7.7%) had abdominal pain, as symptoms of post-embolization syndrome that were managed with conservative therapies. The median hospitalization after each course of treatment was 6 d. All patients were asymptomatic upon discharge from hospital. There were no adverse events that were directly due to IAUS (Table 2).

DISCUSSION

IAUS has previously been shown to be safe and effective in facilitation of TACE to treat malignant liver tumors such as HCC[12-16,23]. Use of IAUS during TACE allows accurate identification of feeding blood vessels, which is difficult with DSA alone, and the frequency of DSA during treatment can be decreased, which reduces the risk of embolization of non-target regions[14]. In addition, IAUS can identify tumors that are not stained through an artery and are fed by a branch of the portal vein, which may provide information comparable to that from CT angiography (CTA)[16].

In DEB-TACE, DEB must be administered selectively because of its effect as a permanent embolic agent, and thus identification of feeding arteries is very important[7]. In addition, DEB-TACE can be simply evaluated during treatment because of the high visibility on CEUS due to the property of the microspheres, unlike conventional TACE using Lipiodol® (Laboratoire Guerbet, Aulnay-Sous-Bois, France). Thus, we evaluated the therapeutic effect of DEB-TACE in 39 lesions in 32 patients with HCC, using IAUS with Sonazoid® as treatment support. The reported CR rate of HCC, including advanced HCC, to DEB-TACE is 12-27%[24-27], but CR on imaging at 6 mo after treatment in our study was achieved for 24 of 26 completely treated lesions (92.3%), and the overall CR rate at 6 mo was 61.5% (24/39).

When two blood vessels are present in an anteroposterior arrangement at the same level, the positional relationship is difficult to identify using two-dimensional imaging with DSA. Moreover, small tumors and tumors with poor blood flow are often not visualized by DSA, but can be visualized by IAUS[12,14,23]. IAUS is also sensitive to hemodynamics and was useful for identification of lesions in our study. Therefore, use of IAUS may have enabled effective treatment.

In particular, in all cases in which residual tumor enhancement was judged to have disappeared on DSA, IAUS showed a residual enhancement in the tumor, and DEB-TACE was repeated until this image disappeared on IAUS. Disappearance of contrast medium within 5-6 heartbeats in fluoroscopy is generally used to indicate completion of DEB-TACE, but this criterion may be insufficient. Continuing treatment until disappearance of the contrast enhancement in the tumor on IAUS is important, and this may have been one reason for the high CR rate in this study.

Manini *et al*[28] showed the efficacy of DEB-TACE for treatment of BCLC A stage HCC patients prior to liver transplantation based on the Milan criteria[29]. The median diameter of the target tumors was 24 mm, similar to that in our study, and achieving CR with initial DEB-TACE was found to be an important prognostic factor. Generally, TACE is used for BCLC B stage (intermediate stage) HCC, and DEB-TACE is frequently indicated for very large HCC and multiple HCCs, as an option to improve the outcome[2]. Our results suggest that reliable DEB-TACE may also increase the CR rate for small tumors, and thus may also improve the therapeutic effect for these tumors.

However, although the effect of intratumoral drug release in DEB-TACE has been reported[6], only one

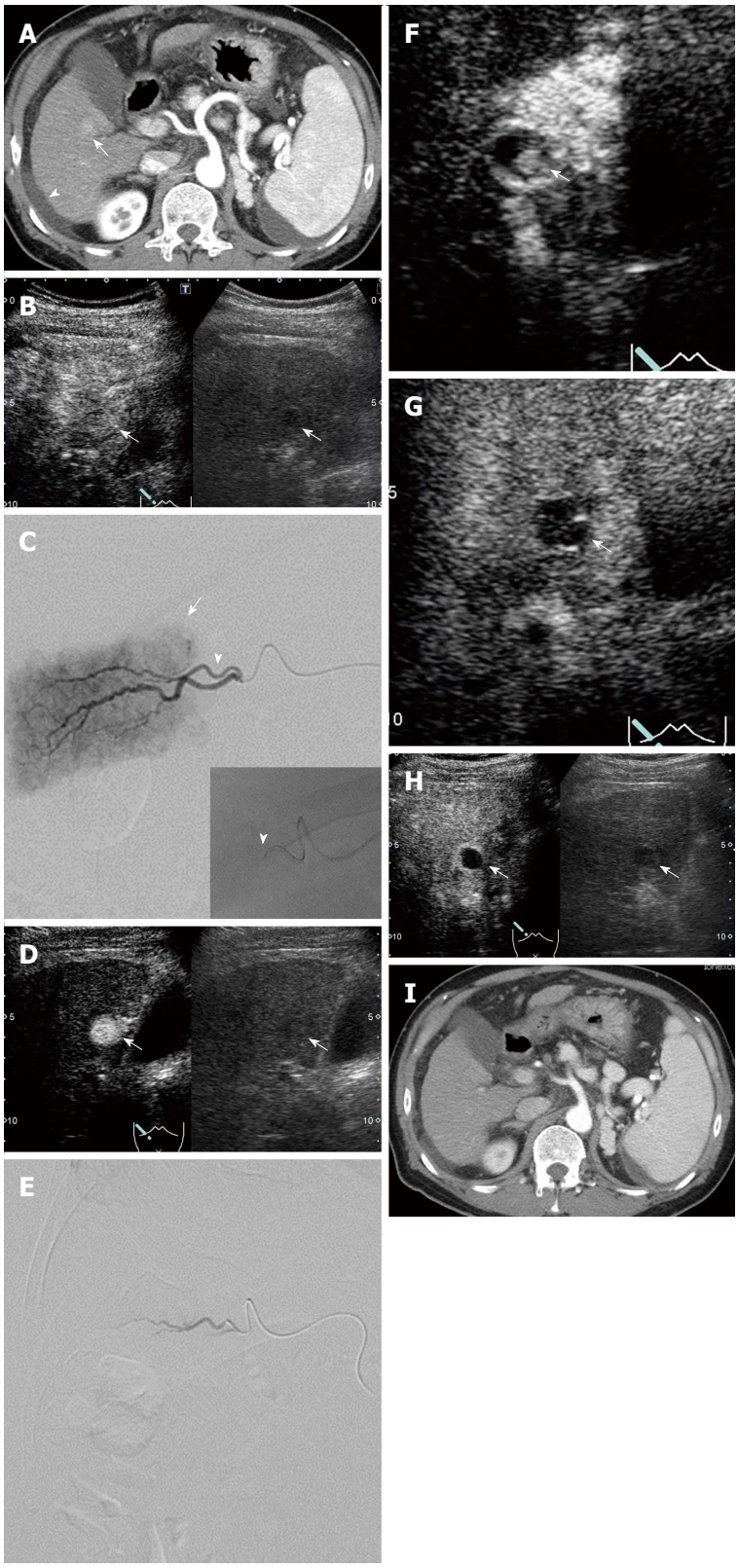

Figure 2 The patient was a 59-year-old male with alcoholic liver cirrhosis. DEB-TACE and transcatheter CEUS (IAUS) using Sonazoid for HCC in S5 with a diameter of 15 mm were performed. A: Dynamic CT in the arterial phase before DEB-TACE showed a hypervascular lesion in S5 (arrow) and ascites (arrow head); B: CEUS in the arterial phase (40 s) before DEB-TACE showed a hyperenhanced lesion in S5 (arrow) (Right image: Monitor mode); C: Digital subtraction angiography (DSA) from a branch of A6 (arrow head) before DEB-TACE showed tumor enhancement (arrow). Insert image: A coaxial microcatheter was advanced in the feeding artery (arrow head); D: IAUS from the branch of A6 before DEB-TACE showed a hyperenhanced lesion (arrow). (Right image: monitor mode); E: DSA from A6 after DEB-TACE (when contrast medium disappeared from the blood vessel within 5-6 heart beats) eliminated the tumor enhancement; F: IAUS from A6 after DEB-TACE showed a residual hyperenhanced area in the tumor (arrow) in spite of elimination of tumor enhancement by DSA. Therefore, DEB-TACE for this lesion was performed repeatedly until the hyperenhanced area disappeared; G: IAUS from the right hepatic artery eliminated the residual area in the tumor (arrow); H: CEUS in the arterial phase (40 s) showed unenhancement lesion in S5 three days after DEB-TACE (arrow) (Right image: monitor mode); I: Dynamic CT in the arterial phase did not show the hypervascular lesion six months after DEB-TACE.

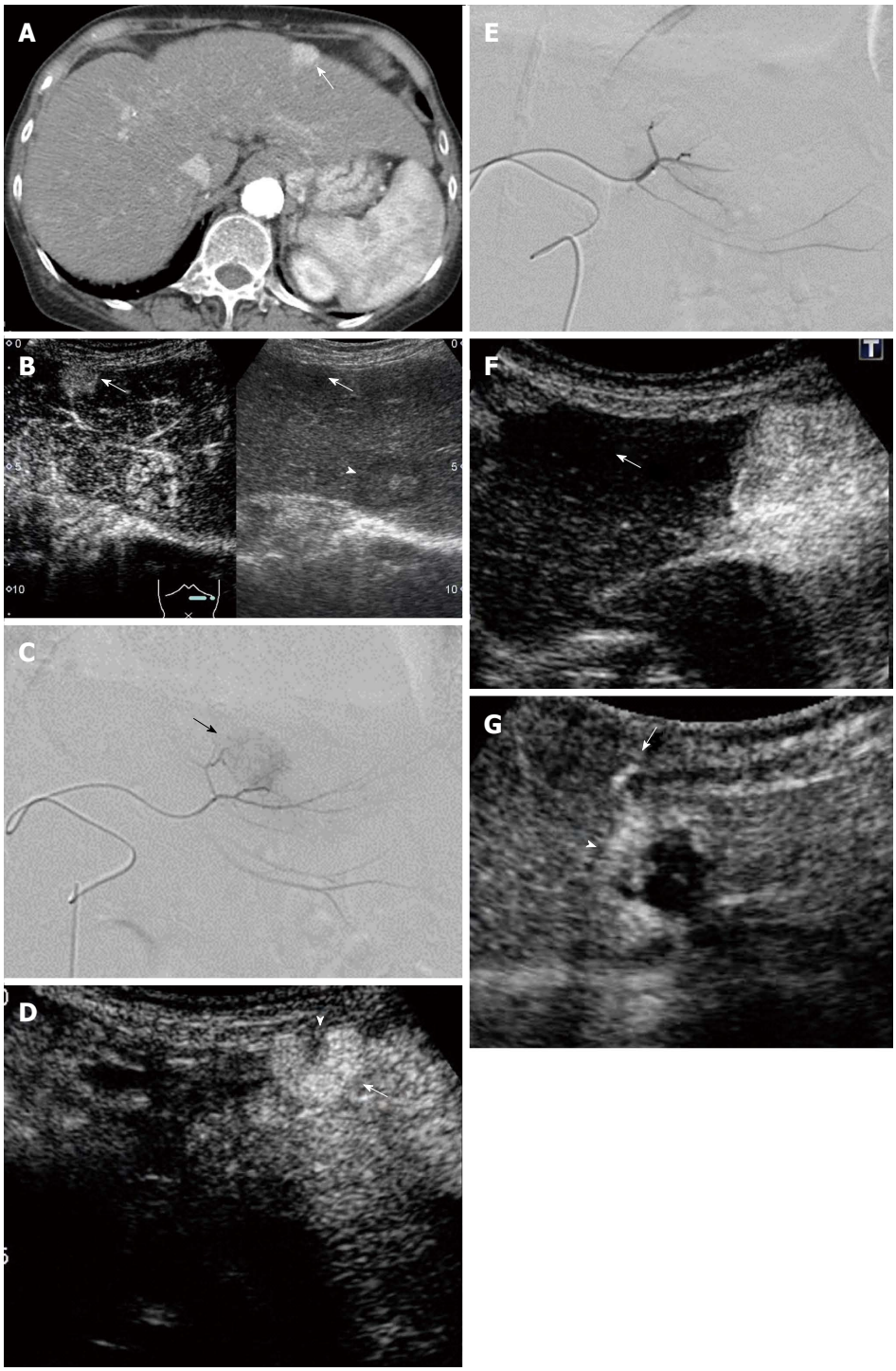

Figure 3 The patient was a 76-year-old female with hepatitis C virus cirrhosis. DEB-TACE and IAUS for HCC in S3 with a diameter of 17 mm were performed. A: Dynamic CT in the arterial phase before DEB-TACE showed a hypervascular lesion in S3 (arrow); B: CEUS in the arterial phase before DEB-TACE showed a hyperenhanced lesion in S3 (arrow) and S2 (arrow head). Radiofrequency ablation was performed for the lesion in S3 after DEB-TACE (Right image: monitor mode); C: DSA from A3 before DEB-TACE showed tumor enhancement (arrow); D: IAUS from A3 before DEB-TACE showed a hyperenhanced lesion (arrow) with a small hypoenhanced area (arrow head), which was not recognized during the procedure. This small hypoenhanced area was recognized with stored video images after DEB-TACE procedure; E: DSA from A3 after DEB-TACE (when contrast medium disappeared from the blood vessel within 5-6 heart beats) eliminated the tumor enhancement; F: The enhanced lesion was disappeared by IAUS from A3 after DEB-TACE (arrow); G: CEUS showed enhancement area in the tumor (arrow head) and extrahepatic feeding artery (arrow) three days after DEB-TACE. It was thought this artery fed the small hypoenhanced area.

lesion changed from PR at one month after treatment to CR at 6 mo on imaging evaluation, suggesting the importance of ensuring CR just after treatment, *i.e.*, treatment should be completed until enhancement in the tumor disappears. Two lesions changed from CR at one month after treatment to PR and PD at 6 mo after treatment, respectively, in 25 lesions judged as CR one month after treatment. For these lesions, peritumoral invasion could not be ruled out before treatment.

DEB-TACE was repeated until the contrast

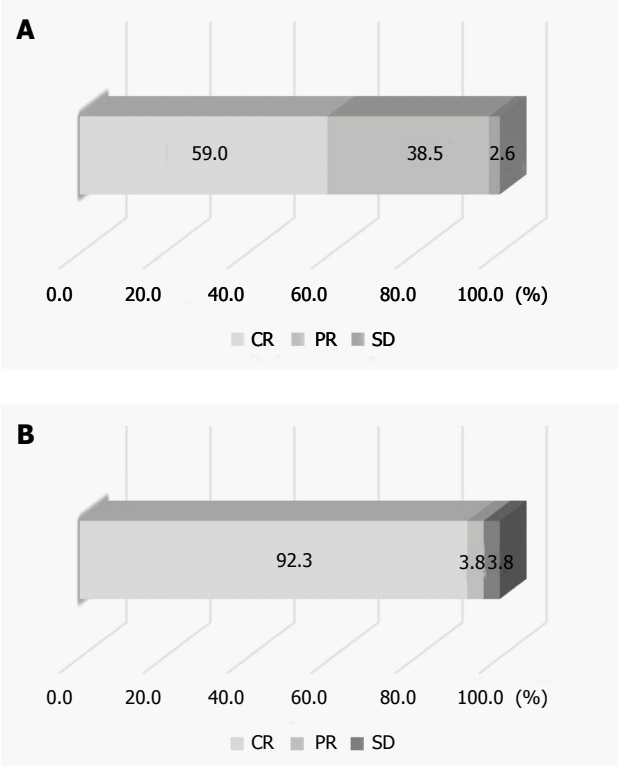

Figure 4 Treatment response. A: The overall complete response (CR) rate of all thirty-nine lesions at one month after treatment; B: The overall CR rate of twenty-six lesions treated completely at six months after treatment. PR: Partial response; SD: Stable disease; PD: Progressive disease.

enhancement in the tumor disappeared on IAUS, but there were no cases with severe postembolization syndrome, such as biloma and liver abscess, and no other adverse events that extended hospitalization. Intravenous administration is generally used for contrast agent for US, but there was no adverse event directly due to IAUS, which suggests that CEUS can be safely used with transarterial administration of the contrast agent Sonazoid®.

Information similar to that provided by IAUS may be acquired using CTA and cone-beam CT. Oblique acquisition may also be performed to define the positional relationship of the tumor on DSA, but the procedure is complicated and time-consuming, and increases the exposure dose and contrast medium volume, all of which are disadvantageous[15,30]. Also, renal function is decreased in many patients with hepatic cirrhosis, and worsens with iodine contrast medium. In contrast, IAUS may reduce the exposure dose and iodine contrast medium volume, which is likely to shorten the treatment time. Furthermore, Sonazoid® can be repeatedly administered[31], which is advantageous when making a judgment using a first IAUS procedure is difficult.

The limitations of this study include the small number of cases and short observation period. Similarly to normal gray-scale US, IAUS may depend on the experience and skill of operating physicians. Moreover, IAUS is difficult to apply for lesions that cannot be visualized in the supine position, for cases with multiple lesions, in obese patients, and for deep lesions. The treatment was incomplete in 13 lesions, even though IAUS was frequently performed during DEB-TACE to identify feeding arteries. Since this study was a pilot study performed early after introduction of IAUS, the operator had limited experience, and the operator of DEB-TACE and rater of IAUS was the same physician, which made the evaluation complex. Thus, the presence of small regions fed by micro-blood vessels in the tumor and other tumor blood vessels may not have been recognized on IAUS, and thus completion of DEB-TACE may not have been judged appropriately (Figure 3). We are planning to perform a long-term study of the effect of IAUS on the therapeutic efficacy of DEB-TACE in a larger number of patients with HCC. Further, in the future, we want to run this future study at multiple centers, and compare conventional TACE and DEB-TACE using IAUS to make more solid conclusions with prospective design. In conclusion, a combination of DEB-TACE with IAUS can improve the therapeutic effects in patients with HCC.

ARTICLE HIGHLIGHTS

Research background

In transarterial chemoembolization with drug-eluting beads (DEB) (DEB-TACE) for hepatocellular carcinoma (HCC), it is particularly important to prevent inflow of DEB into normal hepatic parenchyma and to administer the beads into the tumor artery because of their effect as a permanent embolic material. However, some HCCs are difficult to visualize on digital subtraction angiography for TACE due to the complex blood supply. On the other hand, contrast-enhanced ultrasonography (CEUS) is useful for evaluation of the hemodynamics of hepatic tumors and surrounding hepatic parenchyma in real time. Transcatheter (intra-arterial) CEUS (IAUS) has recently been used in DEB-TACE for HCC, and its safety and efficacy in identifying the feeding artery have been evaluated.

Research motivation

Generally, the complete response (CR) rate of DEB-TACE for small HCC is reported to be low. It is thought that DEB-TACE is mainly performed for giant and multiple HCCs in many facilities. The authors wanted to know the true therapeutic effect of DEB-TACE for small HCCs less than 50 mm by considering whether feeding artery can be selected reliably and whether the timing of completion of treatment is appropriate.

Research objectives

IAUS has recently been used in DEB-TACE for HCCs and metastatic hepatic tumors, and its safety and efficacy in identifying the feeding artery have been evaluated. However, the therapeutic effect using IAUS as support for DEB-TACE in HCC has not been examined. In this study, the authors evaluated the usefulness of IAUS using Sonazoid® in DEB-TACE for HCC.

Research methods

The authors evaluate the identification of feeding arteries and the appropriate timing of completion of DEB-TACE for HCC by IAUS using Sonazoid®.

Research results

DEB-TACE with IAUS can improve the therapeutic effects in patients with HCC. This study includes the small number of cases and short observation period. A same study with much larger number of patients and much longer observation period are awaited.

Research conclusions

IAUS is very useful to obtain CR in HCC treatment with DEB-TACE. IAUS is very useful to obtain CR in HCC treatment with DEB-TACE. In all cases in

which residual tumor enhancement was judged to have disappeared on Digital subtraction angiography (DSA), IAUS showed a residual enhancement in the tumor. Disappearance of contrast medium within 5-6 heartbeats in fluoroscopy is generally used to indicate completion of DEB-TACE, but this criterion may be insufficient. The appropriate treatment using IAUS is possible to obtain CR in DEB-TACE for relatively small HCC. There is a possibility of obtaining CR by appropriate treatment in DEB-TACE for HCC. The appropriate treatment using IAUS is possible to obtain CR in DEB-TACE for HCC. The authors treated HCCs with IAUS using Sonazoid®. In DSA, disappearance of contrast medium within 5-6 heartbeats in fluoroscopy is generally used to indicate completion of DEB-TACE, but this criterion may be insufficient. The appropriate treatment using IAUS is possible to obtain CR in DEB-TACE for HCC. The therapeutic effect of DEB-TACE for HCC may improve.

Research perspectives

In DSA, disappearance of contrast medium within 5-6 heartbeats in fluoroscopy is generally used to indicate completion of DEB-TACE, but this criterion may be insufficient. IAUS is very useful for obtaining CR in DEB- TACE for HCC. The authors are planning to perform a long-term study of the effect of IAUS on the therapeutic efficacy of DEB-TACE in a larger number of patients with HCC. The authors prospectively compare therapeutic efficacy of DEB-TACE with/without IAUS.

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ORIGINAL ARTICLE

Clinical Practice Study

Proton nuclear magnetic resonance-based metabonomic models for non-invasive diagnosis of liver fibrosis in chronic hepatitis C: Optimizing the classification of intermediate fibrosis

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Abstract

AIM

To develop metabonomic models (MMs), using ^{1}H nuclear magnetic resonance (NMR) spectra of serum, to predict significant liver fibrosis (SF: Metavir $\geqslant$ F2), advanced liver fibrosis (AF: METAVIR $\geqslant$ F3) and cirrhosis (C: METAVIR = F4 or clinical cirrhosis) in chronic hepatitis C (CHC) patients. Additionally, to compare the accuracy of the MMs with the aspartate aminotransferase to platelet ratio index (APRI) and fibrosis index based on four factors (FIB-4).

METHODS

Sixty-nine patients who had undergone biopsy in the previous 12 mo or had clinical cirrhosis were included. The presence of any other liver disease was a criterion for exclusion. The MMs, constructed using partial least squares discriminant analysis and linear discriminant analysis formalisms, were tested by cross-validation, considering SF, AF and C.

RESULTS

Results showed that forty-two patients (61%) presented SF, 28 (40%) AF and 18 (26%) C. The MMs showed sensitivity and specificity of 97.6% and 92.6% to predict SF; 96.4% and 95.1% to predict AF; and 100% and 98.0% to predict C. Besides that, the MMs correctly classified all 27 (39.7%) and 25 (38.8%) patients with intermediate values of APRI and FIB-4, respectively.

CONCLUSION

The metabonomic strategy performed excellently in predicting significant and advanced liver fibrosis in CHC patients, including those in the gray zone of APRI and FIB-4, which may contribute to reducing the need for these patients to undergo liver biopsy.

Key words: Metabolomics; Nuclear magnetic resonance spectroscopy; Chronic hepatitis C; Liver fibrosis; Surrogate markers

Core tip: The assessment of liver fibrosis in chronic hepatitis C patients is important to make therapeutic decisions and predict clinical outcomes. Due to various drawbacks related to the use of liver biopsy, individual markers and scores have been validated with feeble accuracy to assess intermediate stages of fibrosis. Our study showed promising results for the metabonomics strategy as a non-invasive tool to distinguish patients with significant fibrosis, advanced fibrosis, and cirrhosis, with sensitivity and specificity values above 95% and high accuracy in the gray zone of aspartate aminotransferase to platelet ratio index and fibrosis index based on four factors, which could avoid a large number of biopsies in these patients.

Batista AD, Barros CJP, Costa TBBC, Godoy MMG, Silva RD, Santos JC, de Melo Lira MM, Jucá NT, Lopes EPA, Silva RO. Proton nuclear magnetic resonance-based metabonomic models for non-invasive diagnosis of liver fibrosis in chronic hepatitis C: Optimizing the classification of intermediate fibrosis. *World J Hepatol* 2018; 10(1): 105-115 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/105.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.105

INTRODUCTION

Approximately 130-170 million people worldwide are chronically infected with the hepatitis C virus (HCV)[1] and an estimated 500000 individuals died in 2010 from virus-related illnesses[2]. In Brazil, the estimated prevalence of hepatitis C is 1450000 cases[3] and about 8000 deaths were due to HCV-related diseases in 2013[4].

The accurate diagnosis of significant fibrosis (SF), advanced fibrosis (AF) and cirrhosis (C) in the liver is important to determine the urgency of treatment, and to monitor complications of the disease. Hepatic histopathology assessment by liver biopsy is still considered the gold standard for evaluating liver fibrosis, but the procedure is invasive and may lead to major complications and death[5]. Sampling error, intra- and inter-observer variability leads to discordant results in 33% of cases[6], thus making biopsy an imperfect reference standard. For these reasons, several non-invasive serological biomarkers of fibrosis have been evaluated. Among them, aminotransferase to platelet ratio index (APRI) and fibrosis index based on four factors (FIB-4) stand out because they are based on readily available laboratory tests in clinical practice[7,8]. These surrogate markers have good accuracy as to excluding significant fibrosis and confirming cirrhosis. However they fail to diagnose intermediate fibrosis[9].

Metabonomics is defined as "a quantitative measure of the dynamic and multiparametric metabolic response of living organisms to pathological or genetic modifications[10]". As a result of homeostasis, the presence of a pathological condition changes the profile of endogenous metabolites, which can be monitored by ^{1}H nuclear magnetic resonance (NMR) spectroscopy[11]. This method seeks to discriminate the samples in

groups according to their biochemical status, thereby associating this status to a given condition[11-12]. The method has been studied in various liver diseases[13], and has been shown to be useful in distinguishing between patients with viral hepatitis and healthy volunteers[14-15] and performing well at identifying complications of liver cirrhosis[16-18]. Recently, our group showed that a partial least squares discriminant analysis (PLS-DA) metabonomic model (MM), based on the H-1 NMR spectroscopy of serum samples, presented a clear separation between 18 patients coinfected with schistosomiasis mansoni and hepatitis B virus (HBV) or HCV and 22 HBV or HCV mono-infected patients, with an accuracy, a predictive ability (Q^2) and a coefficient of determination (R^2) of 100%, 98.1% and 97.5%, respectively[19]. Therefore, the aim of this study was to develop and evaluate MMs, using ^{1}H NMR spectrum of serum samples, as non-invasive markers of significant liver fibrosis, advanced liver fibrosis and cirrhosis in patients with chronic hepatitis C (CHC), and to compare their performance with the APRI and FIB-4.

MATERIALS AND METHODS

Design of the study and patient selection

This was a cross-sectional phase Ⅱ validation diagnostic study[20], with prospective inclusion, by spontaneous demand, of CHC adult outpatients (anti-HCV and HCV-RNA detectable in serum) attended to at the hepatology clinic of the Hospital das Clínicas/ Universidade Federal de Pernambuco (HC/UFPE) between October/2012 and December/2015. Patients who had undergone a percutaneous liver biopsy in the previous 12 mo or had been clinically diagnosed with liver cirrhosis were included.

The clinical diagnosis of cirrhosis was based on characteristic symptoms and signals and/or according to evidence of chronic liver disease and/or portal hypertension on ultrasound (US), such as liver parenchymal heterogeneity, straight borders, reduced liver size, enhanced portal vein dimensions, presence of collateral vessels, splenomegaly, and/or signals of portal hypertension observed on upper gastrointestinal endoscopy, such as the presence of esophageal/gastric varices and/or hypertensive gastropathy.

Those undergoing antiviral treatment or diagnosed with periportal fibrosis induced by schistosomiasis, metabolic, autoimmune or cholestatic liver disease, HBV or HIV co-infection, neoplasia, or with ethanol ingestion > 20 g/d for women and > 30 g/d for men were excluded. All patients signed an informed consent form and the study was approved by the Ethics Committee of the Institution.

Laboratory analysis, determination of APRI and FIB-4

Fasting blood samples were collected from all patients by peripheral vein puncture. The laboratory tests were performed by an automated method. The HCV RNA was detected and the genotype was determined by real-time polymerase chain reaction, using COBAS® AmpliPrep/COBAS® TaqMan® (version 2, Roche, Pleasanton, CA, United States) with a detection limit of 15 IU/mL. The APRI and FIB-4 were calculated as described by Wai *et al*[7] (2003) and Sterling *et al*[8] (2006).

^{1}H nuclear magnetic resonance spectroscopy

Serum samples, stored at -20 ℃, were thawed at room temperature and prepared by adding 200 μL of D_2O to 400 μL of serum. ^{1}H NMR spectra were obtained using a Varian Unity Plus 300 spectrometer, operating at 299.95 MHz, at 300 K. The samples were analyzed using a pulse sequence with suppression of the resonance of water and T2 filter (PRESAT-CPMG), as follows: Pre-saturation time of 2.0 s, acquisition time of 1.704 s, 128 repetitions and spectral width of 4.8 kHz. Spectra were processed using line broadening equal to 0.3 Hz. The signal attributed to the methyl group of the lactate, in δ 1.33 ppm, was used as the internal reference of chemical shift. The baseline of the spectra was corrected manually.

Liver biopsies and allocation of patients

The ultrasonography-guided percutaneous liver biopsies were performed using a 16 G x 90 mm Menghini needle in, at most, 2 punctures. Fragments with at least 15 mm and /or 6 complete portal tracts were included in the analysis. Fibrosis was classified as F0 to F4, in accordance with METAVIR[21], by two experienced pathologists, blinded to the clinical and serological results. The patients were allocated into three groups: SF (METAVIR F ⩾ 2), AF (METAVIR F ⩾ 3) and C (METAVIR F = 4 or clinical cirrhosis).

Multivariate statistical analysis of spectral data and MM development

All spectra were processed using MestreNova software (version 9.0.1, MestreLab Research). The spectra were divided into 250 regions of 0.04 ppm, called bins, used to construct a dataset. The region containing the bins centered between δ 4.52 - 5.12 ppm was excluded so as to eliminate the residual signal of water. The spectra were normalized using the following expression:

$$x = \frac{x_i - \bar{x}}{\sigma}$$

Where x_i is the intensity in each bin, while $(x_1 + x_2 + \ldots\ldots x_n)/n$ is the arithmetic mean of the intensities observed in the bins and σ is the standard deviation.

Principal components analysis (PCA) formalism was initially applied to the dataset, to explore inherent clusters and to identify the presence of outliers. PCA did not present natural grouping in the classes of interest. Then, PLS-DA and linear discriminant analysis (LDA) supervised formalisms were used. Three PLS-DA models were constructed to predict FS, FA and C, respectively, using the MetaboAnalyst 3.0 platform[22]. The models were validated by leave-one-out-cross-

Table 1 Main demographic and laboratory characteristics of 69 chronic hepatitis C patients from Pernambuco/Brazil

Characteristics	Total (n = 69)	
Gender (n, %)		
Male	28	40.60%
Female	41	59.40%
Age (yr)[1]	57.5	± 11.9
BMI (kg/m^2)[1]	27.7	± 4.7
AST (U/L)[2]	51.5	(33.7-88)
ALT (U/L)[2]	54	(32.6-106)
AST/ALT[1]	1.03	± 0.44
GGT (U/L)[2]	81.8	(46.5-155)
Platelets (10^9/mm^3)[1]	191	± 78
Albumin (g/dL)[1]	4.08	± 0.56
Total bilirubin (mg/dL)[2]	0.7	(0.50-1.09)
Alkaline phosphatase (U/L)[1]	94.6	± 49.2
INR[1]	1.09	± 0.14
APRI[2]	0.8	(0.44-2.18)
FIB-4[2]	2.18	(1.29-4.72)
Liver fragment length (cm)[2]	1.5	(1.30-1.80)
Number of portal tracts[2]	15	(12.0-20.0)
Stage of fibrosis (n, %)		
F0	2	2.90%
F1	25	36.20%
F2	14	20.30%
F3	10	14.50%
F4	18	26.10%

[1]Mean ± standard deviation; [2]Median (P_{25}-P_{75}). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; GGT: Gamma glutamyl transferase; INR: International normalized ratio; APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis index based on four factors.

validation (LOOCV) and by permutations tests. In addition, three LDA models were constructed, using the PCA matrix as input data, so as to predict SF, with five principal components (PCs); AF, with four PCs; and C, with five PCs. LDA models were validated by LOOCV, using STATISTICA software (version 10.0, Quest software).

Analysis of the performance of the MMs and of the APRI and FIB-4

For each LDA MM, and for APRI and FIB-4, a 2 x 2 contingency matrix was used to calculate sensitivity (SN), specificity (SP), the positive likelihood ratio (LR+), the negative likelihood ratio (LR-) and accuracy (A). A Receiver Operating Characteristic (ROC) curve was also constructed for APRI and FIB-4.

Statistical analysis

The descriptive and comparative analysis of the data was carried out using STATA (version 12.0, StataCorp, College Station, Texas) and GraphPad Prism software (version 5.0 for Windows, GraphPad Software, La Jolla, California). The qualitative variables were presented as absolute and relative frequencies, and the quantitative variables as means and standard deviation or medians and 25th and 75th percentile. Categorical variables were compared using the χ^2 test, applying Fisher's exact test, when necessary. The Mann-Whitney and Student's t-test were used to compare non-parametric and parametric continuous measurements, respectively. All tests were applied with 95% confidence (P value $\leqslant$ 0.05).

RESULTS

The group studied consisted of 80 CHC patients initially selected. Eleven of the subjects (14%) were excluded due to: diagnosis of periportal fibrosis induced by schistosomiasis in 5 patients, hepatocellular carcinoma in 1, abuse of ethanol in 1 and inadequate liver fragment in 4. Therefore, 69 patients were evaluated, of whom 59.4% were female, with a mean age of 57 ± 12 years. The HCV genotype was determined in 67 patients, while 1b was the most frequent genotype in 36 (53.7%) patients, followed by genotype 3 in 16 (23.9%), genotype 1/1a in 13 (19.4%), genotype 2 in 1 (1.5%) and genotype 4 in 1 (1.5%) patients. The main characteristics of the casuistry are described in Table 1.

Liver biopsy was performed on 54 (78%) patients. There were no serious complications or deaths related to the procedure. The median fragment was 15 mm in length (P_{25}: 13; P_{75}: 18 mm), and there were 15 portal tracts (P_{25}: 12; P_{75}: 20 mm). The METAVIR fibrosis stage was distributed as follows: F0 in 2; F1 in 25; F2 in 14; F3 in 10 and F4 in 3 patients. The diagnosis of cirrhosis was clinically established in 15 (21.7%), thus classified according to the Child-Pugh score: 10 patients Child-Pugh A and 5 Child-Pugh B.

Therefore, 42 (60.9%) patients were classified as SF, 28 (40.6%) as AF and 18 (26.1%) as C. Patients with SF and AF presented a higher mean age, a higher mean value of INR and a higher median value of bilirubin, gamma-glutamyl transferase, APRI and FIB-4, as well as a lower mean platelet count and albumin serum level, when compared with the groups of a lower stage of fibrosis (Table 2).

The PLS-DA MM for SF showed a clear discrimination between the samples with three latent components (Figure 1A). The model presented 100% accuracy, R^2 and Q^2 of 0.98 and 0.91, respectively, when five latent components were used (Figure 1D). The permutation tests, using up to 1000 permutations of the model, indicated that there was no permuted model better than the original one, with observed statistics at P < 0.001 (Figure 1G). The results of the LDA MM for SF are presented in Table 3 and were compared with APRI, with a lower and upper cut-off point of 0.5 and 1.5. The MM showed SN of 97.6% (95%CI: 87.4%-99.9%), similar to APRI cut-off of 0.5, which presented SN of 85.7% (95%CI: 71.5%-94.6%). The LR- of the MM was 0.03 (95%CI: 0.004-0.2), whereas the LR- of APRI values $\leqslant$ 0.5 was 0.3 (95%CI: 0.1-0.7). The MM presented SP of 92.6% (95%CI: 75.7%-99.1%) and LR+ 13.2 (95%CI: 3.5-50.1), similar to APRI cut-off of 1.5, which showed SP of 92.3% (95%CI: 74.9%-99.9%), with LR+ of 5.9 (95%CI: 1.5-23.2) for values > 1.5.

Table 2 Main demographic and laboratory characteristics of 69 chronic hepatitis C patients, in accordance with the fibrosis group, from Pernambuco/Brazil

	Fibrosis group				*P* value
	Significant (≥ F2) *n* = 42		Non-significant (< F2) *n* = 27		
Gender (M/F, %)	16/26	(40.6/59.4)	12/15	(38.1/61.9)	0.600[a]
Age (yr)[1]	62.16	± 9.46	50.22	± 11.86	< 0.0001[b]
BMI (kg/m^2)[1]	28.6	± 4.9	26.3	± 3.8	0.051[b]
AST (U/L)[2]	61.5	(45.5-103)	34	(29.6-47)	< 0 .0001[c]
ALT (U/L)[2]	57.5	(41.7-108)	39.1	(26-81)	0.04[c]
AST/ALT[1]	1.12	± 0.47	0.91	± 0.36	< 0.0001[b]
GGT (U/L)[2]	107.5	(65.8-168)	56	(32.8-82.9)	0.001[c]
Platelets (10^9/mm^3)[1]	162	± 72	239	± 61	< 0.0001[b]
Albumin (g/dL)[1]	3.91	± 0.62	4.35	± 0.30	0.001[b]
Total bilirrubin (mg/dL)[2]	0.72	(0.60-1.30)	0.5	(0.40-0.80)	0.005[c]
Alkaline phosphatase (U/L)[1]	103.3	± 55.6	79.2	± 30.3	0.059[b]
INR[1]	1.13	± 0.17	1.03	± 0.06	0.002[b]
APRI[2]	1.14	(0.75-2.84)	0.48	(0.27-0.79)	< 0.0001[c]
FIB-4[2]	3.45	(2.01-6.19)	1.37	(0.84-1.89)	< 0.0001[c]
	Advanced (≥ F3) *n* = 28		Non-advanced (< F3) *n* = 41		
Gender (M/F, %)	14/14	(50.0/50.0)	14/27	(34.1-65.9)	0.188[a]
Age (yr)[1]	62.23	± 9.42	54.25	± 12.46	0.004[b]
BMI (kg/m^2)[1]	27.6	± 3.9	27.9	± 5.2	0.802[b]
AST (U/L)[2]	79	(52.4-130)	40	(31.4-63)	0.001[c]
ALT (U/L)[2]	62	(42.5-113.3)	43	(29-84.8)	0.078[c]
AST/ALT[1]	1.19	± 0.54	0.93	± 0.33	0.031[b]
GGT (U/L)[2]	110.5	(76.1-159.3)	66.5	(36.2-137.8)	0.005[c]
Platelets (10^9/mm^3)[1]	138	± 65	228	± 63	< 0.0001[b]
Albumin (g/dL)[1]	3.75	± 0.66	4.33	± 0.29	< 0.0001[b]
Total bilirrubin (mg/dL)[2]	0.94	(0.70-1.48)	0.5	(0.44-0.70)	< 0.0001[c]
Alkaline phosphatase (U/L)[1]	99.5	± 38.9	90.9	± 56.1	0.490[b]
INR[1]	1.18	± 0.17	1.03	± 0.07	< 0.0001[b]
APRI[2]	2.13	(0.99-3.75)	0.59	(0.39-0.92)	< 0.0001[c]
FIB-4[2]	4.8	(2.56-9.30)	1.72	(1.10-2.21)	< 0.0001[c]
	Cirrhosis (F4) *n* = 18		Non-cirrhosis (< F4) *n* = 51		
Gender (M/F, %)	7/11	(38.9/61.1)	21/30	(41.2/58.8)	0.865[a]
Age (yr)[1]	63.7	± 11.11	55.29	± 11.51	0.009[b]
BMI (kg/m^2)[1]	27.7	± 3.4	27.7	± 5.1	0.983[b]
AST (U/L)[2]	69	(54.1-101.5)	43.2	(32.5-80)	0.059[c]
ALT (U/L)[2]	54	(36.4-71)	50	(31-114)	0.637[c]
AST/ALT[1]	1.38	± 0.56	0.91	± 0.31	0.003[b]
GGT (U/L)[2]	108.5	(73.8-156.3)	74.5	(41.3-151.2)	0.068[c]
Platelets (10^9/mm^3)[1]	123	± 72	216	± 64	< 0.0001[b]
Albumin (g/dL)[1]	3.41	± 0.63	4.31	± 0.29	< 0.0001[b]
Total bilirrubin (mg/dL)[2]	1.27	(0.70-2.88)	0.6	(0.47-0.80)	< 0.0001[c]
Alkaline phosphatase (U/L)[1]	107.2	± 41.3	89.7	± 51.5	0.202[b]
INR[1]	1.21	± 0.20	1.05	± 0.08	0.003[b]
APRI[2]	2.35	(1.04-4.36)	0.69	(0.40-1.11)	< 0.0001[c]
FIB-4[2]	5.63	(4.37-11.27)	1.85	(1.17-2.38)	0.001[c]

[1]Mean ± standard deviation; [2]Median (P_{25}-P_{75}), [a]χ^2 test; [b]*T* test; [c]Mann-Whitney test. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; GGT: Gamma glutamyl transferase; INR: International normalized ratio; APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis index based on four factors.

The PLS-DA MM for AF, using three latent components, discriminated all the samples, with 100% accuracy, R^2 of 0.98 and Q^2 of 0.93, with five latent components. Permutation tests indicated that the permuted models are not better than the original model (Figure 1B, E and H). The LDA MM for AF showed SN of 96.4% (95%CI: 81.7%-99.1%) and LR- of 0.04 (95%CI: 0.005-0.3), similar to FIB-4 cut-off of 1.45, which showed SN of 89.3% (95%CI: 71.8%-97.7%) and LR- of 0.3 (95%CI: 0.1-0.8) for values ⩽ 1.5. The two methods also presented high SP and LR+, there being observed SP of 95.1% (95%CI: 83.5%-99.4%) and LR+ of 19.8 (95%CI: 5.1-76.5) for MM, and SP of 92.5% (95%CI: 79.6%-98.4%) and LR+ of 10 (95%CI: 3.3-30.3) for FIB-4 cut-off of 3.25 (Table 3).

The PLS-DA MM for C, using three latent components, also adequately discriminated the samples, attaining an accuracy of 84.0% with five latent components. The permutation tests indicated that the original model was not exceeded by any of the permuted models (Figure 1C, F and I). The LDA MM for C showed SN of 100% (95%CI: 81.5%-100%) and LR- of 0.03 (95%CI: 0.002-0.4), similar to APRI cut-off of 1.0, which presented SN of 77.8% (95%CI: 52.4-93.6), with

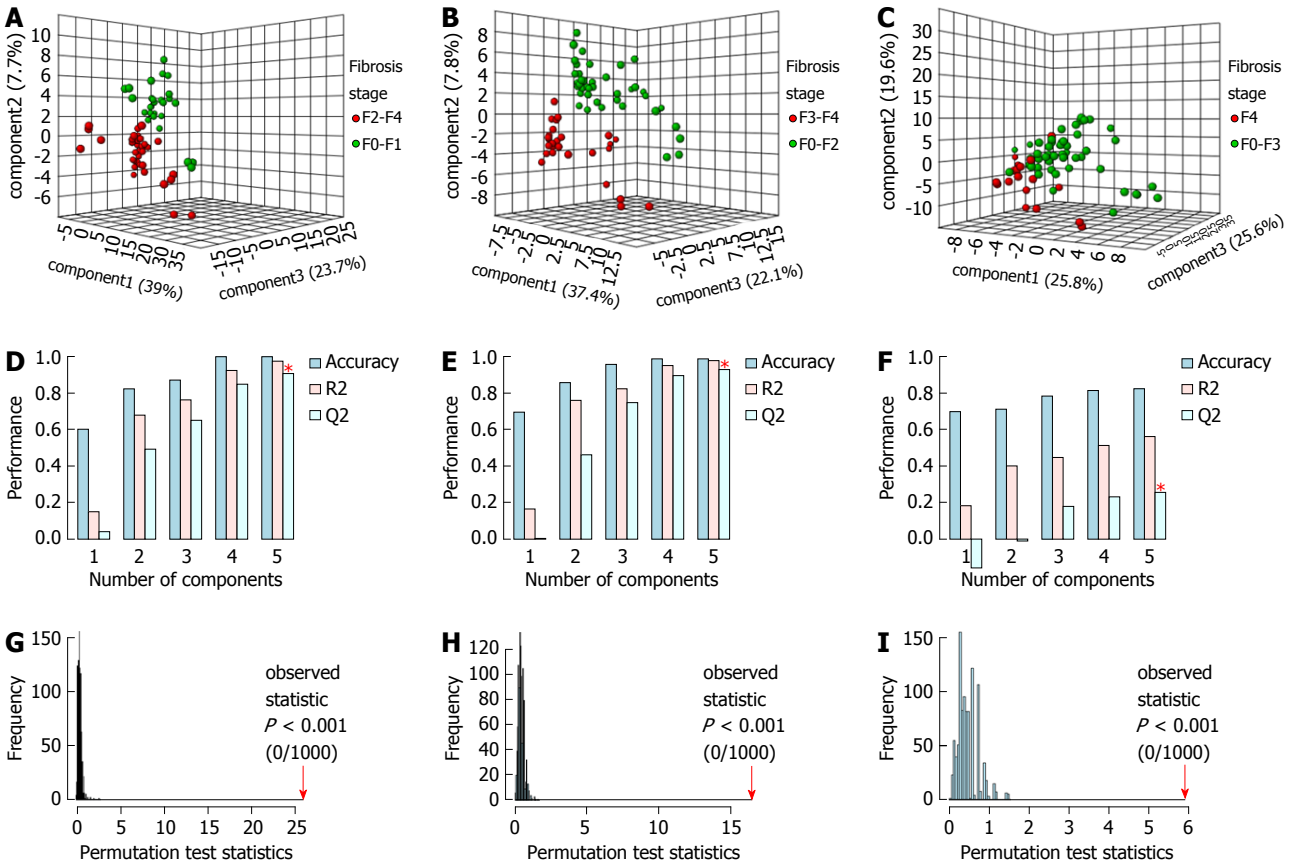

Figure 1 Partial least squares discriminant analysis metabonomic models to predict significant fibrosis (A, D, G) advanced fibrosis (B, E, H) and cirrhosis (C, F, I) in 69 chronic hepatitis C patients from Pernambuco/Brazil. Three-dimensional score plot (A, B, C). Classification of MM using different numbers of latent components, with accuracy = 1.0/ 1.0/ 0.84, R^2 = 0.98/ 0.98/ 0.56 and Q^2 = 0.91/ 0.93/ 0.27, using 5 latent components (D, E, F); permutation tests statistics for 1000 permutations with observed statistic at $P < 0.001$ (G, H, I). MM: Metabonomic models.

LR- of 0.3 (95%CI: 0.1-0.8) for values ⩽ 1.0. The MM presented SP of 98% (95%CI: 89.6%-99.9%), similar to APRI cut-off of 2.0, which showed SP of 82% (95%CI: 68.6 - 91.4). MM showed LR+ of 33.8 (95%CI: 6.9-163.7), which was higher than the APRI values > 2.0, which presented LR+ of 2.8 (95%CI: 1.3-5.9). This result indicates that, by MM, the likelihood of a positive test in the presence of cirrhosis is 33 times more likely than a positive test in the absence of cirrhosis, whereas, by the APRI it is 2.8 times more likely (Table 3).

In general, the MM presented a high accuracy and performance, similar to the APRI to predict SF and C, and similar to FIB-4 to predict AF (Figure 2). The area under ROC curve (AUROC) of APRI to predict SF and C was 0.79 (95%CI: 0.68-0.90) and 0.76 (95%CI: 0.61-0.91), respectively, whereas the AUROC of FIB-4 to predict AF was 0.84 (95%CI: 0.74-0.95) (Supplementary Figure 1).

For the 68 patients with an APRI score, 27 (39.7%) had intermediate test values (> 0.5 and ⩽ 1.5). Of these, 17 (63%) had SF by METAVIR. All these patients were correctly classified by the MM. Likewise, among 25 (36.8%) patients with FIB-4 values in the gray zone (> 1.45 and ⩽ 3.25), the MM correctly identified 4 (16%) with AF. Figure 3 makes a comparison of the extent to which the MMs, APRI and FIB-4 would have correctly avoided the need for a biopsy.

DISCUSSION

In this study, it was observed that the higher the severity of liver fibrosis, the greater the mean age as well as the greater the impairment of liver function. In fact, this findings reflects the natural history of chronic hepatitis C, a fibrosing disease, which progresses to cirrhosis in about 30 years[23].

Our results suggest that the metabonomic strategy can discriminate F0-F1 from F2-F4 patients, F0-F2 from F3-F4 patients and F0-F3 from F4 patients. The MMs developed to predict SF, AF and C in CHC patients presented high performance, with values of SN, SP and an accuracy of above 90%. In practice, considering the confidence intervals, the results would be classified as similar to APRI and FIB-4. However, the MMs presented 100% accuracy for predicting SF and AF in the gray zone of APRI and FIB-4, when these indexes could not determine the absence or presence of significant and advanced fibrosis. If we consider the 39.7% of unclassified and 11.8% of incorrectly classified patients using APRI as a predictor of SF, liver biopsy would have been correctly avoided in 48.5%

Table 3 Linear discriminant analysis metabonomic models, APRI and FIB-4 performances to predict significant fibrosis, advanced fibrosis and cirrhosis in 69 chronic hepatitis C patients from Pernambuco/Brazil

	Biopsy		*P* value	Sensitivity		Specificity		LR+		LR-		A (%)
Significant fibrosis												
Model (n = 69)	F2-F4	F0-F1		(%)	95%CI	(%)	95%CI		95%CI		95%CI	
⩾ F2	41	2	< 0.001[1]	97.6	87.4-99.9	92.6	75.7-99.1	13.2	3.5-50.1	0.03	0.004-0.2	95.7
< F2	1	25										
APRI (n = 68)												
> 0.5	36	13	0.001[2]	85.7	71.5-94.6	50	29.9-0.70	1.71	1.2-2.6	0.3	0.1-0.7	72
⩽ 0.5	6	13										
> 1.5	19	2	0.001[1]	45.2	29.9-61.3	92.3	74.9-99.0	5.9	1.5-23.2	0.6	0.4-0.8	63.2
⩽ 1.5	23	24										
Advanced fibrosis												
Model (n = 69)	F3-F4	F0-F2		(%)	95%CI	(%)	95%CI		95%CI		95%CI	
⩾ F3	27	2	< 0.001[1]	96.4	81.7-99.1	95.1	83.5-99.4	19.8	5.1-76.5	0.04	0.005-0.3	95.7
< F3	1	39										
FIB-4 (n = 68)												
> 1.45	25	24	0.012[1]	89.3	71.8-97.7	40	24.9-56.7	1.5	1.1-2.0	0.3	0.1-0.8	60.3
⩽ 1.45	3	16										
> 3.25	21	3	< 0.001[1]	75	55.1-89.3	92.5	79.6-98.4	10	3.3-30.3	0.3	0.1-0.5	85.3
⩽ 3.25	7	37										
Cirrhosis												
Model (n = 69)	F4	F0-F3		(%)	95%CI	(%)	95%CI		95%CI		95%CI	
F4	18	1	< 0.001[1]	100	81.5-100	98	89.6-99.9	33.8[3]	6.9-163.7	0.03[3]	0.002-0.4	98.6
< F4	0	50										
APRI (n = 68)												
> 1.00	14	16	0.002[1]	77.8	52.4-93.6	68	53.3-80.5	2.4	1.5-3.9	0.3	0.1-0.8	70.6
⩽ 1.00	4	34										
> 2.00	9	9	0.008[2]	50	26.0-74.0	82	68.6-91.4	2.8	1.3-5.9	0.6	0.4-1.0	73.5
⩽ 2.00	9	41										

[1]Fisher's exact test; [2]χ^2 test; [3]Estimated value. A: Accuracy; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis index based on four factors.

of cases. On the other hand, if the MM were used to this end, the biopsy would have been correctly avoided in 95.7% of the patients. Regarding FIB-4 analysis, considering the 36.8% of unclassified and 8.8% of incorrectly classified patients, using this index as the only predictor of AF, biopsy would have been correctly avoided in 54.4% of patients, while the MM would have prevented biopsy in 95.7% of patients (Figure 3).

The most widely indirect methods for the assessment of liver fibrosis in CHC patients in routine clinical practice are the non-commercial serological indexes APRI and FIB-4, and the physical methods, such as liver stiffness measurement, by elastography based on US (transient liver elastography, acoustic radiation force impulse elastography and 2D-shear wave elastography) or based on magnetic resonance imaging. In general, these methods do not distinguish well intermediate stages of fibrosis, although they are increasingly useful in the exclusion of significant fibrosis (< F2) and presence of advanced fibrosis (⩾ F3). The serum levels of the extracellular matrix protein osteopontin are promising for the diagnosis of intermediate fibrosis, with increasing concentration in different stages of fibrosis groups from F0 to F4, progressively and significantly different between the groups, and with an AUROC of 0.977 for the discrimination of F1-F2 from F3-F4 patients[24]. Boursier *et al*[25] proposed the FibroMeter® + FibroScan® (FM+FS) algorithm, based on two fibrosis indexes (significant and advanced fibrosis indexes), from a combination of these two methods by logistic regression. Reliable diagnosis intervals of these two indexes were determined, resulting in a noninvasive classification of fibrosis in six classes. This classification showed an accuracy of 86.7% and using this algorithm, biopsy would be avoided in 100% of patients with significant and advanced fibrosis. However, these methods are based on high cost tests that are not always routinely available, especially in public health services in developing countries.

In fact, the NMR based metabonomics proved to be promising for evaluating the severity of liver disease, cirrhosis and its complications, which reflects the progression of fibrosis[26,27]. Amathieu *et al*[28] correlated the severity of hepatic impairment in 124 patients with chronic alcoholic liver disease, as measured by MELD, using the OPLS-DA model based on ^{1}H NMR spectroscopy of serum samples. The same authors, using OPLS-DA model based on ^{1}H NMR of serum samples, separated 93 patients with compensated alcoholic cirrhosis from 30 patients with acute on chronic liver failure, with good predictability[29]. Furthermore, Jiménez *et al*[30], using OPLS-DA MM, based on ^{1}H NMR spectroscopy of serum samples from cirrhotic patients, were able to distinguish between 39 patients with minimal encephalopathy and 62 patients without encephalopathy, with R^2 = 0.68 and Q^2 = 0.63. A similar finding was described by Qi *et al*[31], who demonstrated that the OPLS-DA model, based on ^{1}H

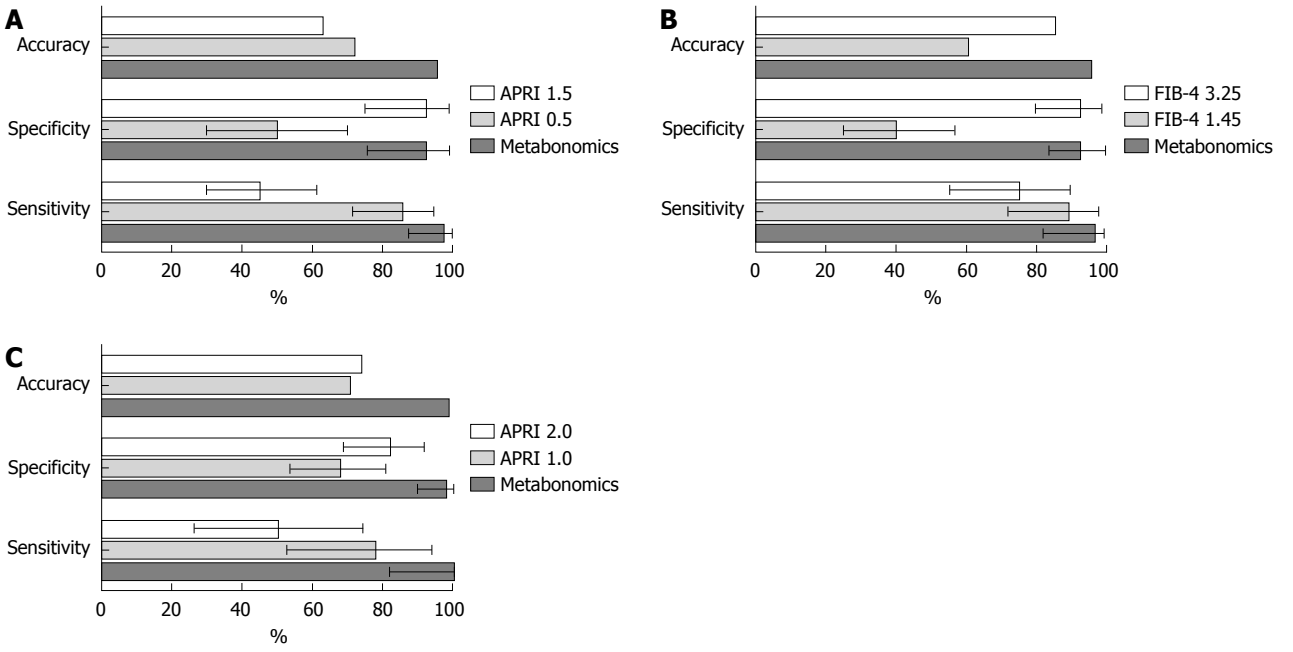

Figure 2 Comparison of performance of the linear discriminant analysis metabonomic models, aspartate aminotransferase to platelet ratio index and fibrosis index based on four factors, in 69 chronic hepatitis C patients from Pernambuco/Brazil. A and C: Performance of metabonomic models (MM) and APRI to predict SF and C; B: Performance of MM and FIB-4 to predict advanced fibrosis. APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis index based on four factors.

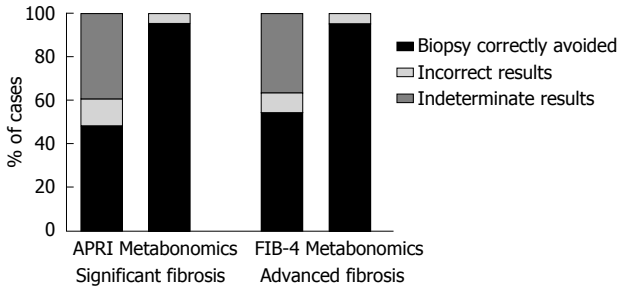

Figure 3 Comparison of biopsy correctly avoided by metabonomic models, aspartate aminotransferase to platelet ratio index and fibrosis index based on four factors, in 69 chronic hepatitis C patients from Pernambuco/Brazil. APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis index based on four factors.

NMR of serum samples, distinguished between 30 compensated HBV cirrhotic patients from 30 patients with decompensated cirrhosis, with 85% accuracy.

Regarding the evaluation of liver fibrosis, the metabonomic and metabolomic strategies have also shown promising results. In fact, Sands *et al*[32] on analyzing ^{1}H NMR plasma spectroscopy of healthy controls and CHC patients, constructed OPLS-DA MMs, using METAVIR and the Enhanced Liver Fibrosis (ELF) score as reference standards. The models distinguished between 6 healthy controls and 34 CHC patients with moderate fibrosis (F1-F2) and 22 with advanced fibrosis (F3-F4) in the validation cohort. Embade *et al*[33] differentiated 27 cirrhotic patients (F4) from 30 patients without fibrosis (F0) using a PLS-DA model, through serum spectral analysis by ^{1}H NMR, in CHC patients, with good predictive power (R^2 = 0.8 and Q^2 = 0.6). Sarfaraz *et al*[34] using serum ^{1}H NMR spectroscopy of 45 CHC patients, showed that F0-F2 and F3-F4 patients were well discriminated with the OPLS-DA MM (R^2= 0.67 e Q^2 = 0.28). Moreover, Gabbani *et al*[35], constructed PLS-models with canonical analysis (PLS-CA), based on serial analysis of serum/plasma and urine NMR spectra in 33 CHC patients, 10 compensated cirrhotic patients and 23 with mild or moderate fibrosis, as measured by biopsy or transient hepatic elastography. The authors demonstrated that the models recognized compensated cirrhosis with better accuracy in plasma/serum samples (68% in plasma, 56% in serum and 50% in urine samples). Herein, the main finding of our study was the correct classification of patients in the gray zone of APRI and FIB-4, who had mainly intermediate fibrosis (METAVIR classification equal to F2).

In conclusion, the metabonomic strategy was able to distinguish between significant liver fibrosis, advanced liver fibrosis and cirrhosis in CHC patients, showing promising results as a non-invasive tool to evaluate liver fibrosis. Furthermore, the method presented high accuracy in the gray zone of APRI and FIB-4, which could avoid large numbers of biopsies in CHC patients.

Due to the small sample size, it was not possible to use one group for external validation. Therefore, further studies with a larger number of patients tested and external validation of the models are necessary, in order to confirm the performance of the MMs, for later incorporation into clinical practice.

ARTICLE HIGHLIGHTS

Research background

Liver fibrosis is the most important prognostic factor in chronic hepatitis C (CHC). The gold standard method for liver fibrosis evaluation is the liver biopsy, which

is invasive and subject to complications and misclassification.

Research motivation

The assessment of significant liver fibrosis is important to make therapeutic decisions and predict clinical outcomes. The serological and physical surrogate methods in hepatic fibrosis evaluation, such as APRI, FIB-4 and hepatic elastography, are good in excluding significant fibrosis and confirming advanced fibrosis, but they fail in diagnosing intermediate fibrosis. The metabonomics strategy is a method that seeks to discriminate biological samples, such as serum, through ^{1}H nuclear magnetic resonance (NMR) spectroscopy, according to their biochemical status, thereby associating this status to a given condition. The method has been studied in various liver diseases, showing to be useful in identifying liver cirrhosis and its complications and with promising results in hepatic fibrosis evaluation in chronic liver diseases.

Research objectives

The aim of this study was to develop and evaluate metabonomic models (MMs), using ^{1}H NMR spectrum of serum samples, as non-invasive tool for assessment of significant liver fibrosis, advanced liver fibrosis and cirrhosis in patients with CHC. Additionally, to compare the performance of the MMs with the serological surrogate indexes APRI and FIB-4 and to investigate the performance of MMs in the gray zone of these serological indexes.

Research methods

This was a cross-sectional phase Ⅱ validation diagnostic study, with prospective inclusion of CHC adult outpatients who had undergone percutaneous liver biopsy in the previous 12 mo or had been clinically diagnosed with liver cirrhosis. The clinical diagnosis of cirrhosis was based on characteristic symptoms and signals and/or according to evidence of chronic liver disease and/or portal hypertension on ultrasound and/or signals of portal hypertension observed on upper gastrointestinal endoscopy. Those undergoing antiviral treatment or diagnosed with other liver disease were excluded. All patients signed an informed consent form and the study was approved by the Ethics Committee of the Institution. Fasting blood samples were collected from all patients by peripheral vein puncture. The laboratory tests were performed by an automated method. The HCV RNA was detected by real-time polymerase chain reaction. APRI and FIB-4 were calculated as described by Wai *et al* (2003) and Sterling *et al* (2006). Serum samples, stored at -20 ℃, were thawed at room temperature and prepared by adding 200 μL of D_2O to 400 μL of serum. ^{1}H NMR spectra were obtained using a Varian Unity Plus 300 spectrometer. The samples were analyzed using a pulse sequence with suppression of the resonance of water and T2 filter (PRESAT-CPMG). Spectra were processed using line broadening equal to 0.3 Hz. The signal attributed to the methyl group of the lactate was used as the internal reference of chemical shift. The baseline of the spectra was corrected manually. The ultrasonography-guided percutaneous liver biopsies were performed using a 16 G x 90 mm Menghini needle in, at most, 2 punctures. Fragments with at least 15 mm and /or 6 complete portal tracts were included in the analysis. Fibrosis was classified as F0 to F4, in accordance with METAVIR, by two experienced pathologists, blinded to the clinical and serological results. The patients were allocated into three groups: SF (METAVIR F $\geqslant$ 2), AF (METAVIR F $\geqslant$ 3) and C (METAVIR F = 4 or clinical cirrhosis). The descriptive and comparative analysis of the data was carried out using STATA and GraphPad Prism softwares. Categorical variables were compared using the Chi-square test, applying Fisher's exact test, when necessary. The Mann-Whitney and Student's *t*-test were used to compare non-parametric and parametric continuous measurements, respectively. All tests were applied with 95% confidence (P value $\leqslant$ 0.05). All spectra were processed using MestreNova software. The spectra were divided into 250 regions of 0.04 ppm, called bins, used to construct a dataset, eliminating the residual signal of water. The spectra were normalized and PLS-DA and LDA supervised formalisms were used. Three PLS-DA models were constructed to predict FS, FA and C, respectively, using the MetaboAnalyst 3.0 platform. The models were validated by leave-one-out-cross-validation (LOOCV) and by permutations tests. In addition, three LDA models were constructed, using the PCA matrix as input data, to predict SF; AF and CCs, and validated by LOOCV, using STATISTICA software. For each LDA MM, and for APRI and FIB-4, a 2 x 2 contingency matrix was used to calculate sensitivity (SN), specificity (SP), the positive likelihood ratio (LR+), the negative likelihood ratio (LR-) and accuracy (A), using liver biopsy as reference standard.

Research results

Sixty-nine patients were evaluated, of whom 59.4% were female, with a mean age of 57 ± 12 years. Liver biopsy was performed on 54 (78%) patients. The median fragment was 15 mm in length (P_{25}: 13; P_{75}: 18 mm), and there were 15 portal tracts (P_{25}: 12; P_{75}: 20). The METAVIR fibrosis stage was distributed as follows: F0 in 2; F1 in 25; F2 in 14; F3 in 10 and F4 in 3 patients. The diagnosis of cirrhosis was clinically established in 15 (21.7%), thus classified according to the Child-Pugh score: 10 patients Child-Pugh A and 5 Child-Pugh B. Therefore, 42 (60.9%) patients were classified as SF, 28 (40.6%) as AF and 18 (26.1%) as C. The PLS-DA MM for SF presented 100% accuracy, R^2 and Q^2 of 0.98 and 0.91, respectively. The results of the LDA MM for SF were compared to APRI and showed SN of 97.6% (95%CI: 87.4%-99.9%) and LR- of 0.03 (95%CI: 0.004-0.2), similar to APRI cut-off of 0.5, which presented SN of 85.7% (95%CI: 71.5%-94.6%) and LR- of 0.3 (95%CI: 0.1-0.7). The MM presented SP of 92.6% (95%CI: 75.7%-99.1%) and LR+ of 13.2 (95%CI: 3.5-50.1), similar to APRI cut-off of 1.5, which showed SP of 92.3% (95%CI: 74.9%-99.9%), with LR+ of 5.9 (95%CI: 1.5-23.2). The PLS-DA MM for AF discriminated all the samples, with 100% accuracy, R^2 of 0.98 and Q^2 of 0.93. LDA MM for AF showed SN of 96.4% (95%CI: 81.7%-99.1%) and LR- of 0.04 (95%CI: 0.005-0.3), similar to FIB-4 cut-off of 1.45, which showed SN of 89.3% (95%CI: 71.8%-97.7%) and LR- of 0.3 (95%CI: 0.1-0.8). The two methods also presented high SP and LR+, there being observed SP of 95.1% (95%CI: 83.5%-99.4%) and LR+ of 19.8 (95%CI: 5.1-76.5) for MM, and SP of 92.5% (95%CI: 79.6%-98.4%) and LR+ of 10 (95%CI: 3.3-30.3) for FIB- 4 cut-off of 3.25. The PLS-DA MM for C also adequately discriminated the samples, attaining an accuracy of 84.0%. The LDA MM for C showed SN of 100% (95%CI: 81.5%-100%) and LR- of 0.03 (95%CI: 0.002-0.4), similar to APRI cut-off of 1.0, which presented SN of 77.8% (95%CI: 52.4 - 93.6), with LR- of 0.3 (95%CI: 0.1-0.8). The MM presented SP of 98% (95%CI: 89.6%-99.9%), similar to APRI cut-off of 2.0, which showed SP of 82% (95%CI: 68.6 - 91.4), with higher LR+ of 33.8 (95%CI: 6.9-163.7), comparing to APRI cut-off of 2.0, which presented LR+ of 2.8 (95%CI: 1.3-5.9). In general, the MMs presented similar performance to APRI e FIB-4. However, their accuracy for predicting SF and AF in the gray zone of APRI and FIB-4 was 100%. Considering the 39.7% of unclassified and 11.8% of incorrectly classified patients using APRI as a predictor of SF, liver biopsy would have been correctly avoided in 48.5% of cases. On the other hand, if the MM were used to this end, the biopsy would have been correctly avoided in 95.7% of the patients. Regarding FIB-4 analysis, considering the 36.8% of unclassified and 8.8% of incorrectly classified patients, using this index as the only predictor of AF, biopsy would have been correctly avoided in 54.4% of patients, while the MM would have prevented biopsy in 95.7% of patients.

Research conclusions

The metabonomic strategy was able to distinguish between significant liver fibrosis, advanced liver fibrosis and cirrhosis in CHC patients, showing promising results as a non-invasive tool to evaluate liver fibrosis. The main finding of our study was the correct classification of patients in the gray zone of APRI and FIB-4, who had mainly intermediate fibrosis (METAVIR classification equal to F2), which could avoid a large number of biopsies in CHC patients.

Research perspectives

It is necessary to perform further studies testing a larger number of patients and with external validation of the models, in order to confirm the performance of the MMs, for later incorporation into clinical practice.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Observational Study

High burden of hepatocellular carcinoma and viral hepatitis in Southern and Central Vietnam: Experience of a large tertiary referral center, 2010 to 2016

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Abstract

AIM

To examine the largest tertiary referral center in southern

and central Vietnam from 2010 to 2016, evaluating epidemiological trends of hepatocellular carcinoma (HCC) and viral hepatitis B-C in this resource-limited setting.

METHODS

We extracted data of patients receiving care from Cho Ray Hospital (Ho Chi Minh City), the largest oncology referral center in southern and central Vietnam, from 2010 to 2016. We collected information on patient age, gender, geographic distribution, and disease characteristics including disease stage, tumor biomarker levels [serum alpha-fetoprotein (AFP), AFP-L3 isoform percentage, and prothrombin induced by induced by vitamin K absence-Ⅱ], and serological testing for hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.

RESULTS

Data from 24091 HCC patients were extracted, with sample demographics comprising mostly male (81.8%) and older age (however with 8.5% younger than 40 years old). This patient sample included a geographic catchment population of 56 million people (60% of the country's total population of 92.7 million), derived from 38 provinces and municipalities in Vietnam. Chronic HBV infection was found in 62.3% of cases, and chronic HCV infection in 26.0%. HBV and HCV co-infection was seen in 2.7%. Cirrhosis was found in an estimated 30% to 40% of cases. Nine percent of patients were not found to have chronic viral hepatitis. Twenty three point two percent of the patients had a normal AFP level. A total of 2199 patients were tested with AFP-L3 and PIVKA Ⅱ over two years, with 57.7% having elevated AFP-L3%, and 88.5% with elevated PIVKA Ⅱ levels. Over this 7-year period, the incidence of HCC increased, with a large proportion of cases (overall 40.8%) presenting initially an advanced stage, not amendable to surgical or locoregional therapy.

CONCLUSION

HCC contributes significant health care burden in southern and central Vietnam, with increasing case volume over this seven-year period. Viral hepatitis likely explains this high HCC prevalence.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Alpha-fetoprotein

Core tip: Hepatocellular carcinoma remains a serious public health issue in Vietnam, and is closely associated with chronic hepatitis B and C virus (HBV and HCV) infections. In one of the largest tertiary referral hospitals in southern and central Vietnam, the clinical volume has been increasing from 2010 to 2016, with most patients having chronic HBV or HCV infections, and most patients initially at an advanced stage, precluding curative treatment. Public health, policy, and institutional efforts are needed to reduce the burden that this disease places on the Vietnamese people in Vietnam.

Nguyen-Dinh SH, Do A, Pham TND, Dao DY, Nguy TN, Chen Jr MS. High burden of hepatocellular carcinoma and viral hepatitis in Southern and Central Vietnam: Experience of a large tertiary referral center, 2010 to 2016. *World J Hepatol* 2018; 10(1): 116-123 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/116.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.116

INTRODUCTION

Hepatocellular carcinoma (HCC) results in significant morbidity and mortality, and has now become the world's second deadliest cancer (after lung cancer)[1] and one of the most common especially in the developing world[2]. HCC is etiologically linked to viral hepatitis, particularly chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. HBV and HCV account for approximately 80% to 90% of HCC cases worldwide, with both infections demonstrating wide regional variability but is known to be highly endemic in Vietnam[3-5]. Although a variety of modalities exist to treat HCC including surgical resection, locoregional ablative therapies, systemic chemotherapy, and liver transplantation, most individuals present later in their disease course and thus are limited in their ability to receive curative treatment[6,7]. HCC is a serious public health issue in Vietnam, with varied reported age adjusted incidence rates but generally thought to be greater than 20 per 100000 people, compared to that experienced in industrialized countries which are estimated to be less than 5 per 100000 people[6,8].

Viral hepatitis is thought to account for many cases of HCC, and epidemiological studies report high HBV and HCV infection rates among Vietnamese people, but have been limited to regional data (provincial or city-wide data) primarily reporting data obtained in northern regions[9-13]. Compounding the issue of high disease prevalence, the burden of disease is suggested to continue to rise in the coming decade, despite a country-wide universal infant immunization program established in 2003, though it has also been reported to have reduced chronic HBV rates[14,15]. It is difficult to ascertain whether efforts in Vietnam have been effective thus far, and challenges associated with reliance of survey-based data, incomplete death reporting, wide geographic dispersal of deaths, and travel between households, and limited testing without systematic screening, are potential limitations to obtaining accurate epidemiological estimates of disease burden in this resource-limited setting[16].

There is no comprehensive national nor regional cancer registry in Vietnam, and limited information in southern and central Vietnam is available compared to the northern regions. Thus, the purpose of this study is to describe and report the experience of Cho Ray Hospital, the largest tertiary referral center in southern and central Vietnam, to better elucidate the HCC disease burden placed on this resource-limited medical system,

as well as geographic and demographic epidemiological trends from 2010 to 2016. We hypothesized that this large referral center, despite being one of the largest in Vietnam, is experiencing an increasing volume of patients with HCC during the study time period. Additionally, as chronic HBV is estimated to account for approximately 50% of all HCC cases, we also hypothesize that the majority of patients with HCC will have comorbid HBV infection, and will comprise a wide range of ages owing to high prevalence of vertical HBV transmission.

MATERIALS AND METHODS

Patient dataset

We conducted a prevalence study across a seven-year time period to examine the public health burden of HCC in southern and central Vietnam, using a large database of patients with liver cancer receiving care at Cho Ray Hospital (CRH), the largest tertiary referral center in Southern and Central Vietnam. CRH is located in Ho Chi Minh City and has a catchment area for HCC referral thought to be 56 million people (approximately 60% of the country's total population of 92.7 million people), including 90% of southern and central Vietnam. This hospital has 1200 inpatient beds and serves approximately 67000 inpatients and 457000 outpatients per year[17]. Patients are cared for by providers of the Liver Cancer Center, composed of 12 medical hepatologists and surgeons. All patients with newly-diagnosed HCC or referred for further management of HCC after diagnosis, were included in this study. Referral for HCC management is generally the reason these patients receive care for HCC at CRH. We collected and analyzed data from January 1st, 2010 through December 31st, 2016. All patients of Vietnamese origin were included for analysis. HCC diagnosis, surveillance, and management was made according to Japan Society of Hepatology clinical guideline recommendations, including imaging characteristics (ultrasonography, computed tomography, and magnetic resonance imaging) with tumor characteristics, in conjunction with Child-Pugh classification stage and tumor marker levels[18].

Patient demographic information was obtained, including age in years, gender (male or female), region of residence (province or municipality), serological testing including serum HBV surface antigen (HBsAg) and anti-HCV antibody, and tumor markers [serum alpha-fetoprotein (AFP), AFP-L3 isoform percentage, and prothrombin induced by induced by vitamin K absence-Ⅱ (PIVKA-Ⅱ, also known as des-gamma-carboxy prothrombin, or DCP)]. Etiology of liver disease was classified as due to viral hepatitis (HBV, HCV, or HBV+HCV infections), or other. Chronic HCV infection was defined as having a positive anti-HCV antibody result. Chronic HBV infection was defined as having a positive HBsAg level. Patients were determined to have cirrhosis based on suggestive imaging findings on ultrasound or computed tomography (CT) imaging with or without evidence of liver chemistry abnormalities [aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, bilirubin]. Staging and management of HCC was based on Vietnamese country-wide guidelines, adapted from the Asia-Pacific Association for the Study of the Liver HCC Guidelines, which has been most recently updated in 2017[18-21]. Local institutional review board approval was obtained for this study. Disease severity was classified as advanced (no treatment possible other than palliative care) *vs* other.

Statistical analysis

Descriptive statistics were generated for this patient dataset, with association testing performed with chi-square for proportions of categorical variables, respectively. Trends testing was performed with Manel-Haenzel tests for categorical variables. All analyses were performed using SAS 9.4 for Windows (Cary, NC, United States).

RESULTS

Sample size and characteristics

After applying exclusion criteria, we extracted data from 24091 Vietnamese patients with HCC from 2010 to 2016. Patient demographic and disease characteristic distributions are summarized in Table 1. An increasing case volume in Cho Ray Hospital was observed during this period (2793 in 2010 with annual increase to 4069 in 2016). The majority of the sample were males (81.8%) and comprised a wide range of ages: A plurality were 50 to 60 years old (29.9%), but a majority of patients were between 40 to 70 years old (72.4%). A notable proportion of patients (8.5%) in the sample were younger than 40 years old.

HCC severity and associated diseases

A large proportion of individuals (9827 patients, 40.8% of total sample), comprising of 8491 (86.4%) males and 1336 (13.6%) females, presented with advanced HCC, only amendable to palliative care (Table 2). In this sample and across years, only 47.0% to 50.5% of patients with HCC had serum AFP levels > 400 ng/mL, otherwise with the next-largest group (ranging 21.6% to 24.0%) had normal levels ⩽ 20 ng/mL. AFP level subgroups did not demonstrate a trend during the study time period (P = 0.33).

New serum tumor biomarker tests AFP-L3 percentage and PIVKA Ⅱ levels were obtained starting in 2015. In 2015, 23.3% of patients received testing with these biomarkers, increasing to 32.2% in 2016. In these patients, 56.4% to 58.6% of patients had an elevated AFP-L3% (greater than 10%, the upper limit of normal), while 88.3% to 88.7% of patients had elevated PIVKA Ⅱ levels (> 40 mAU/mL, the upper limit of normal).

Etiology and associated diseases

Most patients with HCC had available viral hepatitis serologies (89.6%) with HBsAg and anti-HCV antibody testing. Of those tested, 59.7% to 69.9% (overall

Table 1 Percentage distribution of demographic and disease characteristics of patients with primary hepatocellular carcinoma at Cho Ray Hospital, Ho Chi Minh City, Vietnam, 2010 to 2016

Characteristic	2010 (n = 2793)	2011 (n = 3111)	2012 (n = 3349)	2013 (n = 3471)	2014 (n = 3494)	2015 (n = 3804)	2016 (n = 4069)	Total (n = 24091)	P value
Male gender	82.6	82.77	82.23	80.44	81.2	81.2	82.13	81.76	0.17
Age (yr)									
≤ 40	10.31	8.94	9.14	8.58	7.76	7.6	7.77	8.49	< 0.0001
41-50	19.3	18.58	17.44	17.26	17.32	17.43	16.76	17.64	
51-60	30.68	30.57	29.23	30.83	29.28	29.86	29.29	29.92	
61-70	21.48	22.6	24.37	23.22	26.24	26.63	27.89	24.86	
> 70	18.22	19.32	19.83	20.11	19.4	18.48	18.28	19.08	
Advanced disease stage	40.57	43.68	40.52	37.65	40.78	40.75	41.68	40.79	0.76
HBsAg and anti-HCV testing available (n)[1]	n = 2505	n = 2405	n = 2773	n = 3153	n = 3225	n = 3616	n = 3907	n = 21584	< 0.0001
HBV infection	61.72	69.94	66.35	60.1	59.69	60.87	60.23	62.28	
HCV infection	24.79	27.32	28.96	25.06	27.13	25.06	24.98	26	
HBV and HCV coinfection	2.71	2.74	2.52	3.04	2.95	2.41	2.46	2.68	
Non-HBV or HCV liver disease	10.78	0	2.16	11.8	10.23	11.67	12.34	8.97	

[1]HBV infection defined as positive HbSAg level. HCV infection defined as positive HCV antibody level. HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

Table 2 Distribution of serum alpha-fetoprotein levels in patients with hepatocellular carcinoma, 2010 to 2016

AFP level (ng/mL)	2010 (n = 2793)	2011 (n = 3111)	2012 (n = 3349)	2013 (n = 3471)	2014 (n = 3494)	2015 (n = 3804)	2016 (n = 4069)	Total (n = 24091)
< 20	23.24	22.53	21.59	26.22	22.41	22.32	23.99	23.21
> 20-100	15.43	14.14	15.17	13.71	15.00	14.54	14.48	14.62
> 100-200	6.30	5.37	5.11	5.36	6.70	6.34	6.41	5.96
> 200-400	5.87	5.63	5.32	5.30	5.38	5.81	5.97	5.62
> 400	47.65	50.50	50.82	46.99	48.20	49.50	47.53	48.72
Not recorded	1.50	1.83	2.00	2.42	2.32	1.5	1.62	1.88

P value for trend = 0.33. AFP: Alpha-fetoprotein.

62.3%) were found to have chronic HBV infection, while 24.8% to 28.9% (overall 26.0%) had chronic HCV infection. 2.4 to 3.0% (overall 2.7%) of patients had HBV-HCV co-infection. Mono-infection subgroups demonstrated an upward trend during the study period (P < 0.001). 9.0% of the tested sample had negative viral hepatitis makers (negative for both HBsAg and anti-HCV antibody). Cirrhosis was estimated to be present in 30% to 40% of individuals presenting for care, based on presence of hepatic decompensation or suggestive imaging (ultrasound, computed tomography, or magnetic resonance imaging) with hepatic macronodular changes and portal hypertension when available.

Geographic disease distribution

Figure 1 illustrates the geographical distribution from which the sample of HCC patients were derived, which included a total of 38 provinces. Most patients came from Southern Vietnam, with the provinces with most patients including Ho Chi Minh City (3830 patients, 15.89% of total), Tien Giang (1618 patients, 6.72% of total), Dong Nai (1567 patients, 6.50% of total), An Giang (1349 patients, 5.60% of total).

DISCUSSION

We report over 24000 cases of HCC seen over a 7-year period at a tertiary referral hospital, showing an increasing burden of HCC based on the experience of Cho Ray Hospital, with a high frequency of individuals initially presenting with untreatable, advanced disease. Most cases likely resulted from chronic HBV and/or HCV infection. Additionally, we find that men are disproportionately affected compared to women, and that a wide range of ages are affected, including a non-negligible proportion of patients younger than 40 years old (8.5%), which is generally thought to be an uncommon occurrence[2]. This sample entails a broad expanse of southern and central Vietnam, and covers seven recent years. Additionally, AFP tumor markers were elevated in only approximately half of these patients, demonstrating insufficiency of this sole biomarker in appropriate screening for these patients.

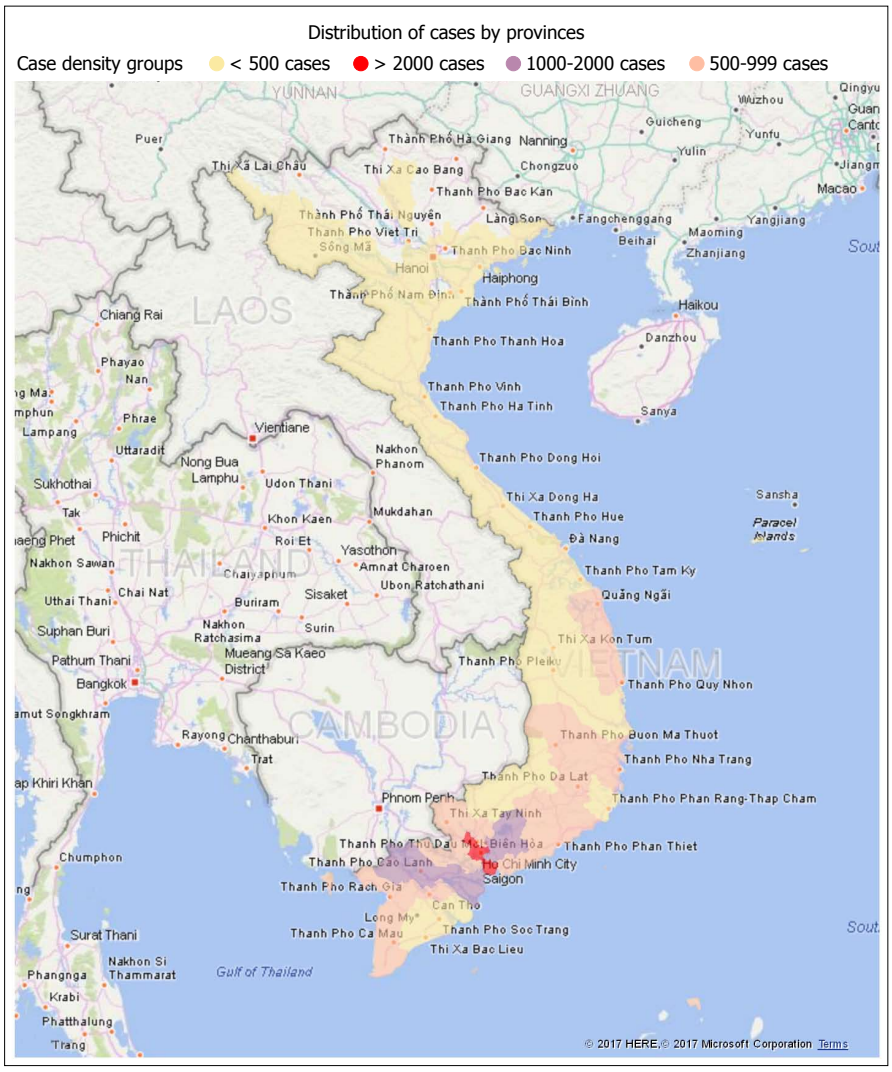

Figure 1 Geographic distribution of patients with hepatocellular carcinoma receiving care at Cho Ray Hospital, Vietnam.

The strengths of this observational study are considerable. Data on viral hepatitis and HCC from Vietnam are rare. To the best of our knowledge, this study represents the largest number of HCC cases reported and analyzed from Vietnam since its reunification in 1975. Cho Ray Hospital is the largest oncology center in southern and central Vietnam. These patients comprise a set of varied demographic and clinical characteristics including viral hepatitis status, disease stage, tumor biomarkers levels, and other indicators were meticulously compiled and analyzed. This data illustrates a clearer image of the markedly severe epidemic that viral hepatitis imposes upon the Vietnamese people, and the marked geographical differences seen between industrialized and rural countries highlights the potential for improved public health with appropriate and sufficient resources.

Our data suggest a potential role for additional tumor biomarker testing with a relatively large proportion of people demonstrating elevated AFP-L3 and PIVKA Ⅱ levels. The Japan Society of Hepatology has recommended HCC surveillance using these biomarkers in supplement to AFP and imaging, and data supports use of these additional biomarkers as a surveillance tool to determine risk of HCC development, in supplement to AFP levels[18,22-24].

Despite these considerable strengths, we are aware of limitations of our findings. Our data were derived from a cross sectional rather than longitudinal database thus limiting data on patient outcomes. As Cho Ray Hospital is a tertiary referral center, many patients had been recently diagnosed with HCC or the clinical suspicion exists before referral. In addition, inadequate knowledge of formal screening and surveillance best practices for HCC among health care providers resulted in late entry to care. Resource limitations in the setting of large patient population-to-provider ratios, access to timely laboratory and imaging testing, and challenges to universal direct-acting antiviral therapies may all play a role in contributing to the high observed disease burden. Additionally, further studies with additional detailed assessment of clinical staging and tumor characteristics with a standardized system (*e.g.*, Child-Pugh or Barcelona Clinic Liver Cancer classification),

prior vaccination/treatment and viral hepatitis details (genotype, viral load), in conjunction with patient outcomes and evaluation for additional etiologies of liver disease such as alcohol use or non-alcoholic steatohepatitis, would enhance our knowledge of anticipated disease burden in Vietnam. Although the catchment area of this medical center is expansive, it does not cover northern Vietnam and thus does not allow for national-level estimates.

Nevertheless, there is much untapped potential and much to be done. Vietnam is a high endemic area for HBV and HCV, with consequent HCC. While lung cancer leads all cancer deaths in Vietnam accounting for 5.95% of all mortality, liver cancer is second of all cancer deaths, accounting for 2.42% but has been increasing at 2.34% annually[25]. To characterize the situation in Vietnam as "epidemic" for HCC compared to the rest of the world would not be an understatement. Additionally, as the window for early-stage HCC diagnosis is thought to be small, measures to quickly identify and provide care linkage, even if on small scales, will likely result in a large magnitude effect given the endemic and epidemic nature of viral hepatitis in Vietnam.

The burden of HCC at the Cho Ray Hospital tertiary referral center is substantial and does not appear to subside in the foreseeable future. Certainly, from a public health perspective, screening for viral hepatitis, both HBV and HCV, with linkage to care is highly warranted. For those not already infected and without natural immunity, HBV vaccinations should be administered and education regarding protecting against HCV transmission should be offered through culturally and linguistically competent messaging. For those who are chronically infected, clinical management and follow up are necessary, but how this occurs should be determined based on future needs assessments and can potentially take the form of augmentation of specialist hepatologists, more education to general providers, or increasing facility access for liver-related diagnostics and care (*e.g.,* elastography, advanced laboratory testing such as tumor markers).

Systematic, institutional, and programmatic efforts all have a role to play in combating HCC, and adoption of birth dose provision through a large-scale childhood HBV vaccination program has been reported to reduce chronic HBV infection rates[15]. Consistent linkage and maintenance of care is paramount, especially as HCC often occurs on the backdrop of chronic diseases (viral hepatitis and cirrhosis) which warrant consistent, regular medical care. Additionally, the need for ongoing and updated information will be important in the coming years to assess progress. A formal, national registry to capture all cases, associated risk factors, and treatment histories collected and analyzed in a standardized manner should allow medical providers and governmental policymakers to better understand high-yield interventional points as well as high-risk populations or sub-populations, to affect the highest impact while minimizing cost.

Additionally, clinical practice standardization is important as data quality in concert with collection will allow for the most accurate public health response. For example, at the institutional level, standardization of disease staging and management will allow for better disease characterization across medical institutions, although it is known that the Barcelona Clinic Liver Cancer (BCLC) staging system is not necessarily utilized by many providers in the Asian-Pacific region, formal staging using the Asia-Pacific Clinical Practice Guidelines or other system will allow for standardization and thus comparability[21,26].

However, specialists in viral hepatitis care and HCC in Vietnam are quite limited. A huge demand for training by the primary care providers and their support personnel exists for the kind of training offered by the Vietnam Viral Hepatitis Alliance where its two annual conferences attracted 350 in its inaugural year, increasing to 500 in its second year. Training both primary care providers along with their support staff so that their knowledge of viral hepatitis and HCC and their practical roles in care are needed. Additionally, health education for the public at large is essential. From research conducted among overseas Vietnamese in the United States, the odds of Vietnamese patients being screened for HBV is 4-fold if the provider recommends it and increases to nearly 9-fold if both the provider and the patient ask for it[27]. We anticipate that the dual targeting of providers and patients will bring about synergy in promoting serological testing for viral hepatitis as the first community-based intervention to stem the tide of HCC in Vietnam.

In the future, trends in chronic liver disease seen in industrialized countries could be expected to be mirrored in Vietnam as well. With globalization of convenience foods that favor taste over nutrition as well as other geopolitical and economy-based factors, concern for obesity and metabolic-associated diseases will likely become a larger issue in Vietnam in the future. From a hepatology perspective, there is known concern about increasing diabetes rates, and given worldwide trends of non-alcoholic fatty liver disease increasing in industrialized countries this may contribute to the growing burden of HCC in Vietnam as well[28-30].

In conclusion, from 2010 to 2016, we find an increasing number of patients receiving HCC care at Cho Ray Hospital, a large tertiary referral center serving southern and central Vietnam, with most patients having an advanced disease stage not amendable to treatment, and additionally these cases are reasonably attributed to chronic HBV or HCV infection. Future efforts in public health efforts to screen, provide linkage to care, to provide surveillance, and to educate health care providers, will all play an important role in curtailing the burgeoning of this disease throughout Vietnam in the coming years.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is currently the world's second deadliest

cancer. HCC is closely linked to viral hepatitis, which in turns has been reported to have high epidemiologic heterogeneity especially in the developing world. There is limited epidemiological data on HCC reported in Vietnam, particularly the southern and central regions.

Research motivation

This study primarily seeks to elucidate the epidemiological characteristics of HCC in southern and central Vietnam. These results have significant policy and research implications in establishing priorities for public health interventions, financial allocations, and driving knowledge acquisition in further large-scale observational studies and potential biomarker testing expansion.

Research objectives

The authors sought to evaluate the burden of HCC and characteristics of patients presenting with HCC, as well as potential disease etiology.

Research methods

The authors conducted an epidemiological observational study from 2000 to 2016, using a large database of patients with liver cancer who receive care at Cho Ray Hospital, the largest tertiary referral center in southern and central Vietnam. Information on patient demographic information, disease staging, and tumor marker results were extracted.

Research results

Analysis was performed on 24091 patients from 2010 to 2016, with increasing disease frequency noted (2793 patients in 2010 to 4069 in 2016). Most patients were male (86.4%), most patients presented with advanced disease (40.8%). Most patients were found to have viral hepatitis (89.6%), with 62.3% with HBV, 26.0% with HCV, and 2.7% with HBV-HCV coinfection. Eight point five percent of patients were younger than 40 years old.

Research conclusions

In the largest epidemiological study conducted for liver cancer in Vietnam to date, we find high and increasing disease burden from 2010 to 2016, which manifests as advanced disease and co-prevalent with viral hepatitis. Demographic patterns suggest higher disease burden on males and disproportionate burden on younger patients.

Research perspectives

These findings emphasize the importance of developing systems and methods to better understand epidemiology of liver cancer, as well as for linkage to care, evaluation, and treatment of both liver cancer and viral hepatitis. Future research should focus on health care services and policy implications for disease screening and treatment outcomes for this population.

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ORIGINAL ARTICLE

Observational Study

Toll-like receptor 4 polymorphisms and bacterial infections in patients with cirrhosis and ascites

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Abstract

AIM

To assess the relationship between the presence of toll-like receptor 4 (TLR4) polymorphisms and bacterial infections in cirrhotic patients with ascites.

METHODS

We prospectively included consecutive patients with cirrhosis and ascites hospitalized during a 6-year period. Patients with human immunodeficiency virus (HIV) infection or any other immunodeficiency, patients with advanced hepatocellular carcinoma (beyond Milan' s criteria) or any other condition determining poor short-term prognosis, and patients with a permanent urinary catheter were excluded. The presence of D299G and/or T399I TLR4 polymorphisms was determined by sequencing and related to the incidence and probability of bacterial infections, other complications of cirrhosis, hepatocellular carcinoma, and mortality during follow-up. A multivariate analysis to identify predictive variables of mortality in the whole series was performed.

RESULTS

We included 258 patients: 28 (10.8%) were carriers of D299G and/or T399I TLR4 polymorphisms (polymorphism group) and 230 patients were not (wild-type group). The probability of developing any bacterial infection at one-year follow-up was 78% in the polymorphism group and 69% in the wild-type group (P = 0.54). The one-year probability of presenting infections caused by gram-negative bacilli (51% *vs* 44%, P = 0.68), infections caused by gram-positive cocci (49% *vs* 40%, P = 0.53), and spontaneous bacterial peritonitis (29% *vs* 34%, respectively, P = 0.99) did not differ between the two groups. The one-year probability of transplant-free survival was 55% in the polymorphism group and 66% in the wild-type group (P = 0.15). Multivariate analysis confirmed that age, Child-Pugh score, active alcohol intake, previous hepatic encephalopathy, hepatocellular carcinoma and serum creatinine were associated with a higher risk of death during follow-up.

CONCLUSION

Genetic polymorphisms D299G and/or T399I of TLR4 do not seem to play a relevant role in the predisposition of cirrhotic patients with ascites to bacterial infections.

Key words: Cirrhosis; Genetic polymorphisms; Toll-like receptor 4; Bacterial infections; Ascites

Core tip: Patients with cirrhosis present a high incidence of bacterial infections. Toll-like receptor (TLR) 4 genetic polymorphisms, particularly D299G, have been previously associated with an increased predisposition to infection in several populations. In the present study, genetic polymorphisms D299G and/or T399I of TLR4 do not seem to play a relevant role in the predisposition to develop bacterial infections or in the prognosis of cirrhotic patients with ascites. Age, serum creatinine, Child-Pugh score, active alcohol intake, previous hepatic encephalopathy and the presence of hepatocellular carcinoma were independent predictive factors of mortality during follow-up.

Alvarado-Tapias E, Guarner-Argente C, Oblitas E, Sánchez E, Vidal S, Román E, Concepción M, Poca M, Gely C, Pavel O, Nieto JC, Juárez C, Guarner C, Soriano G. Toll-like receptor 4 polymorphisms and bacterial infections in patients with cirrhosis and ascites. *World J Hepatol* 2018; 10(1): 124-133 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/124.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.124

INTRODUCTION

Cirrhotic patients, particularly those with decompensated disease, are at high risk to develop bacterial infections. Such infections may in turn precipitate other decompensations of cirrhosis, including renal failure, hepatic encephalopathy, variceal bleeding and acute-on-chronic liver failure (ACLF). As a consequence, bacterial infections have a significant impact on survival in these patients[1-3].

The most common bacteria causing spontaneous bacterial peritonitis (SBP), spontaneous bacteremia and urinary tract infections are enteric gram-negative bacteria (mainly *Escherichia coli*), while gram-positive bacteria are more likely to be the causative agent in cases of pneumonia (*Streptococcus*) and instrumentation-related infection (*Staphylococcus*)[1-3]. The relative importance of gram-positive pathogens has increased in recent years due to norfloxacin prophylaxis and invasive procedures. However, bacterial translocation of gram-negative gut bacteria continues to be an important step in the pathogenesis of infections

in cirrhotic patients, mainly in those with advanced liver insufficiency and portal hypertension[1-4] because of a failure in their local and systemic immune defenses[5].

Toll-like receptors (TLR) are a family of transmembrane receptors found on monocytes, macrophages and neutrophils, and play a key role in the innate immune response. Their main function is the recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), lipoproteins and peptidoglycans[6].

TLR4 in particular recognizes LPS from gram-negative bacilli. The presence of the genetic polymorphisms D299G (rs4986790) and/or T399I (rs4986791) of TLR4 is believed to increase susceptibility to developing infections in some populations[7-10] including cirrhotic patients[10-14]. In a previous retrospective study, an association between the presence of the D299G TLR4 polymorphism and a predisposition to developing bacterial infections in cirrhosis patients was observed[14]. However, this association has yet to be evaluated prospectively.

The aim of this study was to prospectively assess the relationship between the presence of D299G and/or T399I TLR4 polymorphisms and the incidence of bacterial infections in cirrhotic patients with ascites.

MATERIALS AND METHODS

Patients

We prospectively included all cirrhotic patients with ascites hospitalized in the Department of Gastroenterology at Hospital de la Santa Creu i Sant Pau, a tertiary hospital in Barcelona, Spain, from May 2005 to May 2011. We included the patients to the study the first day of admission at the hospital. Biopsy and clinical, analytical and ultrasonographic data were used to diagnose cirrhosis. With the aim of minimizing the effects of confounding factors in the development of bacterial infections, the course of cirrhosis and survival, patients with human immunodeficiency virus (HIV) infection or any other immunodeficiency, patients with advanced hepatocellular carcinoma (beyond Milan's criteria) or any other condition determining poor short-term prognosis, and patients with a permanent urinary catheter were excluded. At admission, we recorded demographic, clinical and analytical characteristics (etiology of cirrhosis, degree of liver insufficiency, renal function), and previous infections and decompensations of cirrhosis.

We obtained blood samples for a posterior genetic analysis of TLR4 polymorphisms on the first day of admission. Patients were assigned to two groups: Subjects with D299G and/or T399I TLR4 polymorphisms (polymorphism group) and subjects without (wild-type group).

The study was approved by the Research Ethics Committee at Hospital de la Santa Creu i Sant Pau. All patients gave consent to be included in the study after receiving appropriate verbal and written information.

Follow-up evaluation

We prospectively determined the incidence and the probability to present infections, other complications of cirrhosis, hepatocellular carcinoma, and mortality during follow-up; and we compared patients from the two groups. Follow-up was performed through regular outpatients visits with a frequency according to patient's clinical condition but at least twice a year, and hospitalizations. A multivariate analysis to identify predictive variables of bacterial infection and mortality in the whole series was performed.

Spontaneous bacterial peritonitis (SBP) was diagnosed on the basis of an ascitic fluid neutrophil polymorphonuclear cell count $\geqslant$ 250/mm^3 with or without positive culture[4]. Bacterascites was defined as the presence of a positive culture with a neutrophil polymorphonuclear count < 250/mm^3 in ascitic fluid[4]. Bacteremia was diagnosed when blood cultures were positive. Conventional criteria were applied for the diagnosis of urinary tract infections, pneumonia, cellulitis and other infections[1]. Secondary bacterial peritonitis and postoperative wound infections were excluded from this analysis, since TLR polymorphisms are unlikely to influence the bacteria responsible for these infections and including them would possibly have biased the analysis of the results.

Genomic DNA extraction and polymorphism genotyping

Genomic DNA was extracted from buffy-coat fraction by using QIAmp DNA blood minikit (Qiagen Inc., Valencia, CA, United States). Sequencing was performed by Macrogen Inc, South Korea using BigDye (Applied Biosystem) chemistry after the PCR-amplified DNA fragment was confirmed. The sequencing primer used for TLR4 Asp299Gly (D299G, rs4986790) was 5'-TGGAATGCTGGAAATCCAGA-3', and for Thr399Ile (T399I, rs4986791) was 5'-CTCTAGAGGGCCTGTGCA-3'.

Statistical analysis

Data are expressed as mean ± SD or frequencies. Results were analysed using the Fisher exact test for qualitative variables. For quantitative parameters the normal distribution was confirmed with Kolmogorov-Smirnov or Shapiro-Wilk tests. We used the nonparametric Mann-Whitney test for non-normally distributed data, and the Student's "*t*" test for normally distributed data. The probabilities of bacterial infections and survival were calculated using the Kaplan Meier method and compared with the log rank test. A multivariate analysis including the variables with a P value < 0.05 in the univariate analysis was performed using Cox proportional hazards regression to identify independent predictive factors of bacterial infection and survival. A P value < 0.05 was considered statistically significant. Statistical analysis was performed using the IBM Corp. Released 2013

Table 1 Baseline characteristics of patients from the polymorphism group and the wild-type group

	Polymorphism group (*n* = 28)	Wild-type group (*n* = 230)	*P* value
Age (yr)	65.93 ± 12.1	64.90 ± 12.7	0.74
Gender (male/female)	17 (60.7)/ 11 (39.3)	128 (55.7)/ 102 (44.3)	0.69
Cause of cirrhosis (%)			0.07
Alcohol	14 (50)	125 (54.3)	
Hepatitis C virus	11 (39.3)	82 (35.7)	
Hepatitis B virus	0 (0)	8 (3.5)	
Alcohol + HCV/HVB	6 (21.4)	18 (7.8)	
Others	3 (10.7)	22 (9.6)	
Active alcoholism (%)	9/20 (45)	80/143 (55.9)	0.84
Diabetes mellitus (%)	9 (32.1)	75 (32.6)	1.00
Child-Pugh score	8.25 ± 1	8.31 ± 1.6	0.95
MELD score	15.29 ± 4.8	15.35 ± 6.2	0.69
Previous decompensations (%)	20 (71.4)	160 (69.6)	1.00
Previous ascites (%)	19 (67.9)	151 (65.7)	1.00
Previous encephalopathy (%)	13 (46.4)	51 (22.2)	0.009
Previous variceal bleeding (%)	7 (25)	54 (23.5)	0.82
Previous spontaneous bacterial peritonitis (%)	3 (10.7)	16 (7)	0.44
Hepatocellular carcinoma (%)	4 (14.3)	26 (11.3)	0.55
Norfloxacin prophylaxis (%)	4 (14.3)	18 (7.8)	0.28
Beta-blockers (%)	10 (35.7)	74 (32.2)	0.68
Diuretics (%)	15 (53.6)	135 (58.7)	0.69
Serum sodium (mmol/L)	135.14 ± 6.3	135.14 ± 9.8	0.78
Serum urea (mmol/L)	9.6 ± 4.1	9.6 ± 9.1	0.18
Serum creatinine (μmol/L)	117.29 ± 59.6	100.6 ± 59.9	0.01
Serum bilirubin (μmol/L)	54.7 ± 59.7	43 ± 34.1	0.64
Serum albumin (g/L)	26.6 ± 5.5	27.9 ± 5.5	0.43
Prothrombin time ratio	1.51 ± 0.24	1.57 ± 0.67	0.65
Ascitic fluid total protein (g/L)	13.9 ± 6.6	15.6 ± 10.7	0.30
First decompensation[1]			
Ascites (%)	19 (67.8)	165 (71.7)	0.66
Encephalopathy (%)	3 (10.7)	6 (2.6)	0.06
Variceal bleeding (%)	4 (14.3)	44 (19.1)	0.79
Infection (%)	2 (7.1)	12 (5.2)	0.65
Cause of current admission[2]			
Ascites (%)	8 (28.6)	101 (43.9)	0.15
Encephalopathy (%)	5 (17.9)	22 (9.6)	0.18
Variceal bleeding (%)	3 (10.7)	31 (13.5)	1.00
Spontaneous bacterial peritonitis (%)	6 (21.4)	33 (14.3)	0.39
Other infection (%)	3 (10.7)	13 (5.7)	0.39
Other (%)	3 (10.7)	30 (13)	1.00

[1]Refers to the first decompensation that patients presented in the past (in patients with previous decompensation) or at present admission (in patients without previous decompensation); [2]Main cause of the hospitalization in which the patient was included to the study. Data are presented as mean ± SD or frequencies (%). MELD: Model for end-stage liver disease; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

IBM SPSS Statistics for Windows, Version 22.0; IBM Corp, Armonk, NY, United States.

RESULTS

Patients' characteristics

From May 2005 until May 2011, 332 cirrhotic patients with ascites were admitted to the Department of Gastroenterology and assessed for inclusion in the study. All patients presented ascites with or without other current complications of cirrhosis, such as bacterial infection, variceal bleeding, hepatic encephalopathy, hepatorenal syndrome, or other admission causes. We evaluated patients with and without previous decompensations of cirrhosis. Seventy-four of the 332 patients were excluded for the following reasons: 9 patients due to HIV infection, one due to variable common immunodeficiency, 45 due to advanced hepatocellular carcinoma, 12 due to other conditions associated with a poor short-term prognosis (two lung neoplasia, one ovarian neoplasia, one bladder neoplasia, one lymphoma, one brain neoplasia, four severe cardiac failure, and two advanced respiratory insufficiency), and 7 due to permanent urinary catheter. Finally, 258 patients were included in the study. The retrospective data of the first 111 patients was previously published in a preliminary study from our group[14].

Analysis of TLR4 polymorphisms showed 28 patients (10.8%) were carriers of D229G and/or T399I polymorphisms. All patients were heterozygous and no homozygous patients were detected. Twenty-five patients (9.7%) had both polymorphisms, one patient was a carrier of the D299G polymorphism only and two were carriers of the T399I polymorphism only. Patients were then separated into two groups: 28 patients (10.8%) were D299G and/or T399I TLR4 polymorphism (polymorphism group) carriers and 230 who were not (wild-type group).

Table 1 summarizes the clinical and analytical characteristics of the both groups at inclusion in the study. We found no statistical differences between the two groups regarding demographics, etiology of cirrhosis, degree of liver insufficiency assessed by the Child-Pugh and MELD scores, previous decompensations of cirrhosis, current medications, type of first decompensation or cause of current admission. However, previous hepatic encephalopathy was more frequent and serum creatinine had a more elevated level in the polymorphism group than in the wild-type group.

The mean follow-up in all patients was 26.6 ± 31.7 mo, 16.9 ± 25.4 mo in the polymorphism group and 27.8 ± 32.2 mo in the wild-type group (P = 0.04). Three patients (10.7%) in polymorphism group and 16 patients (7%) in wild-type group (P = 0.47) were referred to another center to be evaluated for liver transplantation, and therefore censored at that time for the present study. Within the group of patients with alcoholic cirrhosis, 86/140 (61.4%) had active alcohol use at inclusion in the study: 9/14 (64.3%) in the polymorphism group and 77/126 (61.1%) in the wild-type group (P = 1.00). Alcohol abstinence during follow-up in these patients with active alcohol consumption at inclusion was 48/86 patients (55.8%): 4/9 (44.4%) in the polymorphism group and 44/77

Table 2 Overall incidence, number of episodes and causative bacteria of infections in patients from the polymorphism group and the wild-type group during follow-up

	Polymorphism group (*n* = 28)	Wild-type group (*n* = 230)	*P* value
Total infections			
Patients (%)	22 (78.6)	171 (74.3)	0.81
Number of infections	63	437	
Number per patient	2.25 ± 2.40	1.92 ± 2.19	0.46
Infections caused by gram-negative bacilli			
Patients (%)	11 (39.2)	102 (44.3)	0.84
Number per patient	0.86 ± 1.84	0.77 ± 1.25	0.75
Infections caused by gram-positive cocci			
Patients (%)	14 (50)	87 (37.8)	0.22
Number per patient	0.64 ± 0.73	0.60 ± 1.009	0.83

Data are presented as mean ± SD or frequencies (%).

(57.1%) in the wild-type group (*P* = 0.50).

Infections during follow-up

Table 2 shows bacterial infections diagnosed in the two groups during follow-up. Considering all the infections, no statistical differences were observed between the two groups in terms of the incidence and number of infections per patient during the follow-up period, although there was a slight trend to a higher number of infections per patient in the polymorphism group than in the wild-type group. The incidence and the number of infections per patient caused by gram-negative bacilli or gram-positive cocci was similar in both groups although there was a higher, though non-statistically significant, incidence of gram-positive cocci infections in the polymorphism group compared to the wild-type group (50% *vs* 37.8% *P* = 0.22).

Table 3 shows the different types of bacterial infections caused by gram-negative bacilli or gram-positive cocci**.** There were not any statistical differences between both groups regarding the type of infection or the causative bacteria in the different types of infection. A trend for higher incidence of pneumonia was observed in the polymorphism group (17.9% *vs* 8.7%, *P* = 0.08), but this was not statistically significant.

The probability of developing a bacterial infection at one-year of follow-up was 78% in the polymorphism group and 69% in the wild-type group (*P* = 0.54) (Figure 1A). The likelihood of infections caused by gram-negative bacilli after one year was 51% for the polymorphism group and 44% for the wild-type group (*P* = 0.68) (Figure 1B), for infections caused by gram-positive cocci it was 49% *vs* 40% (*P* = 0.53) (Figure 1C), and for spontaneous bacterial peritonitis it was 29% *vs* 34%, respectively (*P* = 0.99) (Figure 1D). Multivariate analysis by Cox regression showed that age (HR 1.023, 95%CI: 1.010-1.035, *P* ⩽ 0.001), MELD score (HR 1.034, 95%CI: 1.010-1.059, *P* = 0.006), and previous hepatic encephalopathy

Table 3 Type of infections in patients from the polymorphism group and the wild-type group during follow-up

	Polymorphism group (*n* = 28)	Wild-type group (*n* = 230)	*P* value
Spontaneous bacterial peritonitis			
Patients (%)	10 (35.7)	81 (35.2)	1.00
Number of spontaneous bacterial peritonitis episodes	15	109	
Number per patient	0.54 ± 0.92	0.47 ± 0.79	0.70
Caused by gram-negative bacilli (%)	0 (0)	20 (8.7)	0.14
Caused by gram-positive cocci (%)	3 (10.7)	24 (10.4)	1.00
Culture negative (%)	12 (42.8)	57 (24.8)	0.06
Bacteremia			
Patients (%)	4 (14.3)	47 (20.4)	0.61
Number of bacteremia episodes	4	57	
Number per patient	0.14 ± 0.36	0.25 ± 0.55	0.41
Caused by gram-negative bacilli (%)	1 (3.6)	26 (11.3)	0.79
Caused by gram-positive cocci (%)	3 (10.7)	31 (13.5)	0.87
Urinary infections			
Patients (%)	13 (46.4)	111 (48.3)	1.00
Number of urinary infection episodes	30	209	
Number per patient	1.07 ± 2.08	0.91 ± 1.42	0.90
Caused by gram-negative bacilli (%)	10 (35.7)	80 (34.8)	0.92
Caused by gram-positive cocci (%)	7 (25)	49 (21.3)	0.74
Other infections			
Patients (%)	9 (32.1)	59 (25.9)	0.50
Pneumonia (%)	5 (17.9)	20 (8.7)	0.16
Bacteriascites (%)	2 (7.1)	15 (6.5)	1.00
Cellulitis (%)	2 (7.1)	13 (5.7)	0.67

Data are presented as mean ± SD or frequencies (%).

(HR 1.570, 95%CI: 1.136-2.169, *P* = 0.006) were associated with a higher risk of infection during follow-up. The presence of TLR4 polymorphisms was not associated with the risk of infection in the univariate analysis or in the multivariate analysis.

Other complications of cirrhosis and survival

The likelihood of suffering hepatic encephalopathy after one year was 43% for the polymorphism group, and 41% for the wild-type group (*P* = 0.97), while the probability of developing variceal hemorrhage after one year was 17% and 12%, respectively (*P* = 0.35). The likelihood of developing a new hepatocellular carcinoma at two-years of follow-up was 7.1% for the polymorphism group and 6.3% for the wild-type group (*P* = 0.87).

The mortality during follow-up was 46.4% (13/28) in the polymorphism group and 46.5% (107/230) in the wild-type group (*P* = 1.00). The causes of mortality were ACLF or liver insufficiency in 46.15% (6/13) in the polymorphism group and 51.4% (55/107) in the wild-type group (*P* = 0.77); infection in 15.4% (2/13) and 20.6 % (22/107) (*P* = 1.00), variceal bleeding in 7.7% (1/13) and 8.4% (9/107) (*P* = 1.00), and hepatocellular carcinoma in 7.7% (1/13) and 5.6 % (6/107) (*P* = 0.56). In the polymorphism group, another three patients (23%) died from other causes unrelated to the liver disease: two from coronary heart

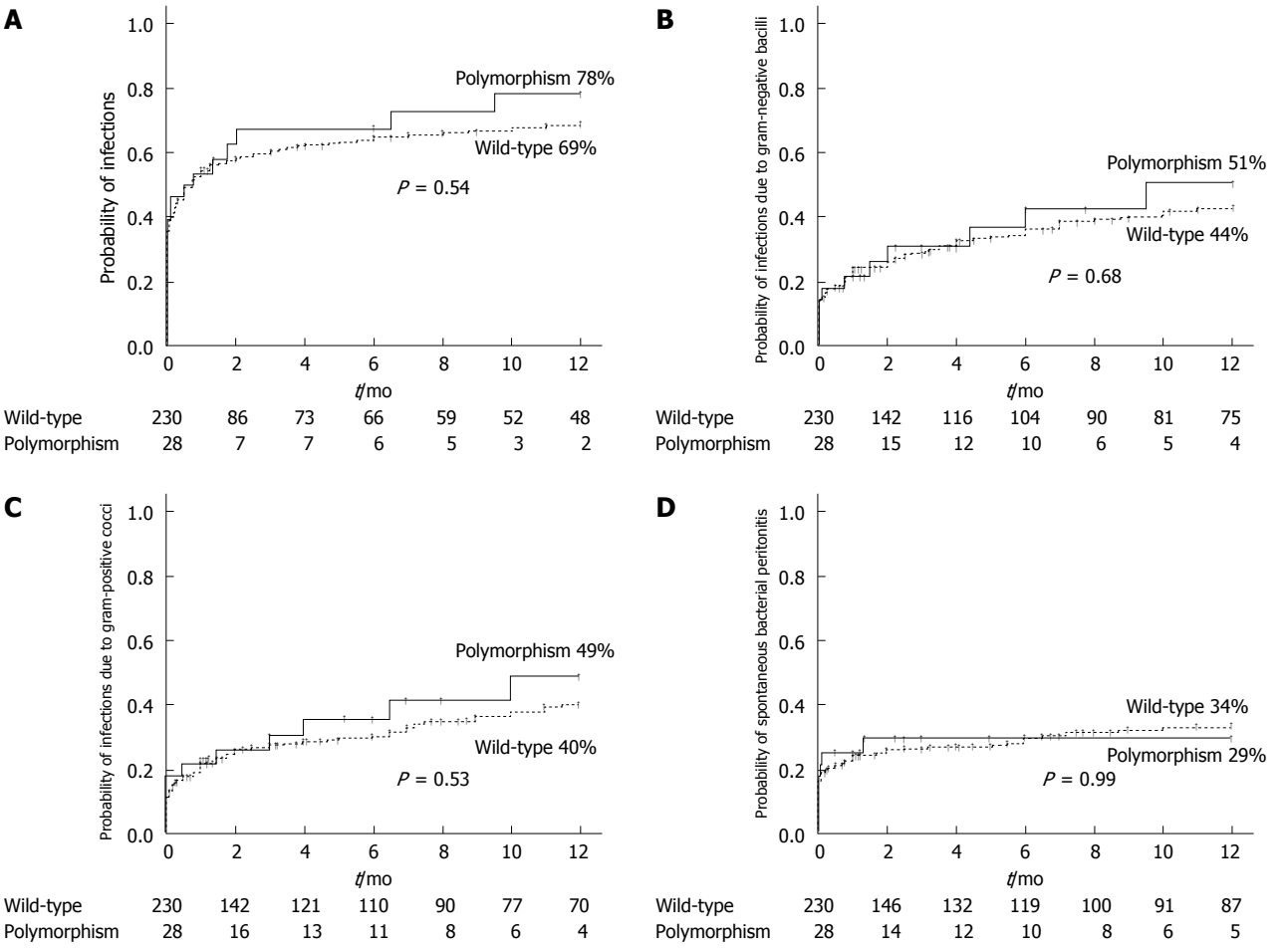

Figure 1 One year-probability of infections in the polymorphism group and in the wild-type group. A: All infections; B: Infections caused by gram-negative bacilli; C: Infections caused by gram-positive cocci; D: Spontaneous bacterial peritonitis.

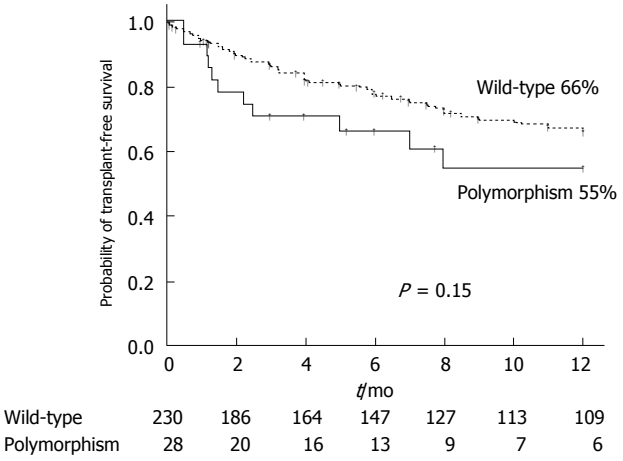

Figure 2 One year-probability of transplant-free survival in the polymorphism group and in the wild-type group.

disease and one from advanced oropharyngeal cancer. In the wild-type group, another fourteen patients (14%) (P = 0.41 with respect to polymorphism group) died from other causes unrelated to the liver disease: five from coronary heart disease and nine from advanced neoplasia (two lung neoplasm, one oropharyngeal cancer, one colon neoplasm, two non-identified advanced neoplasias, one breast cancer, one lymphoma, and one brain cancer). The likelihood of transplant-free survival at one year was 55% for the polymorphism group and 66% for the wild-type group (P = 0.15) (Figure 2), while the figures at two-years of follow up were 48% and 57%, respectively (P = 0.16).

We performed a multivariate analysis to analyse the contribution of baseline characteristics and the presence of TLR4 polymorphisms on the risk of death. In the univariate analysis we found age, HCV (hepatitis C virus) infection, active alcohol intake, Child-Pugh score, MELD score, previous decompensation, previous ascites, previous hepatic encephalopathy, hepatic encephalopathy at admission, hepatocellular carcinoma, treatment with beta-blockers or diuretics, serum creatinine, and serum urea were associated with a higher risk of death during follow-up (Table 4). Multivariate analysis by Cox regression confirmed that age, Child-Pugh score, active alcohol intake, previous hepatic encephalopathy, hepatocellular carcinoma and serum creatinine were associated with a higher risk of death during follow-up. The presence of TLR4 polymorphisms was not associated with mortality in

Table 4 Univariate and multivariate analysis of baseline characteristics regarding the risk of death during follow-up in all patients

Variables	Univariate		Multivariate	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age (yr)	1.042 (1.026-1.056)	< 0.001	1.035 (1.016-1.054)	< 0.001
HCV etiology	1.99 (1.385-2.865)	< 0.001		
Diabetes mellitus	1.583 (1.097-2.283)	0.010		
Active alcohol intake	0.353 (0.230-0.542)	< 0.001	0.568 (0.345-0.935)	0.020
Child-Pugh score	1.170 (1.041-1.317)	0.008	1.263 (1.113-1.432)	< 0.001
MELD score	1.036 (1.007-1.066)	0.010		
Previous decompensation	2.33 (1.473-3.690)	< 0.001		
Previous ascites	2.50 (1.620-3.860)	< 0.001		
Previous encepalophathy	2.794 (1.908-4.092)	< 0.001	2.216 (1.493-3.290)	< 0.001
Hepatocellular carcinoma	2.872 (1.097-4.817)	< 0.001	2.381 (1.411-4.018)	< 0.001
Previous β-blockers	1.727 (1.194-2.498)	0.004		
Previous diuretics	1.957 (1.334-2.869)	0.001		
Serum creatinine (μmol/L)	1.006 (1.004-1.008)	< 0.001	1.005 (1.002-1.007)	< 0.001
Serum urea (mmol/L)	1.026 (1.013-1.040)	< 0.001		
Platelet count ($\times 10^9$/L)	0.997 (0.995-1.00)	0.032		
TLR4 polymorphisms	1.372 (0.770-2.445)	0.280		

HCV: Hepatitis C virus; MELD: Model for end-stage liver disease.

the univariate analysis or in the multivariate analysis.

DISCUSSION

The main finding in the present prospective study was that we failed to show significant differences in the incidence and number of infections, complications of cirrhosis and prognosis between cirrhotic patients with ascites who had D299G and/or T399I TLR4 polymorphisms and patients who were not carriers of these polymorphisms.

Infections in patients with cirrhosis are common and a major cause of morbidity and mortality[4]. Attempts to prevent bacterial infections are therefore reasonable, and successful strategies have been developed in recent years, particularly those using antibiotic prophylaxis[3,4]. Antibiotic prophylaxis, however, mainly in long-term treatments, is not devoid of side effects, especially the development of bacterial resistances[1,3]. This is a serious world-wide problem that decreases the efficacy of prophylactic antibiotics and increases morbidity and mortality, not only in the general population but also in patients with cirrhosis[3]. To minimize bacterial resistance it is important to identify the risk factors for infection in order to develop comprehensive prevention strategies that restrict antibiotic prophylaxis to high-risk groups[3]. Many clinical factors have been associated with an increased risk of infection in cirrhosis, such as high degree of hepatic insufficiency, variceal bleeding, low levels of protein in ascites, prior spontaneous bacterial peritonitis, and hospital admission in the last 3 mo[15].

In addition to clinical factors, the relationship between genetic variants that can modify the immune response and the incidence of infections in cirrhotic patients has gained increasing interest in recent years[11,14,16-18]. Recent studies in patients with cirrhosis have shown that some genetic variants of NOD2 (nucleotide-binding oligomerization domain-containing 2), TLR2 and NDP52 (nuclear dot protein 52 kDa) are involved in the predisposition to spontaneous bacterial peritonitis, probably through alterations at the intestinal barrier and the immune response[16-18].

The presence of the genetic polymorphisms D299G and/or T399I of TLR4 is also thought to modify the immune response to LPS from gram-negative bacilli, and therefore increase susceptibility to infection in patients with cirrhosis[11-14]. In a preliminary retrospective study, it was found that cirrhotic patients with D299G TLR4 polymorphism had more previous infections than wild-type patients[14]. Therefore, the present study was designed to prospectively evaluate whether patients diagnosed with cirrhosis and ascites and D299G and/or T399I TLR4 polymorphisms had a higher risk of bacterial infections during follow-up than wild-type patients. However, we observed a non-significant trend to a higher predisposition to bacterial infections, infections caused either by gram-negative bacilli or by gram-positive cocci in the polymorphism group. Regarding the types of infection, there was a trend to a higher incidence of pneumonia in the polymorphism group, but the incidence of other infections more characteristic of cirrhosis such as SBP, urinary infection or bacteremia was similar in the two groups. The findings of the present study are contradictory with those from our previous preliminary retrospective study. However, we consider the results of this study more reliable because it was prospective and included a higher number of patients.

These negative results may be due to the low prevalence of the polymorphisms evaluated, 10.8% of all patients - a prevalence similar to that in the general population[9,10], an insufficient number of patients studied, and a short follow-up, particularly in the polymorphism group. It should be noted, however, that we prospectively evaluated a relatively high number of patients with decompensated cirrhosis over a 6-year period and with a mean overall follow-up of 26.6 ± 31.7 mo. Moreover, we corrected for the difference in the length of follow-up between the two groups by calculating Kaplan-Meier curves. We consider that, if we failed to show statistically significant differences in the

development of infections under these conditions, the effect, if any, of the studied polymorphisms has little clinical relevance, and their determination should not be included in the design of new preventive strategies.

Our results are in agreement with those of Lee *et al*[19] in patients who underwent a liver transplant. They observed no association connecting D299G and T399I TLR4 polymorphisms with a risk of developing infection or liver disease. Recently, Piñero *et al*[10] also failed to find a relationship between D299G TLR4 polymorphism and the development of infections in patients with cirrhosis and ascites. These findings are probably due to poor functional impact of these polymorphisms and/or the multifactorial and complex nature of the immune response[11].

Patients with polymorphisms of TLR4, the receptor to LPS of gram-negative bacilli, would be expected to present a greater predisposition to infection caused by these bacteria. It is therefore surprising that such patients also had a greater, although not statistically significant, predisposition to infections caused by gram-positive cocci and pneumonia, an infection usually caused by gram-positive cocci, than wild-type patients. Possible explanations could again be the complexity of the immune response, and the association between polymorphisms of TLR4 and other polymorphisms of other PRRs (pattern recognition receptors), such as TLR2 (involved in ligand recognition of gram-positive cocci) or NOD2, which were not evaluated in this study[11].

Inflammation is one of the factors that is increasingly recognized to favor the occurrence of hepatic encephalopathy[20,21]. In a previous study, we reported a greater occurrence of previous hepatic encephalopathy in cirrhotic patients carrying the D299G TLR4 polymorphism than in wild-type group patients[14]. As described by Nieto *et al*[12], cirrhotic patients with D299G and/or T399I TLR4 polymorphisms have less spontaneous production of IL-6 and IL-10 by peripheral monocytes, but a similar production after receptor stimulation compared to wild-type patients. This distinct cytokine production pattern may favor the development of hepatic encephalopathy in cirrhotic patients who are carriers of any of these polymorphisms[12]. In the present study, although previous episodes of hepatic encephalopathy were more frequent in patients with TLR4 polymorphisms than in patients in the wild-type group in agreement with previous data[12,14], this predisposition was not confirmed in the prospective follow-up.

A different inflammatory response could influence the evolution of cirrhosis and survival in patients with TLR4 polymorphisms[22]. In the present study a non-significant trend to higher mortality was observed during the follow-up period in patients with TLR4 polymorphisms than in patients from the wild-type group. Nevertheless, the presence of TLR4 polymorphisms neither in the univariate nor in the multivariate analysis was a predictive factor of mortality. Moreover, we did not observe differences in the cause of mortality between patients with TLR4 polymorphisms and wild-type patients. Most of the independent predictive factors of mortality in the multivariate analysis, such as age, Child-Pugh score, previous hepatic encephalopathy, hepatocellular carcinoma and serum creatinine, coincided with previous studies[23]. Regarding active alcoholism, this was an independent factor of survival probably due to the fact that more than half of patients actively drinking at inclusion in the study remained abstinent during follow-up. This percentage of abstainers was similar to that in previous studies showing that alcohol abstinence improves survival in patients with alcoholic cirrhosis[24]. In contrast, at the time the present study was performed, patients with decompensated cirrhosis due to HCV infection were not usually treated with antivirals.

Hepatocellular carcinoma is associated with inflammation. TLR4 stimulation can induce hepatocarcinogenesis[25] and increase invasiveness of hepatocellular carcinoma[26]. Therefore, a different inflammatory response as a consequence of the presence of TLR4 polymorphisms could influence the development of hepatocellular carcinoma in cirrhotic patients. In the present study, a similar likelihood of developing a new hepatocellular carcinoma was observed in both patients with TLR4 polymorphisms and in wild-type patients, though the follow-up period was too short to accurately evaluate this outcome.

We conclude that the presence of D299G and/or T399I TLR4 polymorphisms in cirrhotic patients with ascites is not a relevant risk factor for the development of bacterial infections and does not seem to significantly modify the evolution of the disease. It would be interesting to study the potential role of other genetic polymorphisms in the susceptibility to infections and the evolution of patients with cirrhosis.

ARTICLE HIGHLIGHTS

Research background

Toll-like receptor (TLR) 4 genetic polymorphisms, particularly D299G, have been previously associated with an increased predisposition to infection in several populations. However, few data regarding the role of these polymorphisms in patients with cirrhosis are available.

Research motivation

Few data regarding the role of TLR4 genetic polymorphisms in patients with cirrhosis are available

Research objectives

The aim of this study was to prospectively assess the relationship between the presence of D299G and/or T399I TLR4 polymorphisms and the incidence of bacterial infections in cirrhotic patients with ascites.

Research methods

The present study was designed to confirm the previous retrospective data and to further explore the relationship between the presence of TLR4 polymorphisms and bacterial infections in cirrhotic patients with ascites. The

authors included consecutive patients with cirrhosis and ascites hospitalized during a 6-year period. The presence of D299G and/or T399I TLR4 polymorphisms was determined by sequencing and related to the incidence of infections during follow-up.

Research results

The authors included 258 patients: 28 (10.8%) were carriers of D299G and/or T399I TLR4 polymorphisms (polymorphism group) and 230 patients were not (wild-type group). The probability of developing any bacterial infection at one-year follow-up was 78% in the polymorphism group and 69% in the wild-type group (P = 0.54). The one-year probability of presenting infections caused by gram-negative bacilli (51% *vs* 44%, P = 0.68), infections caused by gram-positive cocci (49% *vs* 40%, P = 0.53), and spontaneous bacterial peritonitis (29% *vs* 34%, respectively, P = 0.99) did not differ between the two groups. The one-year probability of transplant-free survival was 55% in the polymorphism group and 66% in the wild-type group (P = 0.15).

Research conclusions

The presence of the genetic polymorphisms D299G and/or T399I of TLR4 does not seem to play a relevant role in the predisposition of cirrhotic patients with ascites to develop bacterial infections.

Research perspectives

To study the potential role of other genetic polymorphisms in the susceptibility to infections and the evolution of patients with cirrhosis.

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ORIGINAL ARTICLE

Prospective Study

Effect of transplant center volume on post-transplant survival in patients listed for simultaneous liver and kidney transplantation

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Abstract

AIM

To examine the effect of center size on survival differences between simultaneous liver kidney transplantation (SLKT) and liver transplantation alone (LTA) in SLKT-listed patients.

METHODS

The United Network of Organ Sharing database was queried for patients ⩾ 18 years of age listed for SLKT between February 2002 and December 2015. Post-transplant survival was evaluated using stratified Cox regression with interaction between transplant type (LTA *vs* SLKT) and center volume.

RESULTS

During the study period, 393 of 4580 patients (9%) listed for SLKT underwent a LTA. Overall mortality was higher among LTA recipients (180/393, 46%) than SLKT recipients (1107/4187, 26%). The Cox model predicted a significant survival disadvantage for patients receiving LTA *vs* SLKT [hazard ratio, hazard ratio (HR) = 2.85; 95%CI: 2.21, 3.66; $P < 0.001$] in centers performing 30 SLKT over the study period. This disadvantage was modestly attenuated as center SLKT volume increased, with a 3% reduction (HR = 0.97; 95%CI: 0.95, 0.99; $P = 0.010$) for every 10 SLKs performed.

CONCLUSION

In conclusion, LTA is associated with increased mortality among patients listed for SLKT. This difference is modestly attenuated at more experienced centers and may explain inconsistencies between smaller-center and larger registry-wide studies comparing SLKT and LTA outcomes.

Key words: Kidney transplantation; Center volume; Mortality; Liver transplantation; United network for organ sharing

Core tip: Simultaneous liver kidney transplantation (SLKT) has doubled from 2002-2013. We studied the effect of transplant center volume on survival outcomes. There was a significant survival disadvantage for liver transplant alone (LTA) *vs* SLKT in centers performing 30 SLKT over the study period, although this disadvantage was slightly diminished with increasing center SLKT volume. Therefore, centers with higher transplant volume have a lesser mortality difference in LTA compared to SLKT than those centers with smaller volume.

Modi RM, Tumin D, Kruger AJ, Beal EW, Hayes Jr D, Hanje J, Michaels AJ, Washburn K, Conteh LF, Black SM, Mumtaz K. Effect of transplant center volume on post-transplant survival in patients listed for simultaneous liver and kidney transplantation. *World J Hepatol* 2018; 10(1): 134-141 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/134.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.134

INTRODUCTION

The debate over outcomes of simultaneous liver kidney transplantation (SLKT) *vs* liver transplantation alone (LTA) has intensified since the introduction of Model for End Stage Liver Disease (MELD) into the allocation system for donor livers. An unintentional byproduct of the implementation of the MELD score was an increase in the number of SLKT. From 2002 to 2013, the percentage of SLKT has increased from 4% to 8% of all liver transplants[1], contributing to a shortage of deceased donor kidney grafts for patients on the waitlist for deceased donor kidney transplantation. Since 2007, four guidelines have been proposed for SLKT listing by various societies, including one by the Organ Procurement and Transplant Network (OPTN) and a more recent consensus report by Davis *et al*[2], Eason *et al*[3] and Nadim *et al*[4]. The current recommendations for SLKT include one of the following: (1) Renal replacement therapy (eGFR of 30 mL/min or less) for a minimum of 4-8 wk; (2) proteinuria > 2 g/d; and (3) biopsy-proven interstitial fibrosis or glomerulosclerosis[1,4].

A recent survey studied variations in practice among liver transplant centers in the United States and found that SLKT listing was influenced by center-size rather than aforementioned guidelines[5]. Of the 88 transplant centers that were surveyed, centers that performed greater than 10 SLKT annually were more likely to use lenient dialysis duration (4 wk *vs* 6 or 8 wk). This variability in center practice may contribute to the significant inconsistencies among numerous studies comparing the outcomes of SLKT *vs* LTA, including patient and graft survival[6-9]. A 2015 study using the United Network of Organ Sharing (UNOS) database showed LTA outcomes were inferior to SLKT in all patients listed for SLKT[10], while a 2016 re-analysis of UNOS data found the difference in survival was not statistically significant[11]. Similar to large registry analyses, single-center studies have reported mixed findings on the difference in mortality between SLKT and LTA. Many earlier studies showed no difference between outcomes comparing SLKT to LTA[12-14]; however, a recent single-center study found improved outcomes with SLKT *vs* LTA[15].

Studies have also suggested that larger centers

attain more favorable transplant outcomes, even when involving higher-risk recipients or donors[16,17]. Therefore, the disadvantage of performing LTA in patients listed for SLKT (as reported by some prior studies) could be attenuated at the most experienced programs. However, the effect of transplant center volume on outcome differences between SLKT *vs* LTA has not been evaluated. This study examines the transplant center volume as a potential moderating factor in patients initially listed for SLKT. We hypothesized that the survival disadvantage associated with LTA (compared to SLKT) in patients listed for SLKT would be smaller in more experienced centers performing a greater number of SLKT.

MATERIALS AND METHODS

Data were obtained from the OPTN Standard Transplant Analysis and Research Database[18]. The institutional review board at Nationwide Children's Hospital exempted the study from review (IRB16-01193). The UNOS/OPTN database was queried for all patients ⩾ 18 years of age who were listed for SLKT between February 2002 and December 2015 (post-MELD allocation era), and received either SLKT or LTA. Exclusion criteria were prior transplantation, donation from a non-heart beating donor, living donor liver transplant and receipt of a split liver transplant. The primary outcome was patient survival after LTA *vs* SLKT, among patients listed for SLKT.

Descriptive characteristics of patients meeting inclusion criteria were compared according to the type of transplant (LTA *vs* SLKT) using unpaired *t*-tests for continuous data and χ^2 tests for categorical data. Among patients with known survival time, survival was compared according to transplant type using Kaplan-Meier curves with a log-rank test. Supplemental descriptive statistics and Kaplan-Meier survival curves included stratification of the study sample by tertiles of center SLKT volume, described below. Cases with complete data on covariates were entered in a multivariable Cox proportional hazards model, where the baseline hazard was stratified across transplant centers. In this stratified Cox model, hazard ratios (HRs) represented differences in survival among patients belonging to the same stratum, meaning differences in survival between patients transplanted at the same center. Center volume was primarily defined as the total number of SLKT performed by each center over the study period (2/2002-12/2015). In supplemental analyses, we demonstrate the robustness of our results to using the total number of liver transplants over the study period, or the annual number of SLKT at a given center, as alternative measures of center volume.

In the Cox model, type of transplant (LTA *vs* SLKT) was interacted with continuous center volume to allow the HR of transplant type (*i.e.*, estimated difference in survival between LTA and SLKT) to vary according to center volume[19]. The main effect of total center volume was not estimated in the stratified Cox model, as patients transplanted at the same center shared the same value for overall center volume. For model presentation, volume was centered at 30 total SLKT over the study period, approximately corresponding to the median center in the analytic sample, and divided by 10 (*i.e.*, a value of 0 indicated 30 SLKT performed over the study period; a value of 1 indicated 40 SLKT performed, and so on). Therefore, the main effect (HR) of transplant type described the difference in survival between LTA and SLKT for a center performing 30 SLKT; while the interaction between transplant type and center volume described how this difference was reduced (if the interaction HR was < 1) in more experienced centers.

Covariates in the analysis included recipient age, gender, race, etiology of liver disease, diabetes, dialysis, body mass index (BMI), serum creatinine, serum bilirubin, serum albumin, international normalized ratio (INR), Model for End-stage Liver Disease (MELD) score, and estimated glomerular filtration rate (eGFR) according to Modification of Diet in Renal Disease (MDRD) equation. Hepatic encephalopathy on the wait list, year of transplantation, and liver allograft cold ischemia time were also included. Analyses were performed using Stata/IC 13.1 (College Station, TX: StataCorp LP), and $P < 0.05$ was considered statistically significant.

RESULTS

Study cohort

The analytic sample included 4580 patients listed for SLKT, of whom 393 (9%) received LTA and 4187 (91%) received SLKT. Among these patients, 4573 had known survival time and 4257 had complete data on covariates in the multivariable analysis. There were 121 transplant centers represented in this sample, with a median SLKT volume of 33 over the entire study period [range: 1-278; interquartile range (IQR): 15-62]. The median annual SLKT volume was 3 (range: 0-21; IQR: 2-6). The median center liver transplant volume was 561 over the entire study period (range: 4-2696; IQR: 214-986). Overall mortality occurred in 28% of cases (1287/4580). The Kaplan-Meier plot (Figure 1) and log-rank test ($P < 0.001$) demonstrate worse survival of LTA *vs* SLKT recipients among patients initially listed for SLKT. Actuarial 1, 3 and 5 year survival rates among the LTA and SLKT groups were 68% *vs* 87%, 59% *vs* 79%, and 53% *vs* 72%, respectively. Other characteristics are compared between the 2 types of transplant in Table 1.

Survival implication of transplant type

The main multivariable stratified Cox model is presented in Table 2. At a center performing 30 SLKT over the study period, the model estimates a significant survival disadvantage associated with receiving LTA *vs* SLKT (HR = 2.85; 95%CI: 2.21-3.66;

Table 1 Characteristics of recipients of liver transplant alone or simultaneous liver-kidney transplant

Variable[1]	Cases missing data	Received LTA (n = 393) Mean (SD) or n (%)	Received SLK (n = 4187) Mean (SD) or n (%)	P value[2]
Transplant center SLKT volume	0	107 (± 83)	91 (± 66)	< 0.001
Transplant center LTA volume[3]	0	1187 (628)	1111 (627)	0.024
Age (yr)	0	54.2 (± 9.7)	54.8 (± 9.6)	0.279
Male	0	234 (60%)	2778 (66%)	0.007
Race	0			0.079
White		270 (69%)	2648 (63%)	
Black		47 (12%)	639 (15%)	
Other		76 (19%)	900 (22%)	
Etiology of liver disease	0			0.004
Viral		114 (29%)	1182 (28%)	
Cryptogenic		34 (9%)	330 (8%)	
Autoimmune		31 (8%)	197 (5%)	
NASH		43 (11%)	454 (11%)	
Alcoholic		89 (23%)	982 (23%)	
HCC		28 (7%)	376 (9%)	
AHN		16 (4%)	85 (2%)	
Other		38 (10%)	581 (14%)	
Diabetes	65	123 (32%)	1665 (40%)	0.001
Dialysis	0	109 (28%)	1963 (47%)	< 0.001
BMI (kg/m^2)	5	29.0 (± 5.9)	28.3 (± 5.9)	0.044
Serum creatinine (mg/dL)	5	2.8 (± 2.1)	3.8 (± 2.6)	< 0.001
Bilirubin (mg/dL)	5	8.2 (± 11.7)	5.7 (± 9.2)	< 0.001
Albumin (mg/dL)	6	3.0 (± 0.8)	3.0 (± 0.7)	0.074
INR	5	1.9 (± 1.4)	1.6 (± 0.7)	< 0.001
MELD score	16	25.6 (± 10.5)	25.2 (± 8.7)	0.445
eGFR	5	37.5 (± 27.2)	26.8 (± 22.4)	< 0.001
Hepatic encephalopathy on wait list	31	308 (79%)	2882 (69%)	< 0.001
Liver allograft cold ischemia time	213	6.8 (± 2.6)	6.8 (± 3.5)	0.706
Yr of transplant	0	2009 (4)	2010 (4)	< 0.001

[1]Covariates assessed at wait listing, apart from center volume over study period, hepatic encephalopathy on the wait list, liver allograft cold ischemic time, and year of transplant; [2]P value by independent t-test for continuous variables and χ^2 test for categorical variables; [3]Includes all liver transplants, not limited to LTA among patients listed for SLK. Descriptive characteristics by recipients of liver transplant alone or simultaneous liver-kidney transplant among patients listed for liver and kidney transplant in 2002-2015 (n = 4580). SD: Standard deviation; SLK: Simultaneous liver-kidney transplant; LTA: Liver transplant alone; BMI: Body mass index; INR: International normalized ratio; MELD: Model for end-stage liver disease; eGFR: Estimated glomerular filtration rate.

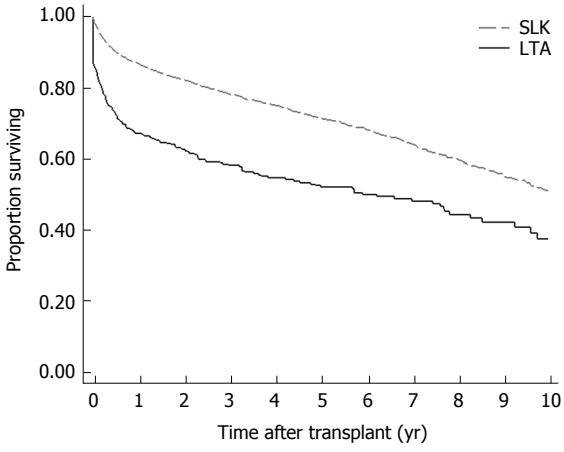

Figure 1 Post-transplant survival according to type of transplant. Kaplan-Meier post-transplant survival curves, according to type of transplant, among patients initially listed for simultaneous liver-kidney transplant. Actuarial 1, 3 and 5 year survival rates among the LTA and SLKT groups were 68% *vs* 87%, 59% *vs* 79%, and 53% *vs* 72%, respectively. LTA: Liver transplantation alone; SLKT: Simultaneous liver kidney transplantation.

P < 0.001). However, a statistically significant modification of this difference was observed as total center SLKT volume increased (interaction HR = 0.97; 95%CI: 0.95-0.99; P = 0.010), meaning that the survival disadvantage of LTA *vs* SLKT was attenuated by about 3% for each additional 10 SLKTs performed by a given center over the study period. Based on this model, estimated differences in survival (HR) between LTA and SLKT are plotted across center SLKT volume in Figure 2. For example, at a center performing a total of 15 SLKT over the study period (approximately the 25th percentile of centers), the HR of LTA compared to SLKT was 2.98 (95%CI: 2.26-3.92; P < 0.001); while at a center performing a total of 60 SLKT over the study period (approximately the 75th percentile of centers), this HR was reduced to 2.61 (95%CI: 2.11-3.23; P < 0.001).

Our findings were consistent when using total liver transplant center volume as a measure of center expertise; with a survival disadvantage for LTA *vs* SLKT at centers performing approximately the median volume (500) of liver transplants over the study period (HR = 2.89; 95%CI: 2.18-3.83; P < 0.001). This disadvantage was diminished at centers that performed more liver transplants over the study

Table 2 Hazard model of survival after liver transplant alone or simultaneous liver-kidney transplant in patients listed for liver and kidney transplant

Variable[1]	HR	95%CI	*P* value
Transplant received			
SLK	ref.		
LTA	2.85	(2.21, 3.66)	< 0.001
Transplant center SLK volume[2]			
Interaction with receiving LTA *vs* SLK	0.97	(0.95, 0.99)	0.010
Age (yr)	1.01	(1.01, 1.02)	< 0.001
Male	1.08	(0.94, 1.24)	0.285
Race			
White	ref.		
Black	1.17	(0.98, 1.39)	0.089
Other	0.79	(0.66, 0.94)	0.007
Etiology of liver disease			
Viral	ref.		
Cryptogenic	0.77	(0.61, 0.98)	0.033
Autoimmune	0.57	(0.41, 0.79)	0.001
NASH	0.79	(0.63, 1.01)	0.060
Alcoholic	0.65	(0.54, 0.77)	< 0.001
HCC	1.04	(0.83, 1.30)	0.721
AHN	1.10	(0.75, 1.63)	0.621
Other	0.77	(0.62, 0.97)	0.024
Diabetes	1.23	(1.08, 1.40)	0.002
Dialysis	1.41	(1.19, 1.67)	< 0.001
BMI (kg/m^2)	0.98	(0.97, 0.99)	0.003
Serum creatinine (mg/dL)	0.97	(0.93, 1.01)	0.092
Bilirubin (mg/dL)	1.00	(0.98, 1.01)	0.394
Albumin (mg/dL)	0.88	(0.81, 0.96)	0.004
INR	0.92	(0.81, 1.05)	0.224
MELD score	1.00	(0.99, 1.02)	0.661
eGFR	1.00	(1.00, 1.01)	0.622
Hepatic encephalopathy on wait list	1.10	(0.94, 1.28)	0.221
Liver allograft cold ischemia time	1.00	(0.98, 1.02)	0.811
Year of transplant	0.98	(0.96, 1.00)	0.107

[1]Covariates assessed at wait listing, apart from center volume over study period, hepatic encephalopathy on the wait list, liver allograft cold ischemic time, and year of transplant; [2]Total number of SLK performed over study period (2/2002-12/2015), centered at 30 procedures, and divided by 10. Multivariable Cox proportional hazards model, with the baseline hazard stratified on the transplant center, of survival after liver transplant alone or simultaneous liver-kidney transplant among patients listed for liver and kidney transplant in 2002-2015 (n = 4257). HR: Hazard ratio; CI: Confidence interval; SLK: Simultaneous liver-kidney transplant; LTA: Liver transplant alone; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; AHN: Acute hepatic necrosis; BMI: Body mass index; INR: International normalized ratio; MELD: Model for end-stage liver disease; eGFR: Estimated glomerular filtration rate.

period (interaction HR = 0.97; 95%CI: 0.94-1.00; P = 0.027). Despite this statistically significant interaction, a survival disadvantage of LTA *vs* SLKT was predicted for centers of all but the highest total liver transplant volumes (Supplemental Figure 1). Finally, the findings were robust when using a measure of annual, rather than total, SLKT volume (Supplemental Table 1; Supplemental Figure 2). Of note, the main effect of annual center volume in the stratified Cox model was not statistically significant (Supplemental Table 1: HR = 1.00; 95%CI: 0.98-1.02; P = 0.940). Therefore, year-to-year fluctuations in SLKT volume within a single center were not associated with survival outcomes of patients originally listed for SLKT.

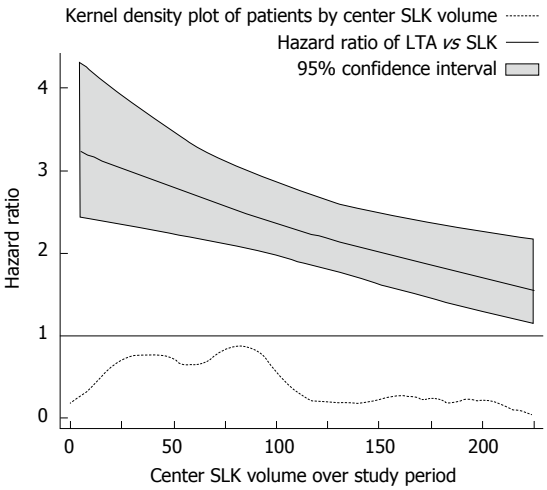

Figure 2 Post-transplant survival according to center volume of simultaneous liver-kidney transplants. Estimated hazard ratios for post-transplant survival, comparing liver transplant alone to simultaneous liver-kidney transplant among patients initially listed for simultaneous liver-kidney transplant, according to center volume of simultaneous liver-kidney transplants. LTA: Liver transplantation alone; SLKT: Simultaneous liver kidney transplantation.

Survival implication of center volume

Supplemental descriptive statistics according to center SLKT volume tertile are presented in Supplemental Table 2. A log-rank test found no difference in survival among patients in the study cohort according to tertile of center SLKT volume over the study period (P = 0.28; Supplemental Figure 3). However, there was marginally less mortality among patients who underwent LTA at larger centers, as illustrated in Supplemental Figure 4 (P = 0.05). The smaller survival difference between SLKT *vs* LTA in larger centers may be partially explained by a survival advantage of total center volume for SLKT-listed patients who received LTA.

DISCUSSION

Using a large national registry we found that center volume influenced the disparity in outcomes between LTA and SLKT, among patients initially listed for SLKT. More experienced centers achieved a smaller difference in mortality between the two types of transplant. With limited data investigating how center volume influences outcomes of multi-visceral organ transplantation, our findings suggest a survival disadvantage for LTA *vs* SLKT recipients at low volume centers, which is partially attenuated at higher volume centers. This influence of center volume on the effect of undergoing LTA after being listed for SLKT may also provide some insight into inconsistencies reported in literature on patients listed for SLKT.

While our study showed center volume influenced survival differences between SLKT and LTA, it is important to compare these findings to existing literature investigating this difference. A recent single-center study found improved overall 1- and 5- year

survival rates among SLKT recipients compared to LTA recipients (92.3% and 81.6% *vs* 73.3% and 64.3% respectively)[15]. On the other hand, a previous single-center study at a larger center found no 1-year survival advantage in LTA *vs* SLKT recipients[13]. Difference in the size of these centers (according to Scientific Registry of Transplant Recipients data from January 2013-June 2015) are consistent with our findings that the survival disadvantage of LTA among patients listed for SLKT is attenuated at larger centers.

Large database studies have also reached incongruous conclusions. Hmoud *et al*[10] recently used the UNOS database to show that LTA outcomes were inferior to SLKT in SLKT-listed patients. However, when comparing SLKT recipients to a propensity-matched subgroup of all liver transplant recipients, Sharma *et al*[11] demonstrated that differences in survival were not clinically significant. By using Cox regression stratified on the transplant center, we attempted to analyze comparable LTA and SLKT recipients (*i.e.*, clusters of recipients transplanted at the same center), while preserving the constraint that all LTA patients must have been listed for SLKT. While our results show smaller differences in survival between LTA and SLKT at more experienced centers, there was no expertise threshold above which LTA outcomes were equal to SLKT outcomes in patients initially listed for SLKT.

With increasing rates of SLKT being performed, it is important to consider center expertise as variable influencing transplant outcomes. Existing literature has explored independent influences of center volume on liver transplant outcomes. A 2011 study indicated that the increased center volume led to reduced allograft rejection and improved recipient survival[16]. More recently, 5130 liver transplants were stratified by number of transplants performed, and transplantation at a higher volume center was associated with lower mortality, length of stay, and costs compared to centers performing fewer transplants[17].

We demonstrated a tendency to perform fewer LTA in patients listed for SLKT at larger centers, which could be due to multiple reasons. Compared to smaller centers, larger transplant centers have distinct advantages including a dedicated and experienced organ procurement team and adequate organ transportation and storage facility. Additionally, the increased number of transplants performed may result in a technical advantage and increased experience to adequately address intra-operative and post-procedural complications. The combination of adequate ancillary staff, resources, and patient referrals enable increased SLKT listing and subsequent transplantation at large programs. It is possible that higher LTA mortality at smaller centers was related to patients who could not wait for multi-organ transplantation; and that high volume centers are able to better manage this patient population. These non-measurable factors may influence center specific outcomes, as programs are dependent on outcomes measures to continue to expand their transplant practice.

With the rise in SLKT, there has been an unintentional reduction in available kidney donors candidates afflicted with end-stage renal disease (ESRD). Due to this concomitant single organ donation, experts have suggested stricter criteria for the allocation of two allografts, especially considering limited access to kidneys compared to livers[6,13,20,21]. Recently, Cheng *et al*[22] outlined an important distinction of utility *vs* urgency based practice, where each SLKT resulted in a reduction of 1-year allograft lifespan to provide sicker patient populations access to dual organ transplantation. Our results indicate that patients listed for SLKT have worse outcomes when only receiving a liver allograft, indicating further discussion regarding standardizing national guidelines for SLKT listing is required. We recognize there is a real need for dual organ transplantation as the OPTN recently proposed a change in SLKT guidelines; however, improving the current allocation system between the ESRD and SLKT population is also needed[23-25]. Our study suggests when implementing national change, patients listed for SLKT should be evaluated with stricter criteria to ensure individuals listed for SLKT obtain both organs.

The current analysis is limited in several aspects, including the potential exclusion of confounding variables, missing data, and data entry errors. We were unable to assess important variables such as the duration of dialysis or renal impairment, biopsy proven renal interstitial fibrosis, or proteinuria. Although these factors influence the SLKT listing process, our focus was on post-transplant mortality differences between LTA and SLKT groups. Additionally, patients who received a LTA rather than SLKT may have had worsening clinical status, which could inherently bias estimating the difference in survival between the two procedures. Finally, while we used center volume as a measure of expertise, it is important to note it was not possible to assess peri-operative and post-operative management of patients as well as long-term medical management.

In summary, we demonstrated that centers with higher transplant volume achieve smaller difference in mortality with LTA as compared to SLKT among patients initially listed for SLKT. This finding may help reconcile controversy in the literature regarding center size and outcomes of LTA. These findings further demonstrate the need for standardization of SLKT listing guidelines.

ARTICLE HIGHLIGHTS

Research background

There has been an increase in the number of simultaneous liver kidney transplantation (SLKT) performed over the past decade. Recently, it has been noted that SLKT listing was influenced by center-size rather than by guidelines. Inconsistent outcomes of SLKT *vs* liver transplantation alone (LTA) have been reported.

Research motivation
The effect of transplant center volume on outcome differences between SLKT *vs* LTA has not been evaluated. As such, the authors examined transplant center volume as a potential moderating factor in patients initially listed for SLKT.

Research objectives
The authors hypothesized that the survival disadvantage associated with LTA (compared to SLKT) in patients listed for SLKT would be smaller in more experienced centers performing a greater number of SLKT.

Research methods
The United Network of Organ Sharing database was queried for patients ⩾ 18 years of age listed for SLKT between February 2002 and December 2015. Post-transplant survival was evaluated using stratified Cox regression with interaction between transplant type (LTA *vs* SLKT) and center volume.

Research results
Overall, 393 of 4580 patients (9%) listed for SLKT underwent LTA. Mortality was higher among LTA recipients (180/393, 46%) than SLKT recipients (1107/4187, 26%). The Cox model predicted a significant survival disadvantage for patients receiving LTA *vs* SLKT (HR: 2.85; 95%CI: 2.21-3.66) in centers performing 30 SLKT over the study period. This disadvantage was modestly attenuated as center SLKT volume increased, with a 3% reduction (HR: 0.97; 95%CI: 0.95-0.99) for every 10 SLKs performed.

Research conclusions
LTA is associated with increased mortality among patients listed for SLKT. This difference is modestly attenuated at more experienced centers and may explain inconsistencies between smaller-center and larger registry-wide studies comparing SLKT and LTA outcomes.

Research perspectives
The findings of this study may help to reconcile the current controversy regarding center size and outcomes of LTA. Future research should focus on the apparent need for standardization of SLKT listing guidelines.

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META-ANALYSIS

Vitamin D levels do not predict the stage of hepatic fibrosis in patients with non-alcoholic fatty liver disease: A PRISMA compliant systematic review and meta-analysis of pooled data

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Author contributions: Saberi B, Dadabhai AS and Mullin GE contributed to the data evaluation, manuscript and figure preparation, editing and final submission; Shinohara RT and Wang L provided biostatistics methodology oversight, as well as data extractions, meta-analysis with pooled data figure preparation and manuscript preparation and editing; Nanavati J provided informatics methodology support, conducted the literature search, and reviewed and edited the manuscript.

Conflict-of-interest statement: None.

Data sharing statement: The authors of the following studies were contacted to obtain their approval to share data in order to systematically evaluate their data for our meta-analysis: Drs. Patel, Luger, Barchetta, Anty, Bril, and Targher.

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Abstract

AIM

To investigate the relationship between 25-hydroxy-vitamin D [25(OH)D] levels and fibrosis stage in patients with non-alcoholic fatty liver disease (NAFLD).

METHODS

Two individual reviewers identified relevant studies using the PubMed, EMBASE, Cochrane, and Scopus databases. Inclusion criteria were as follows: (1)

Studies that evaluated adults with NAFLD and serum or plasma 25(OH)D levels; and (2) assessed fibrosis stage using liver biopsy. A rigorous analysis yielded six articles as having sufficient data to employ in evaluating the association of serum vitamin D levels in patients with NAFLD based on their liver fibrosis stage by histopathological analysis. The lead investigators of each of the six studies were contacted and the data were collected. To meta-analyze vitamin D levels in F0-F2 *vs* F3-F4 fibrosis, a random-effects meta-analysis fit using restricted maximum likelihood was applied. To examine trends across each stage of fibrosis with respect to vitamin D levels, a meta-regression was performed. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 937 subjects from six studies were included in the final analysis to evaluate the association of serum vitamin D levels in patients with NAFLD based on their liver fibrosis stage by histopathological analysis. The lead investigators of each of the six studies were contacted and the data were collected. First, the investigators performed a meta-analysis to compare serum vitamin D levels in patients with NAFLD with stage F0-F2 compared to F3-F4, which did not show significance [meta-estimate of the pooled mean difference = -0.86, P = 0.08 (-4.17, 2.46)]. A meta-regression evaluation of serum vitamin 25 (OH)D levels across the individual stages (F0-F4) of fibrosis did not show an association for the six included studies.

CONCLUSION

Low vitamin D status is not associated with higher stages of liver fibrosis in patients with NAFLD.

Key words: Vitamin D; 25-hydroxyvitamin D; Liver fibrosis; Meta-analysis; Nonalcoholic fatty liver disease; Non-alcoholic steatohepatitis

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a condition that can progress to cirrhosis, hepatic failure, and liver cancer. Vitamin D sufficiency is impaired in the advanced stages of liver disease and in NAFLD. However, our systematic review of the literature and meta-regression confirms that the serum 25-hydroxyvitamin D levels in patients with NAFLD are not associated with the severity of hepatic fibrosis.

Saberi B, Dadabhai AS, Nanavati J, Wang L, Shinohara RT, Mullin GE. Vitamin D levels do not predict the stage of hepatic fibrosis in patients with non-alcoholic fatty liver disease: A PRISMA compliant systematic review and meta-analysis of pooled data. *World J Hepatol* 2018; 10(1): 142-154 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/142.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.142

INTRODUCTION

Non-Alcoholic fatty liver disease (NAFLD) represents a growing epidemic that requires better understanding in order to develop new therapeutic targets[1]. The definition of NAFLD is based upon the presence of ⩾ 5% hepatic steatosis without having etiologies, such as alcohol[2]. As one of the most prevalent causes of liver disease worldwide, the importance of NAFLD is gaining prominence in the medical literature and in the press. The prevalence of NAFLD is estimated to be 6% to 35% worldwide and 10% to 35% in the United States, increasing parallel to diabetes and obesity[3-5]. Based on these studies, it is estimated that between 75 million to 100 million individuals are at-risk of having NAFLD in the United States. NAFLD is a condition which has a range of manifestations from steatosis, nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma[1]. The number of adults with NASH on the liver transplant list has grown by a factor of three, and NASH is the 2nd most common etiology of liver disease in patients who are awaiting liver transplantation[6].

Vitamin D is well known for its physiologic role in mineral and skeletal homeostasis[7]. Ultraviolet light from sun exposure transforms 7-dehydrocholesterol, into pre-vitamin D3, which is converted into vitamin D3 (cholecalciferol). Vitamin D controls the expression of genes linked to various processes including immunomodulation which may be highly pertinent to chronic liver disease. Vitamin D has numerous properties that modulate injury, tissue remodeling, fibrogenesis, and chronic inflammation, which may prevent the progression of chronic liver disease[8,9]. Vitamin D has immunomodulatory actions that include the attenuation of interleukin-2, interferon-γ, and interleukin-12, which drive pro-inflammatory T-helper-1 (Th1) response[10] (Figure 1). Vitamin D upregulates anti-inflammatory T-helper-2 (Th2) cytokines and induces regulatory T cells (Tregs)[11].

Vitamin D has a number of potential roles for favorably altering the course of NAFLD (Figure 2), while it also improves the secretion and tissue sensitization to insulin[12]. The adipocyte is felt to be an important contributor to the pathogenesis of NAFLD. Vitamin D deficiency promotes adipocyte proinflammatory cytokines (adipokines), which are elevated in individuals with obesity, metabolic syndrome, and NAFLD, and are felt to contribute to disease[13,14]. Furthermore, vitamin D has been shown to upregulate adiponectin-an adipocyte-derived hormone. Adiponectin improves insulin sensitivity and prevents atherogenesis, which is decreased in those with obesity, metabolic syndrome, and NAFLD[15]. Vitamin D has been shown to inhibit hepatic inflammation and attenuates liver fibrosis in animal models[16]. Thus, the relationship of vitamin D deficiency to NAFLD pathogenesis merits careful analysis.

Numerous reports have revealed that patients

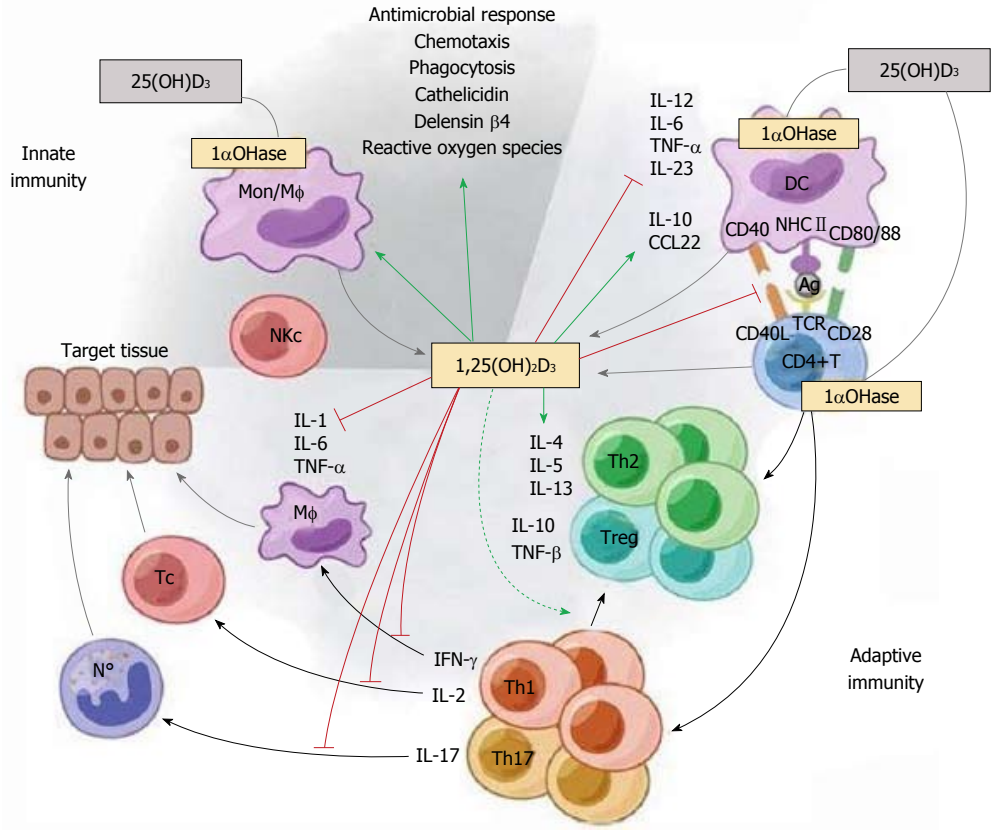

Figure 1 The immunomodulatory effects of 1,25(OH)$_2$D$_3$. 1,25(OH)$_2$D$_3$ targets different players of the innate and adaptive immune compartment. 1,25(OH)$_2$D$_3$ stimulates innate immune responses by enhancing the chemotactic and phagocytotic responses of macrophages, as well as the production of antimicrobial proteins such as cathelicidin. On the other hand, 1,25(OH)$_2$D$_3$ also modulates adaptive immunity. At the level of the APC (like the DC), 1,25(OH)$_2$D$_3$ inhibits the surface expression of the MHC-Ⅱ-complexed antigen and co-stimulatory molecules, in addition to the production of the cytokines IL-12 and IL-23, thereby indirectly shifting the polarization of T cells from a Th1 and Th17 phenotype towards a Th2 phenotype. In addition, 1,25(OH)$_2$D$_3$ directly affects T cell responses, by inhibiting the production of Th1 cytokines (IL-2 and IFN-γ) and Th17 cytokines (IL-17 and IL-21), and by stimulating Th2 cytokine production (IL-4). Moreover, 1,25(OH)$_2$D$_3$ favors Treg cell development *via* modulation of DCs and by directly targeting T cells. Finally, 1,25(OH)$_2$D$_3$ blocks plasma cell differentiation, IgG and IgM production, and B cell proliferation. Reproduced with the permission of the Nature Publishing Group[52].

with chronic liver disease from different etiologies had low vitamin D status[17-21]. In particular, liver diseases heralded by autoimmune or chronic inflammatory states appear to be worsened in the setting of vitamin D deficiency. In a pooled data meta-analysis, we recently showed that in nine of the 12 studies on mono-infected or co-infected patients with chronic hepatitis C, stages three and four fibrosis were associated with profound 25-hydroxyvitamin D deficiency and the associated odds ratio (OR) and the 95% confidence interval (CI) were 1.88 (1.27, 2.77)[22]. The total heterogeneity, I^2, was 66.94%, thus indicating that there was substantial heterogeneity between studies[22].

A recent meta-analysis supports the contention that individuals with NAFLD with and without non-alcoholic steatohepatitis (NASH) are more prone to have hypovitaminosis D[23]. Wang *et al*[23] extracted data from 29 studies and reported that subjects with NAFLD had decreased 25-hydroxyvitamin D and were 1.26 times more likely to be vitamin D deficient. Individuals with inflammatory disease (NASH) have also been reported to have decreased levels of 25(OH)D. In support of our prior findings for chronic hepatitis C, recent studies have suggested that vitamin D levels are further decreased in advanced stages of fibrosis[24-26]. However, limitations have been observed regarding the criterion used to diagnose NAFLD, clinical variation in disease severity among the study groups, and inconsistency in defining vitamin D deficient states[9].

A number of investigations have attempted to link vitamin D status to histological disease activity and fibrosis of NAFLD[26-32]. Jaruvongvanich *et al*[33] systematically reviewed the literature to determine if vitamin D status was associated with NAFLD disease activity or fibrosis score and extracted data from six included studies involving 974 NAFLD subjects[33]. These investigators did not find a difference in the serum 25-hydroxyvitamin D levels among NAFLD patients with high histologic activity *vs* low, nor high fibrosis score *vs* low. In light of these findings, the investigators concluded that vitamin D status was not related to the histologic activity of NAFLD. In their study, Jaruvongvanich *et al*[33] did not assess the association of vitamin D levels across each precise stage of liver fibrosis based on liver biopsy in patients NAFLD.

In the current study, we determined the relationship between serum vitamin D status relative to the precise degree of hepatic fibrosis. Based on the histopath-

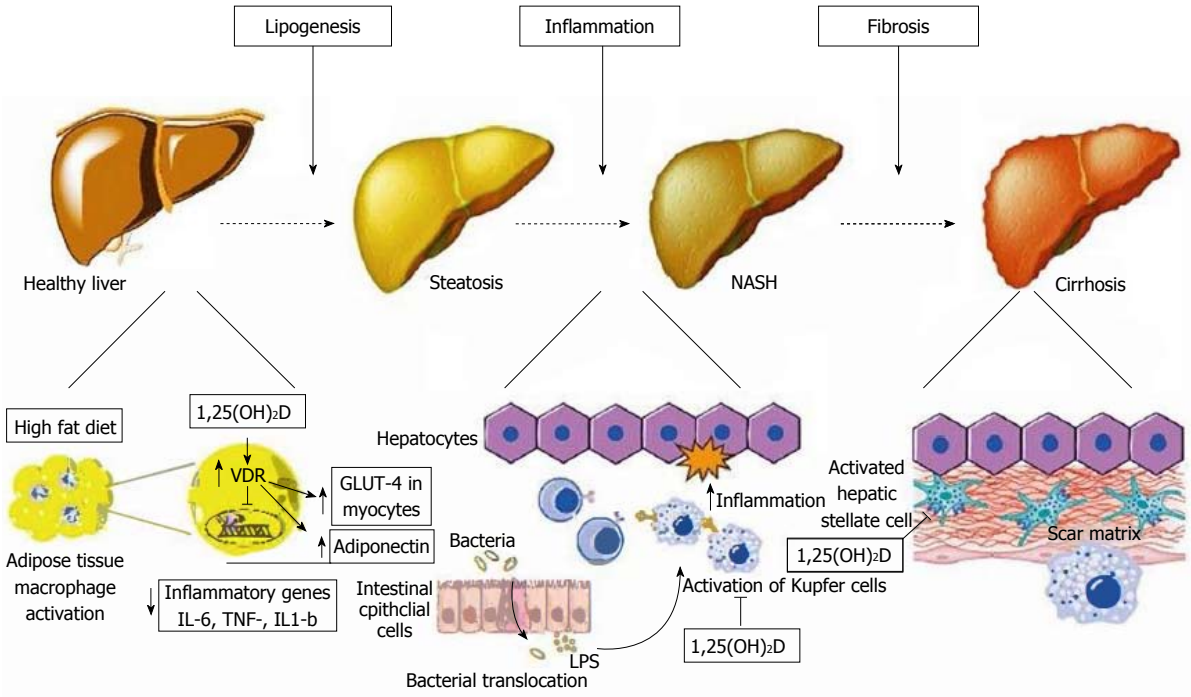

Figure 2 Schematic representation of metabolic, anti-inflammatory, and anti-fibrotic effects of vitamin D on hepatocytes and non-parenchymal hepatic cells (hepatic stellate cells, Kupffer cells) in non-alcoholic fatty liver disease. Left: At the initial stage of lipogenesis, 1,25(OH)D acts on adipocytes and inhibits NF-κB transcription, known as the pro-inflammatory "master switch", and thus inhibits the expression of the inflammatory cytokines IL-6, TNF-α, and IL-1β. It also increases adiponectin secretion from adipocytes and enhances GLUT-4 receptor expression in myocytes, both of which improve insulin resistance; Middle: Increased gut permeability allows the translocation of bacterial pathogens which can activate Toll-like receptors (TLR) on Kupffer cells. 1,25(OH)D downregulates the expression of TLR-2, TLR-4, and TLR-9 in these cells, thus ameliorating inflammation; Right: 1,25(OH)D acts on hepatic stellate cells by binding to VDR, which reduces the proliferation of these cells that play a major role in inducing fibrosis. VDR: Vitamin D receptor; TLR: Toll-like receptor; LPS: Lipopolysaccharide. Reproduced in compliance with Creative Commons in PubMed Central Open Access to Reproduced with the permission of the Baishideng Publishing Group Inc[9].

ological staging in patients with NAFLD, we performed a systematic review and meta-analysis[34].

MATERIALS AND METHODS

Literature search

The present meta-analysis was performed according to the Cochrane Collaboration and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statements[35]. Applicable studies were identified by a library literature search using the PubMed, Embase, Cochrane, and Web of Science databases by utilizing the PRISMA checklist from its inception to March 2017, then updated in September 2017. "Present a full electronic search strategy for at least one database, including any limits used, so that it could be repeated" and the Cochrane review reporting guidelines (6.6.2.2). The mesh terms for PubMed were as follows: "Non-alcoholic fatty liver disease", "Vitamin D", and "Liver cirrhosis". Also, the studies cited by the selected articles were searched for further pertinent studies. The details of the search strategy were prepared by the informationist (JN) in collaboration with the authors (Saberi B, Dadabhai AS and Mullin GE), as shown in Table 1.

Study selection

In the first phase, two separate reviewers carefully reviewed the abstract of the studies. When there was an agreement between two reviewers that a study fit the inclusion and exclusion criteria (Table 2), the article was then selected for further assessment. When there was a disagreement between the two reviewers, a third reviewer determined whether the study met the criteria for inclusion. Once the abstracts were included, the text was then carefully reviewed and data extraction was completed by at least two of the reviewers. The flowchart of the included studies is shown in Figure 3.

Data extraction

A total of six studies were included for extraction, which was performed by two independent reviewers (GM, BS) based on data quality, sufficiency, and relevance. Disagreements were resolved by a third reviewer (TS) to reach a consensus. The following data were extracted: last name of the first author, demographic information of patients, publication year, population, sample size, BMI, ALT, study design, method of vitamin D measurement, vitamin D levels in control and subjects, stage of fibrosis based on liver biopsy, and association of serum vitamin D level and fibrosis stage (Table 3). We then contacted the investigators of each study and collected the details of their data regarding serum vitamin D level measurements based on the stages of liver fibrosis (Tables 4 and 5). The

Table 1 Search results of vitamin D and non-alcoholic fatty liver disease

Database/search	Search terms	Search results
EMBASE		
1	("liver cirrhosis"/exp OR cirrhosis: ti, ab OR cirrhoses: ti, ab OR fibrosis: ti, ab OR fibroses: ti, ab)	
2	("vitamin D"/exp OR "25 hydroxyvitamin d"/exp OR "vitamin d": ti, ab OR "ergocalciferols": ti, ab OR "ergocalciferol": ti, ab OR "25 hydroxy vitamin d": ti,ab OR "25 hydroxyvitamin d": ti, ab OR "25 hydroxy d": ti, ab OR "25(OH)D": ti, ab OR "25-hydroxyvitamin d 2": ti, ab)	
3	("nonalcoholic fatty liver"/exp OR "Non-alcoholic Fatty Liver": ti, ab OR "nonalcoholic fatty liver": ti, ab OR "Non-alcoholic Fatty Livers": ti, ab OR "nonalcoholic fatty livers": ti, ab OR "NAFLD": ti, ab OR "NASH": ti, ab OR "nonalcoholic steatohepatitis": ti, ab OR "nonalcoholic steatohepatitides": ti,ab OR "fatty liver"/de OR "fatty liver": ti, ab OR "Steatohepatitis": ti, ab OR "Steatosis of Liver": ti, ab OR "Liver Steatosis": ti, ab OR "Liver Steatoses": ti, ab OR "hepatic steatosis": ti, ab OR "hepatosteatosis": ti, ab)	
4	1 and 2 and 3	199
Web of science		
1	("Non-alcoholic Fatty Liver" OR "nonalcoholic fatty liver" OR "Non-alcoholic Fatty Livers" OR "nonalcoholic fatty livers" OR "NAFLD" OR "NASH" OR "nonalcoholic steatohepatitis" OR "fatty liver" OR Steatohepatitis OR "Steatosis of Liver" OR "Liver Steatosis" OR "Liver Steatoses" OR "hepatic steatosis" OR "hepatosteatosis")	
2	("liver cirrhosis" OR cirrhosis OR cirrhoses OR fibroses OR fibrosis)	
3	("vitamin d" OR "ergocalciferols" OR "ergocalciferol" OR "25 hydroxy vitamin d" OR "25 hydroxyvitamin d" OR "25 hydroxy d" OR "25(OH)D" OR "25-hydroxyvitamin d 2")	
4	1, 2 and 3	69
Cochrane		
1	MeSH descriptor: [Non-alcoholic Fatty Liver Disease] explode all trees	181
2	MeSH descriptor: [Liver Cirrhosis] explode all trees	2462
3	MeSH descriptor: [Vitamin D] explode all trees	2907
4	"Non-alcoholic Fatty Liver" or "nonalcoholic fatty liver" or "Non-alcoholic Fatty Livers" or "nonalcoholic fatty livers" or "NAFLD" or "NASH" or "nonalcoholic steatohepatitis" or "fatty liver" or Steatohepatitis or "Steatosis of Liver" or "Liver Steatosis" or "Liver Steatoses" or "hepatic steatosis" or"hepatosteatosis": ti, ab, kw	1470
5	"liver cirrhosis" or cirrhosis or cirrhosis or fibrosis or fibroses: ti,ab,kw	13273
6	"vitamin d" or "ergocalciferols" or "ergocalciferol" or "25 hydroxy vitamin d" or "25 hydroxyvitamin d" or "25 hydroxy d" or "25(OH)D" or "25-hydroxyvitamin d 2": ti,ab,kw	6061
7	1 or 4	1470
8	2 or 5	13273
9	3 or 6	6722
10	7 and 8 and 9	13
PubMed		
1	(("Non-alcoholic Fatty Liver Disease"[Mesh] OR "Non-alcoholic Fatty Liver"[tw] OR "nonalcoholic fatty liver"[tw] OR "Non-alcoholic Fatty Livers"[tw] OR "nonalcoholic fatty livers"[tw] OR "NAFLD"[tw] OR "NASH"[tw] OR "nonalcoholic steatohepatitis"[tw] AND "Fatty Liver"[Mesh: noexp] OR "fatty liver"[tw] OR Steatohepatitis[tw] OR "Steatosis of Liver"[tw] OR "Liver Steatosis"[tw] OR "Liver Steatoses"[tw] OR "hepatic steatosis"[tw] OR "hepatosteatosis"[tw])	
2	("vitamin d"[mh] OR "vitamin d"[tw] OR "ergocalciferols"[tw] OR "ergocalciferol"[tw] OR "25 hydroxy vitamin d"[tw] OR "25 hydroxyvitamin d"[tw] OR "25 hydroxy d"[tw] OR "25(OH)D"[tw] OR "25-hydroxyvitamin d 2"[tw])	
3	("liver cirrhosis"[mh] OR cirrhosis[tw] OR cirrhoses[tw] OR fibrosis[tw] OR fibroses[tw])	
4	1 and 2 and 3	56
		Search results
	Total	337
	Duplicated	101
	Final total	236

methodologies utilized by the authors to assess the severity of fibrosis score are shown in Tables 6 and 7.

Statistical analysis

Statistical computations were conducted in R (Version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria, 2016)[36] and RevMan 5.3 (The Cochrane Collaboration, 2014). In several studies, the mean and variance of vitamin D levels in the combined F0-F2 and F3-F4 fibrosis stage groups were unavailable in combined form despite multiple attempts from the authors; hence, the vitamin D levels were estimated using Monte Carlo simulations assuming vitamin D levels were normally distributed with the reported parameters for each fibrosis stage. For the meta-analysis of the comparisons between low fibrosis (F0-F2) *vs* high fibrosis (F3-F4), a random-effects meta-analysis fit using a restricted maximum likelihood (REML) was then fit using the metafor package in R. To assess associations across each fibrosis level, a meta-regression fit *via* REML was conducted using the metan and metareg functions in RevMan 5.3. $P < 0.05$ was considered statistically significant[36]. The risk of publication bias across the included studies for all outcome measures was assessed by the construction of funnel plots.

RESULTS

Study selection

The search strategy utilized medical subject headings (MeSH) terms used to identify articles that evaluated

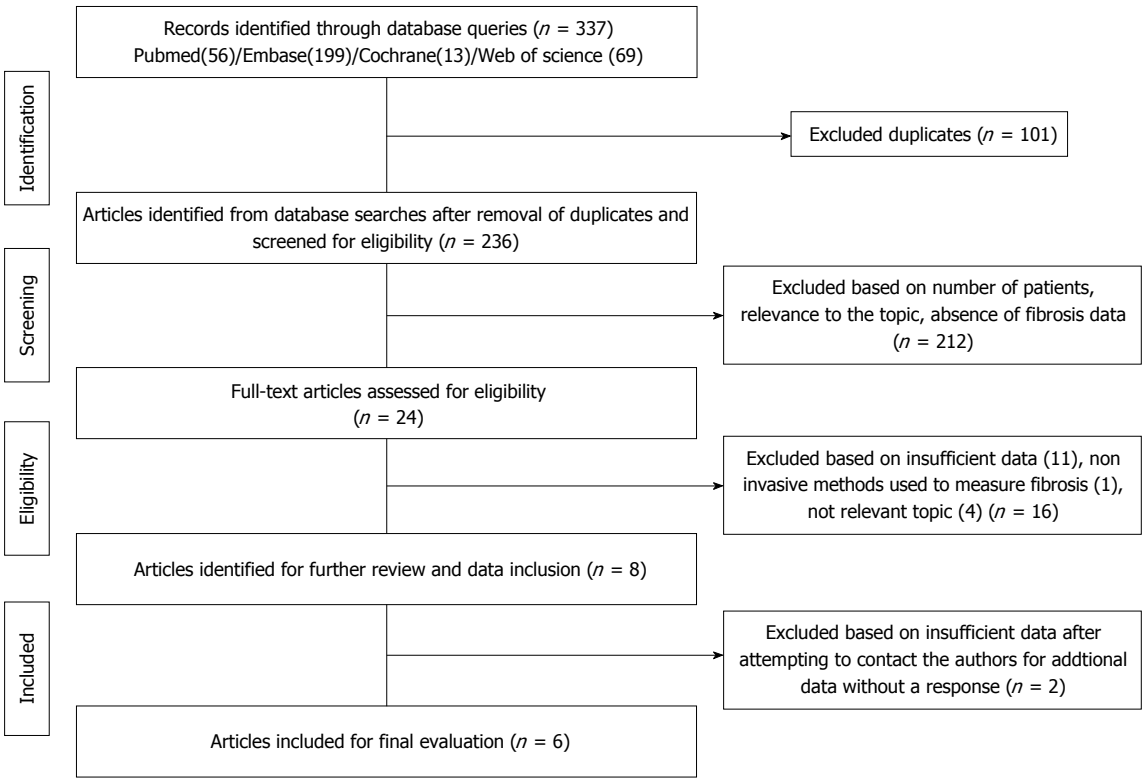

Figure 3 Flowchart illustrating the process for the selection of the included articles. Three hundred and thirty-seven articles were identified using PubMed (*n* = 56)/EMBASE (*n* = 199)/Cochrane (*n* = 13)/Web of Science (*n* = 69) search engines. A detailed evaluation of the articles by at least two independent reviewers (total of three) assessed the sufficiency of data, the method of fibrosis qualification, and relevance to the topic in order to narrow the studies to six.

Table 2 Inclusion and exclusion criteria of studies on vitamin D in non-alcoholic fatty liver disease

Inclusion criteria
Patients ⩾ 18 yr
Studies that evaluated vitamin D in NAFLD
Studies that evaluated the liver fibrosis stage, only based on liver biopsy
Studies that reported serum or plasma 25(OH)D levels
Exclusion criteria
Age < 18 yr
Liver diseases other than NAFLD
Studies that used non-invasive methods to evaluate liver fibrosis
Studies with inadequate data

25-OH(D): 25-hydroxyvitamin D; NAFLD: Nonalcoholic fatty liver disease.

serum vitamin D levels in patients with NAFLD based on the severity of liver fibrosis stage. Three hundred and thirty-seven articles were identified by PubMed (*n* = 56), EMBASE (*n* = 199), Cochrane (*n* = 13), and Web of Science (*n* = 69) search engines and one hundred and one duplicates were removed. Two independent reviewers provided a detailed evaluation of the articles assessed. This evaluation included data adequacy, criterion used to measure fibrosis, and overall pertinence to streamline for qualitative synthesis (Figure 3). All studies were cross-sectional. Table 2 summarizes the baseline characteristics, including the year of study, country, gender, population, BMI, Mean ALT (IU/L), vitamin D levels in NAFLD, and control patients. We then contacted investigators for the included studies and collected detailed data on vitamin D levels (Median or interquartile ranges; IQRs) based on the stage of fibrosis 4 (F0-F4). Out of the eight studies eligible for quantitative synthesis, we were able to gather and assemble vitamin D levels for each fibrosis stage category in a total of six studies (Tables 3-5). Based on this information on serum vitamin D levels, we then performed a quantitative synthesis across the six studies by performing a meta-analysis comparing F0-F2 (low fibrosis) *vs* F3-F4 (high fibrosis) groups and a meta-regression for the five categories of liver fibrosis (F0-F4).

Definition of vitamin D levels

Vitamin D status is based upon serum 25(OH)D values but this remains controversial. The most stable and plentiful metabolite of vitamin D in human serum, 25(OH)D, has a half-life of about 3 wk, making it the most suitable indicator of vitamin D status[37]. The lower limit of normal was defined as being less than 30 ng/mL, thus serum 25(OH)D lower than 30 ng/mL defined insufficiency. Deficient serum vitamin D was defined by some investigators as 25(OH)D < 20 ng/mL while others used < 10 ng/mL as the cutoff. During the data extraction, we discovered that two of the studies did not use ng/mL to express serum 25(OH)D. Instead, the unit used was nmol/L to express serum

Table 3 Characteristics of patients' studies for vitamin D status in non-alcoholic fatty liver disease

Citation	Patel *et al*[32]	Luger *et al*[30]	Barchetta *et al*[28]	Anty *et al*[27]	Dasarthy *et al*[24]	Bril *et al*[29]	Nelson *et al*[31]	Targher *et al*[26]
Year	2016	2016	2012	2016	2014	2015	2016	2007
Country	United States	Austria	Italy	France	United States	United States	United States	Italy
Subjects (M, F)	293 (195, 98)	50 (10, 40)	45 (22, 23)	398 (64, 334)	187 (51, 136)	239 (204, 35)	190 (89, 101)	120 (80, 40)
Population	Suspected NAFLD undergoing liver biopsy	Gastric bypass patients	Suspected NAFLD	Morbidly obese referred for bariatric surgery	Biopsy proven NAFLD, normal controls	Overweight patients	Biopsy proven NAFLD	Biopsy proven NAFLD
Mean BMI	36.1 ± 7.8	43.8 ± 4.3	30.5 ± 5.5	42.8 ± 5.0	35.7 ± 7.0	34.6 ± 0.4	35.6 ± 10.8	26.3 ± 2.0
Subjects	NAFLD	All	NASH	All	NAFLD	NASH	NAFLD	NAFLD
Mean ± SD ALT IU/L	66.5 ± 51.2	36.4 ± 20.8	87.5 ± 46.6	35.2 ± 24.5	45.9 ± 30.0	64.0 ± 4.0	77.0 ± 48.2	105 ± 42.0
Subjects	NAFLD	All	NASH	Morbidly Obese	NAFLD	NASH	NAFLD	NAFLD
Study design	Cross sectional	Cross sectional	Cross sectional	Cross sectional	Cross sectional	Cross sectional	Cross sectional	Cross sectional
Vitamin D analysis	CLIA	Not described	CLIA	CLIA	CLIA	CLIA	GC-MS	CLIA
Mean/SD 25(OH)D (ng/mL), (*n*) subjects	27.6 ± 11.8	15.6 ± 5.6	22.0 ± 12.4	19.2 ± 9.0	21.2 ± 10.4	21.8 ± 1.0	20.9 ± 4.0	20.4 ± 8.8
Mean/SD 25(OH)D (ng/mL) Non-NAFLD Controls	27.9 ± 12.8	NA	52.9 ± 11.02	21.5 ± 10/2	35.7 ± 6.0	24.5 ± 2.1	NA	30.0 ± 6.0
P value; NAFLD *vs* controls	0.878	NA	Not significant	0.13	< 0.01	0.18	NA	< 0.001

n: Number of subjects; 25-OH(D): 25-hydroxylvitamin D; SD: Standard deviation; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; CLIA: Chemiluminescence; GC-MS: Gas chromatography mass spectroscopy; M: Male; F: Female.

Table 4 Relationship of vitamin D to liver fibrosis in non-alcoholic fatty liver disease

Author/year/Ref	(*n*), 25-OH(D) Mean ± SD F0	(*n*), 25-OH(D) Mean ± SD F1	(*n*), 25-OH(D) Mean ± SD F2	(*n*), 25-OH(D) Mean ± SD F3	(*n*), 25-OH(D) Mean ± SD F4	*P* value
Patel *et al*[32], 2016	(39) 24.4 ± 10.4	(78) 26.5 ± 8.9	(55) 29.1 ± 12.5	(63) 30.7 ± 14.1	(9) 20.2 ± 20.2	0.028
Targher *et al*[26], 2007	(16) 20.8 ± 8.4	(10) 14.4 ± 9.2	(7) 10.0 ± 10.0	(6) 6.0 ± 10.8	0	0.01
Anty *et al*[27], 2016	(50) 20.04 ± 7.81	(233) 19.91 ± 9.12	(98) 18.28 ± 9.58	(15) 16.71 ± 9.86	(2) 25 ± 10.18	0.01
Luger *et al*[30], 2016	(2) 15.6 ± 5.2	(30) 15.2 ± 6.0	(8) 15.6 ± 4.4	(4) 17.6 ± 7.6	(2) 20.4 ± 4.4	0.792
Bril *et al*[29], 2015	(61) 20.5 ± 10.4	(75) 24.2 ± 15.1	(22) 20.8 ± 12.1	(22) 25.5 ± 12.2	(5) 21.1 ± 6.9	0.27
Barchetta *et al*[28], 2012	(1) 20.5	(10) 23.5 ± 14.4	(7) 16.25 ± 6.1	(6) 28.8 ± 14.9	(1) 17.3	0.56

n: Number of subjects; 25-OH(D): 25-hydroxylvitamin D; SD: Standard deviation; F0-F4: Severity score of hepatic fibrosis.

25(OH)D. Vitamin D insufficiency was defined as below the lower limit of normal (< 80 nmol/L).

Association between vitamin D deficiency and the severity of liver disease

Six included studies were cross-sectional analyses. A meta-analysis was conducted to compare the 25(OH) serum levels in patients with NAFLD according to the fibrosis stage (F0-F2 *vs* F3-F4) using a random-effects model. The results are shown in the Forest plot in Figure 4. We found no difference in the serum vitamin D levels according to high *vs* low severity of hepatic fibrosis in subjects with NAFLD [(meta estimate mean difference = -0.86 (-4.17, 2.46)], I^2 (total heterogeneity /total variability): 50.0%, χ^2 = 9.95, df = 5, *P* value = 0.08]. The forest plot (Figure 4 and Supplemental Figure 1) also demonstrates heterogeneity among the six studies. The funnel plot in Figure 5 shows some asymmetry, thereby suggesting a limited publication bias within the studies. The NAFLD subjects in two of the eight relevant studies from the qualitative synthesis had significantly lower serum 25(OH)D in controls when compared to those with NAFLD[24,26].

We then further categorized the patients into five groups based on the stage of their fibrosis from F0-F4 (Table 4) and conducted a meta-regression, and found no association (*P* = 0.86, Supplementary Figure 2) between fibrosis stage and vitamin D levels across the six studies.

DISCUSSION

We examined the peer-reviewed literature of reports of NAFLD patients for an association of serum vitamin D with the stage of liver fibrosis by conducting a systematic review and meta-analysis. A total of eight cross-sectional studies underwent a full article review and were included for qualitative synthesis. We contacted the investigators of each study and collected details of their data regarding serum vitamin D level measurements based on the specific stage of liver fibrosis. Investigators from six of the eight included studies provided sufficient data to perform a quantitative analysis on a total of 937 subjects with the diagnosis of NAFLD. First, we performed a meta-

Table 5 Relationship of vitamin D to liver fibrosis in non-alcoholic fatty liver disease by high *vs* low fibrosis score

Author	(*n*), 25-OH(D) Mean ± SD F0-F2	(*n*), 25-OH(D) Mean ± SD F3-F4
Patel *et al*[32], 2016	(172) 26.9 ± 10.7	(72) 29.4 ± 15.4
Targher *et al*[26], 2007	(33) 16.6 ± 10.0	(6) 6.0 ± 10.8
Anty *et al*[27], 2016	(381) 19.5 ± 9.1	(17) 17.7 ± 10.0
Luger *et al*[30], 2016	(40) 15.2 ± 5.6	(6) 18.6 ± 6.4
Bril *et al*[29], 2015	(158) 22.3 ± 13.2	(27) 24.7 ± 11.5
Barchetta *et al*[28], 2012	(7) 20.2 ± 11.07	(18) 26.7 ± 14.2

n: Number of subjects; 25-OH(D): 25-hydroxylvitamin D; SD: Standard deviation; F0-F4: Severity score of hepatic fibrosis.

Table 6 Levels of sIL-2R, ALT, and HBV DNA in the sera of patients with chronic HBV infection (mean ± SD)

Study	Fibrosis stage used
NASH Clinical Research Network Scoring System Definition	Seven stages:
Kleiner *et al*[53], 2005	F0: No fibrosis F1a: Mild zone 3 sinusoidal fibrosis F1b: Moderated zone 3 sinusoidal fibrosis F1c: Peri-portal sinusoidal fibrosis F2: Zone 3 sinusoidal fibrosis and peri-portal sinusoidal fibrosis F3: Bridging fibrosis F4: Cirrhosis
Brunt *et al*[54], 1999	Stage 1: Zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present Stage 2: Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis Stage 3: Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis Stage 4: Cirrhosis

NASH: Nonalcoholic steatohepatitis.

analysis comparing 25(OH)D levels in subjects with high *vs* low stages of fibrosis (F0-F2 *vs* F3-F4). This association was not statistically significant [meta-estimate pooled mean difference= -0.86, P = 0.08 (-4.17, 2.46)]. These results were consistent with the findings by Jaruvongvanich *et al*[33] who reported that there was no difference in serum 25-hydroxyvitamin D levels among 974 NAFLD subjects across the same six studies. In their study, Jaruvongvanich *et al*[33] compared the high *vs* low histologic activity of NAFLD [pooled mean difference =-0.93 (-2.45, 0.58), I^2 = 0%], and likewise, for the high *vs* low fibrosis score [pooled mean difference=0.88 (-2.65, 4.42), I^2=64%][33]. They concluded that vitamin D status was not related to the histologic activity of NAFLD. We also conducted a meta-regression to determine whether there was an association between serum vitamin D levels and stage of liver fibrosis (F0-F4) in NAFLD. As shown in Table 4, there are conflicting reports with three studies demonstrating significance

Table 7 Methodology for grading of hepatic fibrosis utilized by the authors of the six included studies

Study	Fibrosis stage used
Anty *et al*[27], 2016	Kleiner *et al*[53], 2005
Barchetta *et al*[28], 2012	Brunt *et al*[54], 1999
Bril *et al*[29], 2015	Kleiner *et al*[53], 2005
Luger *et al*[30], 2016	Kleiner *et al*[53], 2005
Patel *et al*[32], 2016	Kleiner *et al*[53], 2005
Targher *et al*[26], 2007	Brunt *et al*[54], 1999

($P < 0.05$)[26,27,32] and three finding no association ($P > 0.05$)[28-30]. Our meta-regression did not find an association between vitamin D level and fibrosis stage across the studies.

As mentioned earlier, NAFLD encompasses a histological spectrum that encompasses a wide range of pathology. Hepatic steatosis, inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma are all possible consequences of NAFLD, and can even coexist in the same patient. It is well documented that a proportion of patients with NASH with liver inflammation will develop fibrosis, with this stage progressing over time from F0 to F4[38]. In a meta-analysis of patients with NAFLD, the proportion of fibrosis for stage 0 (35.8%), stage 1 (32.5%), stage 2 (16.7%), 3 (9.3%), and 4 (5.7%) respectively[39]. Patients with NASH and baseline F0 fibrosis had an estimated annual fibrosis progression rate of 0.14 stages (95%CI, 0.07-0.21 stages), corresponding to 1 stage progression over 7.1 years for patients with NASH (95%CI, 4.8-14.3)[39]. It is well known that the major risk factors for NAFLD include obesity, insulin resistance, dyslipidemia, diabetes mellitus, and metabolic syndrome[38].

Vitamin D receptors (VDR) are expressed abundantly in the liver had have diverse consequences on metabolism which include the regulation of genes involved in glucose and lipid metabolism, and immunomodulation[40]. Low vitamin D has been reported to be strongly associated with insulin resistance[41]. Previous studies have estimated links between vitamin D and the development of NAFLD through various mechanisms that were recently reviewed by Eliades *et al*[9]. Vitamin D action on adipocytes and downregulates inflammatory cytokines IL-6, TNF-α and IL-1β through NF-κB pathway. Vitamin D also enhances the GLUT-4 receptor expression in myocytes, and also improves insulin utilization by increasing adiponectin secretion from adipocytes. Vitamin D downregulates the expression of various toll receptors in kupffer cells, thereby lessening inflammation caused by bacterial translocation (Figure 2)[9].

Also, researchers have noted vitamin D to have antifibrotic properties, as well as its involvement in the pathophysiology of liver fibrosis. The main cell involved in development of fibrosis in NAFLD is hepatic stellate cell (HSC). The HSCs become activated by losing their characteristic vitamin A droplets. Activated

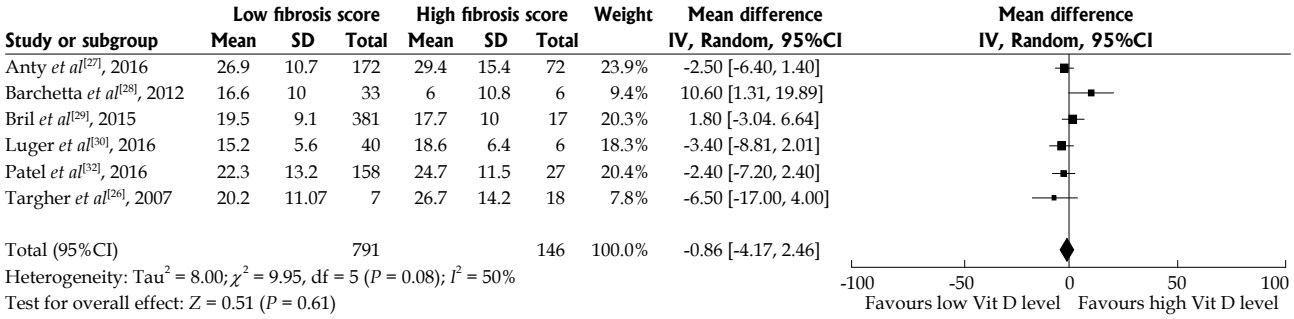

Figure 4 Random effects pooled the mean difference of 25-hydroxyvitamin D levels in nonalcoholic fatty liver disease patients with high and low fibrosis scores. A meta-analysis of the pooled data of the six included studies according to fibrosis scores of low F0-2 *vs* high F3-4. Figure 4 illustrates the forest plot of the results of the six included studies, with 95%CI, and the overall effect (under the random-effects model) with 95%CI are illustrated in this forest plot. The six included studies[26-30,32] assessed the association of 25-hydroxyvitamin D among patients with nonalcoholic fatty liver disease (NAFLD). We used a random-effects model to assess the pooled data in a meta-analysis as previously described[36]. The statistical heterogeneity was not significant with I^2 of 37.8% ($P_{heterogeneity}$ = 0.0766); however, we observed a trend towards high heterogeneity. We found no difference in 25-hydroxyvitamin D among NAFLD patients with high (F3-4) *vs* low (F0-2) fibrosis, with the summary effect size of 0.95 representing mean differences between F0-2 and F3-4 NAFLD patients. Overall, our analysis confirmed that there was no association between serum 25-hydroxyvitamin D and low *vs* high fibrosis score in NAFLD patients from the six included studies.

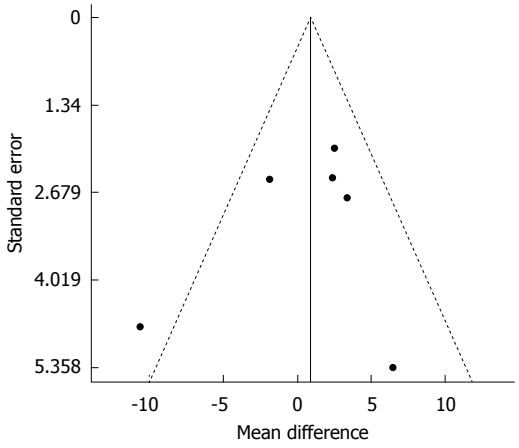

Figure 5 Funnel plot of standard error by differences in Means for 25(OH)D. We analyzed the data for a possible publication bias. The circles represent observed published studies. The funnel plot was asymmetric, thereby suggesting a possible publication bias.

HSCs then produce an extracellular matrix, which leads to fibrosis and cirrhosis[42]. It is thought that the effect of vitamin D on the liver is complex but by binding to HSC VDR it reduces proliferation of these cells which play a major role in inducing fibrosis. It is known that liver nonparenchymal cells, including HSCs, express fully functional VDR, which has led many researchers to consider the vitamin D pathway as a possible modulator of liver fibrosis[43,44]. Ding *et al*[45] demonstrated that administration of the synthetic VDR agonist Calcipotriol ameliorated liver fibrosis in a standard mouse model of a Carbon Tetrachloride (CCL4) hepatic injury. Interestingly, they also showed that liver fibrosis was discovered in mice who have a genetic deletion of VDR, which strongly supports its role in hepatic homeostasis. Furthermore, activation of VDR signaling interferes with a wide range of transforming growth factor-beta (TGFβ)/SMAD)-dependent transcriptional responses on pro-fibrotic genes in HSCs[45].

In addition to the suggested mechanistic link between vitamin D and NAFLD, various clinical cohorts have shown the association of vitamin D and fibrosis in fatty liver disease patients. In a study by Nelso *et al*[31] 190 biopsy-proven NASH adults in the Non-alcoholic Steatohepatitis Clinical Research Network (NASHCRN) cohort were reviewed. The results demonstrate an independent association between serum 25-hydroxyvitamin D, increased NASH histological activity, and the presence of fibrosis. Although epidemiologic studies are promising in showing the association between low vitamin D levels and chronic liver disease, such as NAFLD, this study suggests that the current literature has a dearth of evidence to establish causality between vitamin D and the histopathologic stage of liver fibrosis[8]. Some of the recent studies raised doubts regarding a causal link between vitamin D deficiency and non-skeletal health outcomes reviewing prospective studies and clinical trials, thereby suggesting that having a vitamin D deficiency is a predictor rather than the cause of the disease[46]. Well-designed prospective randomized clinical trials are needed to better understand the influence the oral intake (food and supplement) of vitamin D to the point of sufficiency on disease progression in NAFLD patients.

A few clinical trials using small numbers of study subjects have evaluated the effect of vitamin D supplementation in patients with NAFLD. These studies should be interpreted with caution, given the small sample sizes and short course of follow up. In a small double-blind, placebo-control trial study, NAFLD patients were randomly assigned to receive vitamin D (50000 IU every 14 d for 4 mo) *vs* placebo[47]. The period of 4 mo was used as the benchmark for analysis of results. The authors reported that the serum levels of liver chemistries, homeostatic model assessment for insulin resistance (HOMA-IR), or grades of hepatic steatosis as measured by ultrasound, were not at variance (vitamin D *vs* placebo)[47].

In a more recent study, a 12-wk, randomized, controlled, double-blind trial was conducted on 120 NAFLD patients randomly assigned to three groups. Each patient received 25 μg calcitriol (n = 37), 500 mg calcium carbonate, plus 25 μg calcitriol (n = 37) or placebo (n = 36) every day following a weight-loss program. Serum insulin and HOMA-IIR significantly reduced in subjects who received vitamin D compared to control group. Adjusting to the baseline measurements, the patients who received vitamin D showed a significant decrease in ALT and stage of fat, as evaluated by liver ultrasound following 12 wk of intervention[48]. In another small, clinical, double-blind, placebo-controlled trial on patients with NAFLD and type 2 diabetes from Italy, there was no significant difference found between patients who received 24 weeks of vitamin D *vs* placebo in terms of primary endpoint, hepatic fat fraction (HFF) measured by MRI, nor hepatic outcomes, such as liver enzymes, CK18, and Fatty Liver Index (FLI)[49]. Most of these studies evaluated markers of inflammation and degree of fat, but not the degree of fibrosis, except for the clinical trial by Corte *et al*[50] which studied 41 pediatrics patients who were enrolled to receive docosahexanoic acid (DHA) and vitamin D *vs* placebo. All patients had a liver biopsy diagnosing NAFLD at the beginning of the study. Furthermore, patients on the treatment arm also received liver biopsy at completion. The combination of vitamin D and docosahexaenoic acid treatment reduced the nonalcoholic fatty liver disease activity score (NAS) in the treatment group[50]. These investigators reported a reduction of the activation of HSC and fibril-forming collagen but not fibrosis score in the treatment group. Moreover, the ALT and HOMA-IR were all decreased with treatment[50]. A meta-analysis of seven clinical trials of vitamin D supplementation with 452 participants concluded that Vitamin D supplementation did not affect a number of markers associated with insulin resistance such as triglycerides, total-, LDL-cholesterol, FPG, insulin, HOMA-IR, AST, ALT, and BMI[51].

Finally, hepatic inflammatory processes, such as NASH, are known to deplete 25(OH)D levels and promote oxidative stress and other mediators, which contribute to progressive fibrogenesis and resistance to supplementation with vitamin D[51,52].

There are a number of noteworthy limitations to this meta-analysis. The included studies in the meta-analysis are all cross-sectional studies. Observational research is not enough to conclude a causal link between vitamin D and severity of liver disease. Randomized controlled trials will provide complementary evidence concerning such an association. If the benefits are not reproduced in randomized trials, then the relationship between vitamin D and NAFLD is probably the result of confounding or physiological events involved in these disorders[46]. There was heterogeneity among the included patient population in the studies. The BMI was variable among the studies, and particularity patients included in the study by Targher had a mean BMI of 26.3 that was significantly lower than others[26]. The evaluation of the stage of fibrosis is usually made through NASH clinical trial research network scoring system (Table 6)[53]. In two of the six included studies in the meta-analysis, Tragher and Barchetta used the liver fibrosis staging system developed by Brunt *et al*[54], which is slightly different from the NASH clinical trial research network scoring system (Tables 6 and 7). Our study was not adjusted for other confounders of metabolic syndrome, such as diabetes, obesity, and insulin resistance. Moreover, our study did not evaluate other factors that can affect vitamin D levels such as diet, circadian rhythm and season. Studies have shown that serum vitamin D levels are higher in individuals who use diet high in: dairy products, fatty fish and vitamin D supplementation. Vitamin D is directly associated with sun exposure and the serum levels of vitamin D is lower in winters[55].

In summary, prior studies have illustrated that vitamin D may be involved in the pathogenesis of NAFLD. However, in this meta-analysis, we found no evidence that the progression of fibrosis in subjects with NALFD is linked to low vitamin D status. These data are consistent with the aforementioned failure of clinical trials using vitamin D supplementation to improve NAFLD.

ARTICLE HIGHLIGHTS

Research background

Vitamin D is a hormone and a vitamin combined that appears to effects cells throughout the body and impart abundant health benefits. There are many studies on its potential role in modifying chronic liver disease. Given the escalating prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide, we studied the literature for the association of vitamin D serum levels and progression of scar tissue formation in NAFLD.

Research motivation

The goal of a systematic review is to pull together the peer-reviewed literature and then apply standardized guidelines to extract the papers that used proper methodology. In this instance, we sorted through 337 papers to find relevant peer-reviewed manuscripts of sufficient quality to provide scientific evidence about the association between vitamin D level and hepatic fibrosis.

Research objectives

The primary objective was to determine whether there was an association of serum vitamin D and the degree of scar tissue in the liver.

Research methods

We followed international guidelines for the Preferred Reporting Items for Systematic Reviews and Meta-Analyses in systematically analyzing the 337 articles with duplicate screening and extraction by the authors. The authors contacted investigators of previous papers to report crucial data not stated in their manuscripts. An expert biostatistician assisted with data analysis by using Cochrane RevMan 5 software.

Research results

We discovered that only six of the 337 studies presented sufficient data to be included in the meta-analysis. We did not find an association of serum vitamin D with the degree of liver scarring in NAFLD.

Research conclusions

We applied advanced methodologies to determine the relationship between stages of liver scarring and serum vitamin D levels. We observed that serum vitamin D was not associated with liver scar tissue accumulation irrespective of the phase of hepatic injury. In February 2017, we reported in *World Journal of Hepatology* that there was an association between the degrees of scar tissue formation in chronic Hepatitis C with the serum level of vitamin D. Given that vitamin D appears to have a strong influence on immunity and wound healing, it is still possible that supplemental vitamin D to normal levels could help prevent liver disease progression in NAFLD. Interventional trials would be best suited to explore this possibility. This study further elucidated that serum vitamin D does not appear to be associated with the stage of liver scar tissue accumulation. Application of meta-regression permits an analysis of the individual phases of liver scar tissue formation in association with the serum levels of vitamin D. This meta-analysis utilized data synthesis and statistical inquiry to study whether the degree of liver scarring is associated with serum vitamin D status, and found no association. Clinicians should bear in mind that many patients with nonalcoholic fatty liver disease are obese and have lower serum vitamin D levels than non-obese subjects due to sequestration into adipose tissues. Thus, supplementation with vitamin D3 to sufficient levels should be considered.

Research perspectives

When conducting a meta-regression, there may be crucial data that is unavailable that does require proactive investigation by researchers for analysis. Careful meta-analyses can help the scientific community to integrate evidence across studies. Systematic reviews and synthesis, as in our paper, should employ vigilance in data extraction and make efforts to contact the authors of relevant prior works to obtain further information about missing data, statistical analysis, and to clarify methods. As in our paper, acknowledgment of authors who cooperate with the provision of information for systematic review and synthesis should be noted in the resulting manuscript.

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META-ANALYSIS

Epigenetic basis of hepatocellular carcinoma: A network-based integrative meta-analysis

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Abstract

AIM

To identify the key epigenetically modulated genes and pathways in HCC by performing an integrative meta-analysis of all major, well-annotated and publicly available methylation datasets using tools of network analysis.

METHODS

PubMed and Gene Expression Omnibus were searched for genome-wide DNA methylation datasets. Patient clinical and demographic characteristics were obtained. DNA methylation data were integrated using the Ingenuity Pathway Analysis, a software package for visualizing and analyzing biological networks. Pathway enrichment analysis was performed using IPA, which also provides literature-driven and computationally-predicted annotations for significant association of genes to curated molecular pathways.

RESULTS

From an initial 928 potential abstracts, we identified and analyzed 11 eligible high-throughput methylation datasets representing 354 patients. A significant proportion of studies did not provide concomitant clinical data. In the promoter region, *HIST1H2AJ* and *SPDYA* were the most commonly methylated, whereas *HRNBP3* gene was the most commonly hypomethylated. *ESR1* and *ERK* were central genes in the principal networks. The pathways most associated with the frequently

methylated genes were G-protein coupled receptor and cAMP-mediated signalling.

CONCLUSION

Using an integrative network-based analysis approach of genome-wide DNA methylation data of both the promoter and body of genes, we identified G-protein coupled receptor signalling as the most highly associated with HCC. This encompasses a diverse range of cancer pathways, such as the PI3K/Akt/mTOR and Ras/Raf/MAPK pathways, and is therefore supportive of previous literature on gene expression in HCC. However, there are novel targetable genes such as *HIST1H2AJ* that are epigenetically modified, suggesting their potential as biomarkers and for therapeutic targeting of the HCC epigenome.

Key words: Network analysis; Hepatocellular carcinoma; Methylation

Core tip: Hepatocellular carcinoma (HCC) is a high-fatality cancer with limited screening biomarkers and therapeutic options. It arises in the context of chronic liver disease, having accumulated epigenetic changes over time. The goal of this study was to perform an integrative network-based meta-analysis of all genome-wide DNA methylation data in HCC. Using bioinformatics tools, we identified the most important aberrantly methylated genes and associated pathways. G-protein receptor signaling was the most significantly associated with HCC based on differential methylation of involved genes, which is consistent with the implication of the Ras/Raf/MAPK and mTOR pathways. The identification of novel epigenetically modified genes such as *HIST1H2AJ* within known pathways suggests targeting of the epigenome as a potential therapeutic avenue for HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) arises in the context of chronic liver disease, where there is ongoing injury over decades. HCC incidence in North America has been increasing in recent years, in the setting of a higher prevalence of cirrhosis secondary to hepatitis C and fatty liver disease[1]. It is the fifth most common cancer worldwide, and five-year survival is the second worst worldwide among all cancers at 8.9%. HCC is often diagnosed at later stages, and there is an inability to tolerate chemotherapy in patients with cirrhosis[1]. Curative treatment with resection, radiofrequency ablation or transplantation is only possible in early stage disease[2]. When diagnosed at a later stage, the first-line chemotherapeutic agent is sorafenib, which extends survival only by 3 mo[2]. Several other trials of chemotherapeutic regimens have been developed based on studies of genomic and transcriptomic data, with no further improvement in overall survival[3]. There is therefore a dire need to better understand HCC pathogenesis, elucidate screening biomarkers in patients at risk, and develop more optimal therapeutic agents.

Epigenetic changes are of significant interest to this malignancy, given that it results from mutations accumulating over time with exposure to various insults such as viral hepatitis, alcohol or fatty liver. Epigenetic modifications are heritable states of gene expression without altering DNA sequences[4]. They encompass processes such as DNA methylation, histone modifications, non-coding RNAs and nucleosome positioning. These changes are passed along faithfully to daughter cells during cell division[4]. Among these, DNA methylation has been the most studied, regulating gene expression through a stable silencing mechanism[5]. Covalent modification by DNA methyltransferases of cytosine residues with methyl groups in CpG dinucleotides occurs preferentially at the 5′ end in promoter regions. Transcriptional gene silencing results from this through two mechanisms: steric hindrance of transcription factors being able to access their cognate binding sites on gene promoters[5], and direct binding of methyl CpG binding domain containing proteins to the methylated DNA causing transcriptional repression[6]. The recent advent of genome-wide methylation analysis has enabled an appreciation of methylation status in genes of interest to cancer: Hypermethylation of tumor suppressor genes, hypomethylation of oncogenes, and methylation of repetitive elements[7]. The extensive reprogramming of the epigenome in cancer has led to a growing interest in epigenetic therapy. Specifically in HCC, aberrant DNA methylation of tumor suppressor gene promoters has been documented[8]. These epigenetic changes have been closely correlated with disease stage and clinical outcome[9]. There has been significant variability in the reported frequency of hypermethylated loci in HCC[10-12]. CDKN2A is methylated in 30%-70% of HCCs[10,12,13], RASSF1A in up to 85%[11,12], GSTP1 in 50-90%[14] and MGMT in 40%[15]. DNA methylation loci have also been reported as significantly enriched in the signaling networks of cellular development, gene expression, cell death, and cancer[16].

Our study represents the first comprehensive network-based attempt to integrate all relevant, publicly available, high-throughput genome-wide DNA methylation data to better understand the epigenetic landscape in HCC. Network and pathway analysis tools can enable identification of the most commonly

Table 1 Datasets used for the meta-analysis

No	GEO Accession	PubMed ID	HCC samples	Adjacent tissue samples
1		21500188	13	12
2		24306662[1]	45	45
3		25376292[1]	22	22
4	GSE59260[2]	25945129	8	8
5	GSE29720[2]	21747116	12	12
6	GSE18081[2]	20165882	20	20
7	GSE37988	22234943	62	62
8	GSE44970	24012984	20	8
9	GSE54503	23208076	66	66
10	GSE57956	25093504	59	59
11	GSE60753[2]	25294808	27	27

[1]Only genes are reported, without information on CpG sites; [2]Differential methylation data analysis performed in-house.

methylated genes across studies and associated pathways, and propose novel treatment options using network-based analysis[17,18].

The goal of this study was to identify key epigenetically modulated genes and pathways in HCC by integrating all major, well-annotated and publicly available methylation datasets datasets using tools of network analysis.

MATERIALS AND METHODS

Data collection, analysis and database compiling

Genome-wide methylation profiles related to HCC samples were downloaded from published datasets (PubMed, http://www.ncbi.nlm.nih.gov/PubMed) using the following MeSH terms: "{["methylation"(MeSH Terms) or "methylation" (all fields)] and ["carcinoma, hepatocellular" (MeSH terms) or ["carcinoma" (all fields) and "hepatocellular" (all fields)] or "hepatocellular carcinoma" (All Fields) or ["hepatocellular" (all fields) and "carcinoma" (all fields)]} and ["humans" (MeSH Terms) and English (lang)]. All entries on PubMed since 2002, which represents the advent of high-throughput profiling, were considered for inclusion. A second search was performed using Gene Expression Omnibus (GEO), a public functional genomics data repository containing genome-wide methylation profile array data (https://www.ncbi.nlm.nih.gov/geo). This search was performed using the following MeSH terms {["methylation" (MeSH Terms) or methylation (all fields)] and ["carcinoma, hepatocellular" (MeSH terms) or hepatocellular carcinoma (all fields) and "Homo sapiens" (porgn) and "Homo sapiens" (porgn) and "Homo sapiens" (porgn)] and "Homo sapiens" (porgn)}, covering all HCC high-throughput methylation profiling datasets comparing HCC to adjacent non-tumoral tissue.

The study workflow is illustrated in Figure 1A. Results were retrieved from both databases: GEO and PubMed. The exclusion criteria listed in Figure 1A were applied to identify papers reporting quantitative results of methylation profile performed on HCC patients and the relative adjacent tissue as control.

Available patient data, including etiology of liver disease (HCV, HBV, alcohol, fatty liver disease) on the basis of which the HCC tumors developed, presence of cirrhosis, the Model for End-stage Liver Disease score (MELD score, an assessment of the severity of liver dysfunction), tumor histology, stage of cancer, alpha-fetoprotein (AFP) level, overall and recurrence-free survival following treatment were also documented.

We identified 928 abstracts retrieved by the search on PubMed and 233 results were obtained from GEO. The flow chart outlining the selection process is detailed in Figure 1A. Details regarding the 11 included studies[8,19-30], together with the information on number of samples per group, per study are provided in Table 1.

Table 2 Availability of clinicopathological information on the 11 datasets used for the integrative analysis of genome-wide DNA methylation

Clinical-pathological information	*n* = 11	%
Cirrhosis status in HCC samples	6	55
Child-Pugh/MELD Score	3	27
HCC Etiology	10	91
Alphafetoprotein level	5	45
Tumor grade	5	45
Tumor stage	5	45
Survival data	3	27

Demographic and clinical characteristics

Demographic and clinical patient information pertaining to each dataset are presented in Tables 2 and 3.

Only 6 out of 11 papers (55%) included details regarding presence/absence of cirrhosis, and 10 out 11 papers (91%) provided details regarding the etiology of liver disease. MELD or Child-Pugh score were provided in 27% of papers. Less than half of the selected publications, 5 out of 11 (45%), included details regarding the stage of cancer, although all studies were performed using hepatectomy patient samples. The same trend is identified for information regarding the histologic grade of the tumor (well-, moderately-, or poorly-differentiated tumors). Only 5 out of 11 papers (45%) had alpha-fetoprotein levels available. Overall survival and HCC recurrence statistics as follow-up data were available in 3/11 studies (27%).

Genomic region selection

For the final network-based integrative analysis, we selected 11 datasets (Table 1). Out of these, raw data from eight studies were available on the GEO website (https://www.ncbi.nlm.nih.gov/geo/). Except for the GSE60753[25] dataset, for which we have performed our own analysis with R[31] (due to comparison between sample groups required being different from the main paper), we selected the CpG sites or genes reported to be hyper-or hypo- methylated in the corresponding

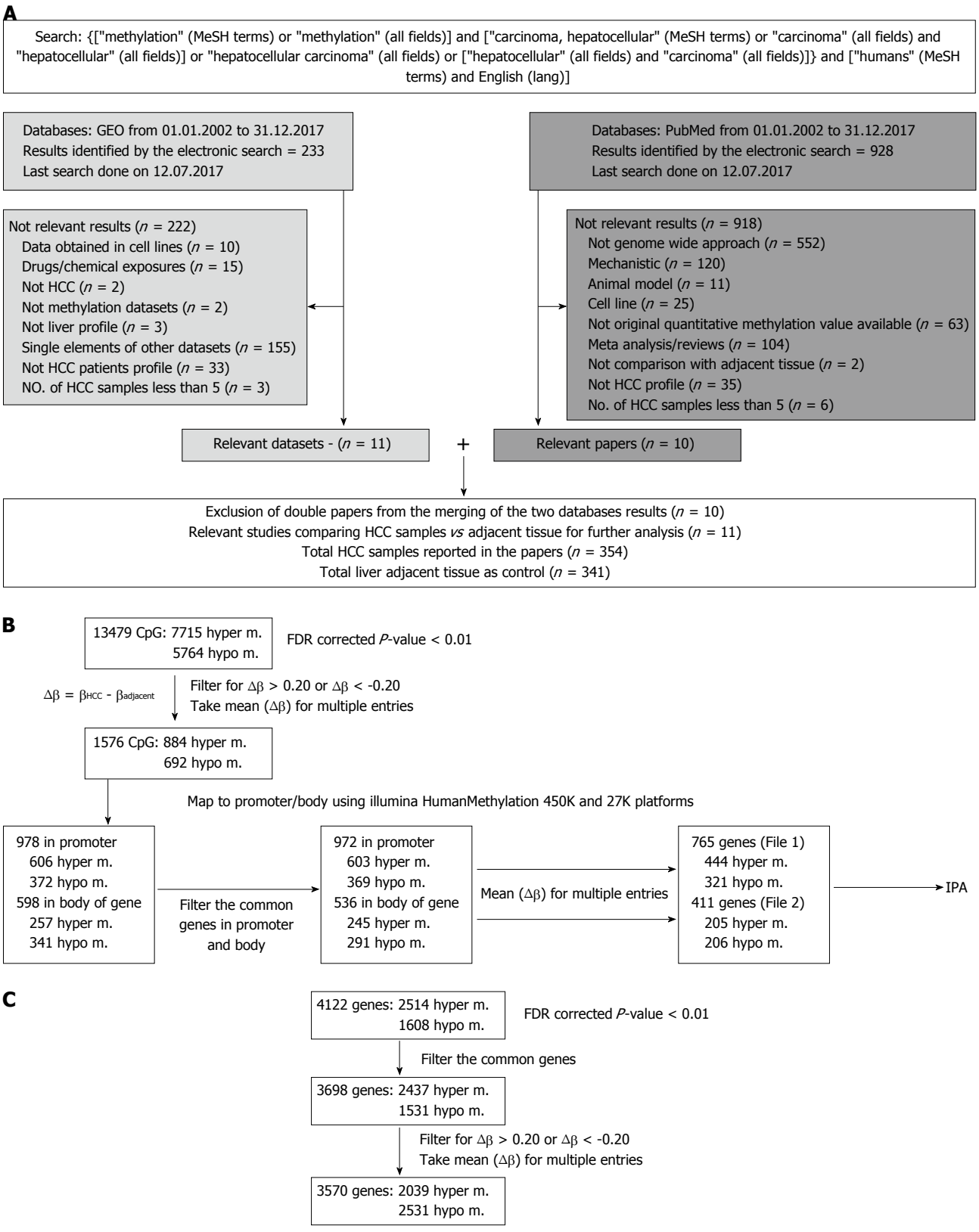

Figure 1 Flow chart. A: Workflow of Data collection, analysis and database compiling; B: Bioinformatics flow for selecting differentially methylated CpG sites from HCC meta-analysis; C: Bioinformatics flow for selecting differentially methylated genes from HCC meta-analysis. HCC: Hepatocellular carcinoma.

publications. In 6/11 datasets, CpGs and the mapped genes were selected, whereas in the remaining 5/11 datasets, only the genes without CpG sites were found. Therefore, we separated our analysis into two parts: (1) Taking into account approximately 13500 CpG sites provided or obtained with R analysis (Figure 1B); and (2) considering only the 4122 differentially methylated genes without information on the corresponding CpG

Table 3 Clinicopathological information of the individual datasets and methodology used for methylation analysis

Dataset	Year	PMID	GEO dataset	HCC (*n*)	Controls (*n*)	Liver cirrhosis in HCC samples	Etiology of liver disease (*n*)	Method
1	2011	21500188		13	12	Y (12)	HBV (3), HCV (4), alcoholic (6)	Human methylation 27 DNA analysis bead-chip
2	2014	24306662		45	45	Y (120), N (34)	HBV (149), HCV (1), nonviral (4)	Illumina GoldenGate Methylation Beadarray Cancer Panel I
3	2014	25376292		22	22	N/A	HBV (1), HCV (9), alcohol (4), other (8)	Infinium Humanmethylation 27 Beadchip
4	2015	25945129	GSE59260	8	8	N/A	HBV-HCV-(8)	Nimblegen Human DNA Methylation 3 x 720K CpG Island PI
5	2011	21747116	GSE29720	12	12	N/A	N/A	Agilent-017075 human hg 18 promoter 800-200
6	2010	20165882	GSE18081	20	20	Y (20)	HCV (20)	Illumina Golden Gate Methylation Beadarray Cancer Panel I
7	2012	22234943	GSE37988	62	62	N/A	HBV-HCV-(7), HBV + HCV-(36), HBV-HCV+(6), HBV + HCV+(13)	Illumina Human Methylation27 Beadchip
8	2013	24012984	GSE44970	20	8	N/A	HCV (8)	Human Methylation27 Beadchip
9	2013	23208076	GSE54503	66	66	Y (48), N (17), missing (1)	HBV-HCV-(19), HCV (19), HBV (13), HBV + HCV (4), missing (11)	Infinium Human Methylation 450K Beadchip
10	2014	25093504	GSE57956	59	59	Y (37), N (21)	HBV+(36), HBV-(23)	Intinium Ilumanmethylation27 Beadchip
11	2014	25294808	GSE60753	27	27	Y (26), N (1)	HBV (1), HCV (7), alcohol (9), other (10)	Iinfinium -450K Human Methy lation Beadchip

sites (Figure 1C). In both cases, the genomic region is considered differentially methylated between HCC tissue and the adjacent non-tumoral sample, if the FDR[32] corrected *P*-value < 0.01. Furthermore, we filtered out everything that did not satisfy the criteria: $\Delta\beta \geqslant 0.20$ or $\Delta\beta \leqslant -0.20$, where $\Delta\beta = \beta_{HCC} - \beta_{adjacent}$ was the difference in methylation between above specified groups. When the CpG sites were considered, the Illumina HumanMethylation450K and 27K platforms were used for mapping to the genes. When multiple sites or genes were found having the same sense of differential methylation, the mean value of Δβ was calculated. The CpGs in the 5'UTR, 1st Exon, TSS200, TSS1500 or in CpG islands were considered in the promoter and all other CpGs were considered to be in the body of the gene.

Pathway and network analysis

Two final lists of differentially methylated genes corresponding to CpGs in the promoter (*n* = 765, Supplementary Table 1) or to the body of the gene (*n* = 411, Supplementary Table 2) and their corresponding mean (Δβ) were uploaded into IPA (Ingenuity Systems®, www.ingenuity.com). Based on the manually-curated Ingenuity Knowledge Base derived from experiments and findings published in top peer-reviewed journals, IPA identifies a series of canonical pathways, diseases and functions or networks associated with the molecules in the input list. For each of these, a *P*-value is calculated with the right-tailed Fisher's exact test[33], which takes into account the number of focus molecules (input genes) in the network and the total number of molecules in the IPA database that could be included in the corresponding networks.

RESULTS

Based on datasets with known CpG sites, the most frequently hypermethylated genes in the promoter region included *HIST1H2AJ,* which is a histone protein and *SPDYA,* a cell cycle regulator known to trigger transition from G1 to S phase. The *HRNBP3* gene, an RNA-binding protein, was the most commonly hypomethylated in the promoter region. Further details are provided in Supplementary Tables 1 and 2.

Using the differentially methylated CpG sites in the promoter

Canonical pathways: The most significantly associated pathways with our list of differentially methylated genes are given in Table 4. G-protein coupled receptor signaling, Transcriptional Regulatory Network in Embryonic Cells, cAMP-mediated signaling were the top hits.

Diseases and functions: Not surprisingly Cancer, Organismal Injury and Abnormalities were identified as some of the top diseases and functions. Among different types of cancer, abdominal cancer (*P* = 1.7E-210), digestive system cancer (*P* = 7.88E-14), abdominal carcinoma and digestive organ tumor (*P* = 8.58E-13–1.76E-12) were listed. Cellular development (*P*-value=3.22E-03–2.66E-08), growth and proliferation (*P* = 3.22E-03–6.99E-06) were the most important molecular and cellular functions associated with HCC methylated data.

Networks: Among the most statistically and biologically significant networks associated with the genes

Table 4 Top canonical pathways identified by IPA for the genes corresponding to CpG sites in promoter

Ingenuity canonical pathways	-log (*P*-value)	Molecules
G-protein coupled receptor signaling	3.84E+00	DRD5, GNA11, VIPR2, ADCY5, ADRB1, CNR1, PIK3R5, NPY1R, FPR1, FFAR3, NFKBID, MC2R, PDPK1, GRM4, MC3R, CXCR2, PRKAR1B, DRD4, PDE6B, HCAR2, DUSP4, PTGDR
Transcriptional regulatory network in embryonic stem cells	3.42E+00	MYF5, SIX3, PAX6, GBX2, CDYL, FOXD3, ONECUT1, FOXC1
cAMP-mediated signaling	3.22E+00	DRD5, VIPR2, ADCY5, ADRB1, CNR1, NPY1R, FPR1, FFAR3, MC2R, GRM4, MC3R, CXCR2, PRKAR1B, DRD4, PDE6B, HCAR2, DUSP4, PTGDR

Table 5 Canonical pathways identified by IPA for the genes with methylation differences in the body of the gene in hepatocellular carcinoma

Ingenuity canonical pathways	-log (*P*-value)	Molecules
Aryl hydrocarbon receptor signaling	2.48E+00	GSTM1, CCND2, TFF1, ALDH1L2, GSTM2, TP73, GSTP1, ALDH3A1
G-protein coupled receptor signaling	2.03E+00	CAMK2B, RGS7, GABBR1, PDE4D, ADCY2, PDE1C, ADRA1D, NPR3, PDE10A, GRM6, PRKCG
cAMP-mediated signaling	1.73E+00	CAMK2B, RGS7, GABBR1, PDE4D, ADCY2, PDE1C, NPR3, PDE10A, GRM6

differentially methylated in the promoter region, three of them captured our attention: Organismal Development, Organismal Injury and Abnormalities, Cellular Development (Figure 2A), Lipid Metabolism, Small Molecule Biochemistry, Cell Death and Survival (Figure 2B) and Cell-to-Cell Signaling and Interaction, Drug Metabolism, Small Molecule Biochemistry (Figure 2C). We noted that networks 1 and 3 were mainly formed by the hyper-methylated genes, whereas network 2 is constituted by both hyper- and hypo-methylated genes approximately equally.

Using the differentially methylated CpG sites in the body of the gene

Canonical pathways: G-protein coupled receptor signaling and cAMP-mediated signaling were among the top 20 hits (Table 5), which was similar to the pathways generated by the genes with methylated CpG sites in the promoter region.

Diseases and functions: Our study shows that DNA methylation in HCC patients leads to the same diseases and functions, regardless of the CpG site position (promoter or body), with cancer and, in particular, abdominal/digestive system cancer among the top listed by IPA.

Networks: From the top detected networks, the Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance (Figure 3A) and Drug Metabolism, Glutathione Depletion in Liver, Small Molecule Biochemistry (Figure 3B) are closely related to HCC.

Validation using only the reported differentially methylated genes

Having identified the differentially methylated genes corresponding to the CpG sites in the promoter or the body of the genes through this integrative meta-analysis, we wanted to verify how many of these overlapped with the reported differentially methylated genes in those studies that did not include information on CpG sites. The Venn diagram in Figure 4 shows 165 genes reported genes in common with CpG sites in the promoter and 82 genes in common with CpG sites in the body (Supplementary Table 3).

DISCUSSION

The literature on the epigenome in HCC has grown since the advent of tools permitting genome-wide methylation analysis. Epigenetic changes in HCC arise in the context of various etiologies of chronic liver disease, and have been revealed to contribute to tumorigenesis and cancer progression. Therapeutic targeting of HCC has not been as successful as in other malignancies, and requires exploration of a different approach[34,35]. Given that this cancer is driven by various known environmental factors, targeting epigenetic changes in HCC represents a potentially promising therapeutic avenue[36].

The current study is the largest network-based integrative meta-analysis of all publicly available genome-wide DNA methylation data in HCC, with 354 HCC samples represented. These HCCs had arisen mainly in the context of viral hepatitis B and C, with only a few occurring in patients with alcoholic cirrhosis. Therefore, the literature on methylation in HCC is heavily weighted towards epigenetic changes from viral infection, and the aberrantly methylated genes in our analysis will likewise be influenced by the greater proportion of hepatitis B and C. Clinical information regarding tumor grade, disease stage, and survival were only available in around half of the datasets, thereby limiting the ability to correlate with histopathological characteristics and disease outcome. The genome-wide DNA methylation datasets included in our integrative analysis were published from 2010

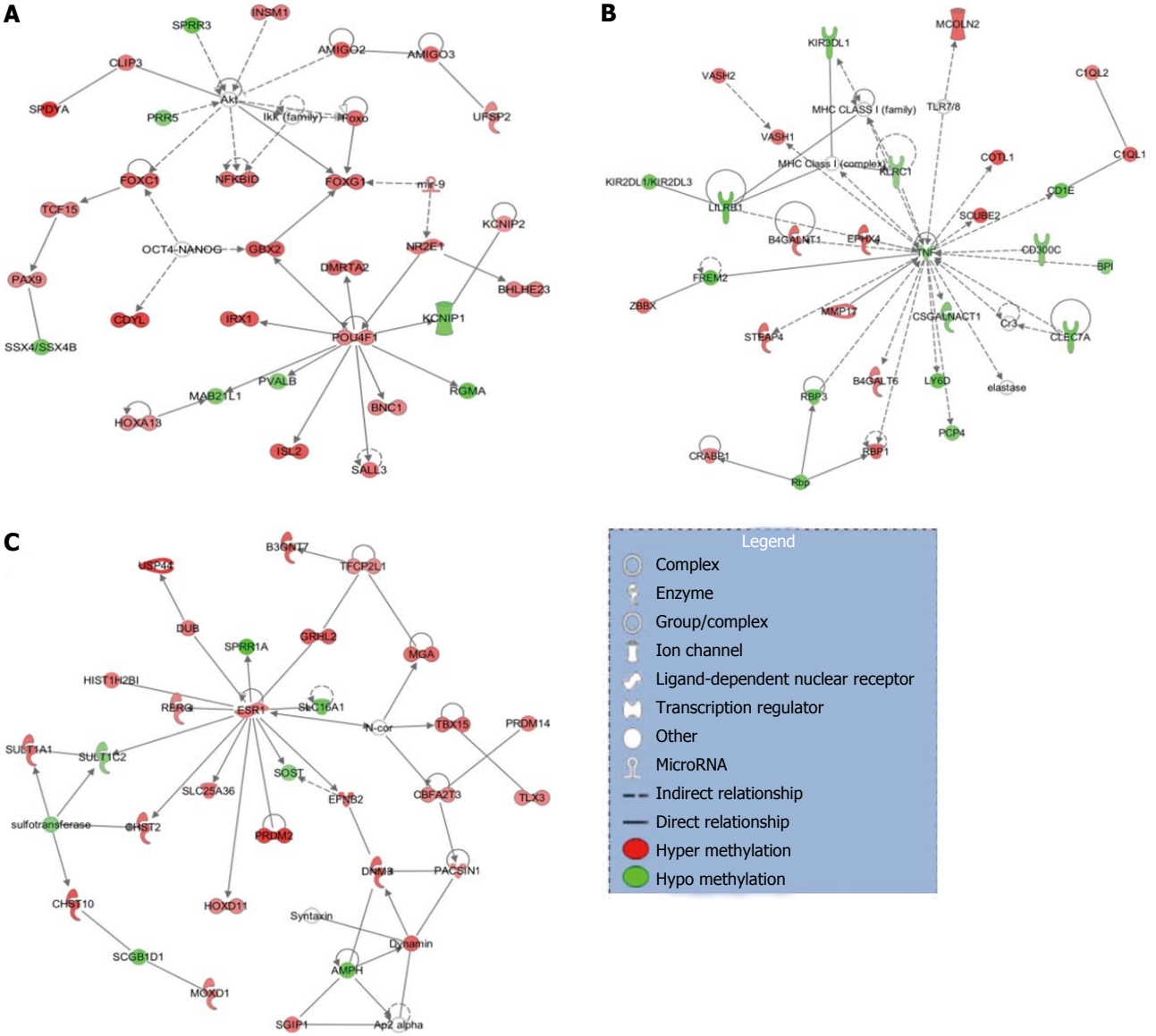

Figure 2 Networks associated with differentially methylated CpG sites in the promoter regions of genes. A: Organismal development, organismal injury and abnormalities, cellular development; B: Lipid metabolism, small molecule biochemistry, cell death and survival; C: Cell-to-cell signaling and interaction, drug metabolism, small molecule biochemistry.

to 2015.

Network-based tools offer a different and unique perspective into the key genes and pathways implicated in disease pathogenesis and progression[37]. Network-based medicine is critical to a broader understanding of HCC, whose pathogenesis has been difficult to elucidate given the multiplicity of underlying liver disease etiologies[30]. Epigenetic changes impact genetic networks, and a network-based integrative meta-analysis is ideally suited to integrating and exploring effects of networks on disease pathogenesis[38]. Using IPA, we performed this integrative analysis in order to identify the most commonly aberrantly methylated genes and associated pathways. The most commonly hyper- and hypomethylated genes were identified. These included *HIST1H2AJ,* which is a histone-coding cell cycle gene previously also identified as hypermethylated in patient lung adenocarcinoma samples[37] and head and neck squamous cell carcinomas[39]. In a study investigating the genetic-and-epigenetic cell cycle network in HeLa cancer cells, methylation of *HIST1H2AJ* (among other genes) was found to result in cell proliferation and anti-apoptosis through NFκB, TGF-β, and PI3K/Akt/mTOR pathways[40]. *HIST1H2AJ* has not previously been highlighted as a gene of interest in HCC, which is a novel finding of our integrative analysis of methylation datasets. This illustrates the power of integrating all available high-throughput data to better understand important genes in cancer. *SPDYA,* a cell cycle regulator known to trigger transition from G1 to S phase, was differentially hypermethylated. The *HRNBP3* gene, an RNA-binding protein, was the most commonly hypomethylated, as had been reported in

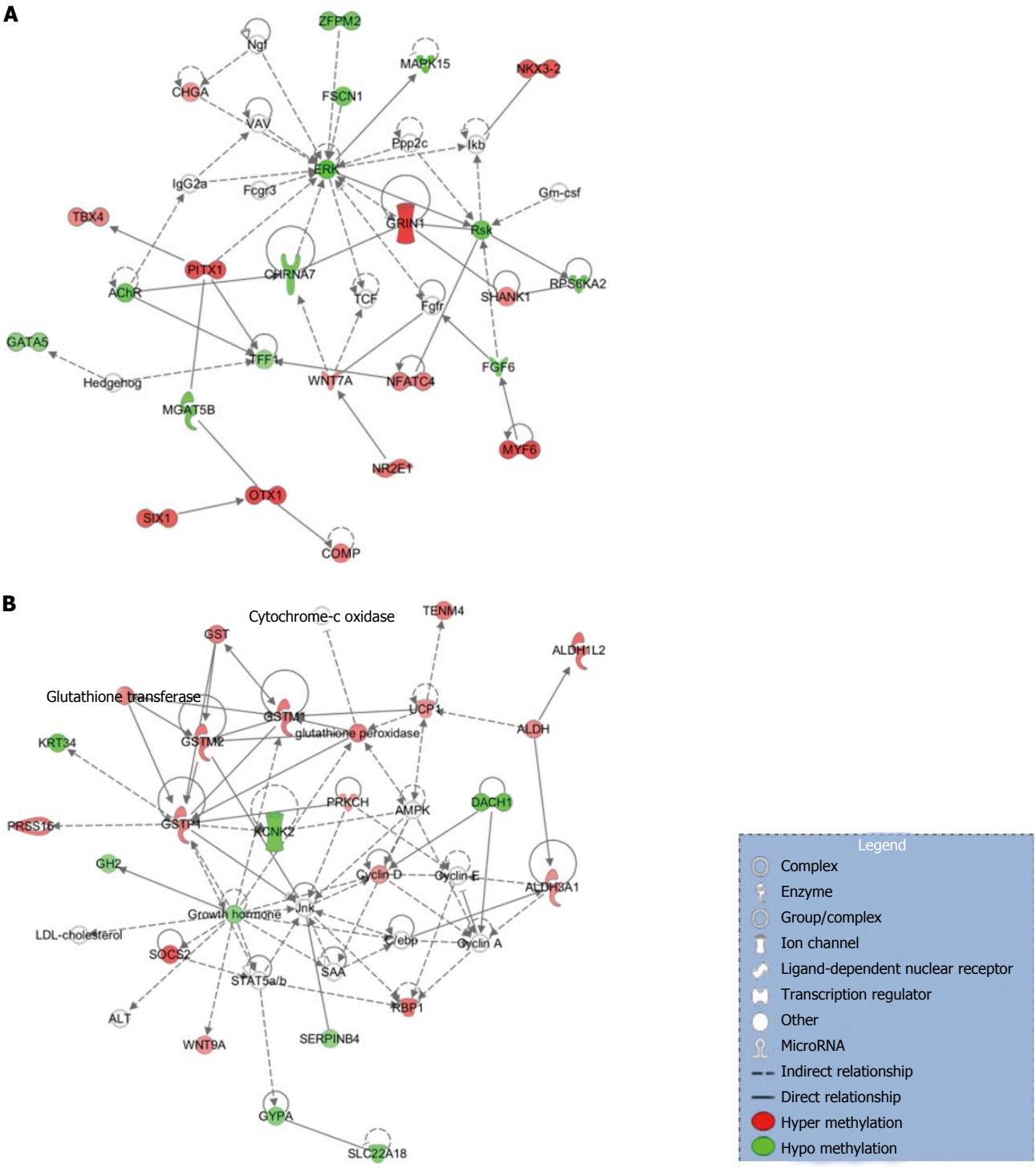

Figure 3 Networks associated with differentially methylated CpG sites in body of the gene. A: Cellular assembly and organization, cellular function and maintenance; B: Drug metabolism, glutathione depletion in liver, small molecule biochemistry.

the integrative analysis of epigenetic data by Song *et al*[16] in 2012. One would thereby anticipate increased gene expression of *HRNBP3* in HCC. These genes with differential methylation have not previously been highlighted in the HCC literature, and serve as potential new biomarkers and therapeutic targets[41].

Using IPA, we then determined the most commonly affected networks in HCC.

G-protein coupled receptor signaling, Transcriptional Regulatory Network in Embryonic Cells, cAMP-mediated signaling were the top hits, which was in perfect agreement with the work of Song *et al*[16], wherein they used IPA to analyze methylation profiling for a set of 27 HCC tumors compared with 20 normal patients. G-protein coupled receptor signalling is common to various principal pathways known to be implicated in HCC, including the PI3K/Akt/mTOR and Ras/Raf/MAPK pathways based on genomic and gene

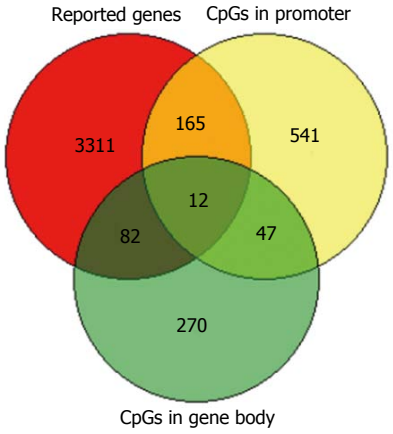

Figure 4 Venn diagram intersecting three lists: (1) reported differentially methylated genes in HCC from studies not providing information on the corresponding CpG sites; (2) identified differentially methylated genes in HCC corresponding to CpG sites in promoter; and (3) identified differentially methylated genes in HCC corresponding to CpG sites in body of the gene.

expression analyses. Therefore, our results reinforce the biological rationale of targeting these pathways. We also elucidated the crosstalk between proteins within the networks of interest to HCC. This analysis revealed ESR1 and ERK to be proteins central to they key networks.

A unique aspect of our study was the analysis of methylation at CpG sites in both the promoter and body of genes. Whereas methylation in the gene promoter is known to cause transcriptional repression, methylation in the body has the opposite effect, promoting gene expression. A novel finding was that the genes with the greatest differential methylation in the promoter were the same as those with the greatest differential methylation in the body, further confirming the importance of these genes. We were also able to validate the identity of several genes with data on CpG sites within datasets without such data available.

Limitations of our study include the lack of methylation data on individual HCC samples. Given the relatively recent advent of genome-wide methylation analysis methods, with the earliest dataset in HCC being released in 2010, this analysis was representative of only 354 samples in comparison to a similar number of non-cancerous liver samples. Nonetheless, our study is the largest integrative network-based analysis of DNA methylation in HCC. Clinicopathological characteristics such as grade, stage and survival were available only for half of the datasets, thereby limiting the ability to correlate these data points with the most aberrantly methylated genes. Finally, these data were most representative of hepatitis B and C, as described above.

In conclusion, our integrative analysis of genome-wide DNA methylation represents the largest such study in HCC. By integrating all genome-wide DNA methylation data with network-based tools, we have systematically elucidated the landscape of epigenetic DNA modifications in HCC and identified novel potential biomarkers and targetable genes within known pathways of interest to HCC. Therapeutic targeting of the epigenome in HCC is a potential avenue to address this malignancy that arises in the context of various etiologies of chronic liver disease.

ARTICLE HIGHLIGHTS

Research background

The advent of high-throughput technologies in epigenetics has led to improved characterization of methylation status and its impact on development of Hepatocellular carcinoma (HCC).

Research motivation

HCC is a malignancy that arises in the context of ongoing liver injury from various causes, such as hepatitis B, hepatitis C, alcoholic and non-alcoholic liver disease. Therefore, epigenetic changes are very likely to contribute to the pathogenesis of this malignancy.

Research objectives

We aimed to identify the key epigenetically modulated genes and pathways in HCC by performing an integrative meta-analysis of all major, well-annotated and publicly available methylation datasets using tools of network analysis.

Research methods

PubMed and Gene Expression Omnibus were searched for genome-wide DNA methylation datasets. Patient clinical and demographic characteristics were obtained. DNA methylation data were integrated using the Ingenuity Pathway Analysis, a software package for visualizing and analyzing biological networks. Pathway enrichment analysis was performed using IPA, which also provides literature-driven and computationally-predicted annotations for significant association of genes to curated molecular pathways.

Research results

From an initial 928 potential abstracts, we identified and analyzed 11 eligible high-throughput methylation datasets representing 354 patients. A significant proportion of studies did not provide concomitant clinical data. In the promoter region, *HIST1H2AJ* and *SPDYA* were the most commonly methylated, whereas *HRNBP3* gene was the most commonly hypomethylated. *ESR1* and *ERK* were central genes in the principal networks. The pathways most associated with the frequently methylated genes were G-protein coupled receptor and cAMP-mediated signalling.

Research conclusions

Using an integrative network-based analysis approach of genome-wide DNA methylation data of both the promoter and body of genes, we identified G-protein coupled receptor signalling as the most highly associated with HCC. This encompasses a diverse range of cancer pathways, such as the PI3K/Akt/mTOR and Ras/Raf/MAPK pathways, and is therefore supportive of previous literature on gene expression in HCC. However, there are novel targetable genes such as *HIST1H2AJ* that are epigenetically modified, suggesting their potential as biomarkers and for therapeutic targeting of the HCC epigenome.

Research perspectives

Our integrative analysis of genome-wide DNA methylation represents the largest such study in HCC. By integrating all genome-wide DNA methylation data with network-based tools, we have systematically elucidated the landscape of epigenetic DNA modifications in HCC and identified novel potential biomarkers and targetable genes within known pathways of interest to HCC. Therapeutic targeting of the epigenome in HCC is a potential avenue to address this malignancy that arises in the context of various etiologies of chronic liver disease.

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CASE REPORT

Contrast uptake in primary hepatic angiosarcoma on gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging in the hepatobiliary phase

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Author contributions: Hayashi M, Abe K and Ohira H designed the report; Hayashi M wrote the paper; Hayashi M, Matsuoka N, Kashiwagi M and Kanno Y treated the patient; Kawana S and Hashimoto Y performed an autopsy and analyzed histological findings; Sekino H and Hiroshi I made the radiological diagnosis; Okai K, Takahashi A and Ohira H critically reviewed the manuscript.

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Abstract

Primary hepatic angiosarcoma is the most common malignant mesenchymal tumor of the liver. It has a poor prognosis and various appearances on magnetic resonance (MR) images. We report a case of hepatic angiosarcoma with a characteristic appearance on gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MR imaging in the hepatobiliary phase. A 72-year-old man was admitted with a complaint of abdominal pain. Gd-EOB-DTPA-enhanced MR imaging revealed a liver tumor that

showed slight hyperintensity in the hepatobiliary phase. These findings suggested Gd-EOB-DTPA uptake in the tumor. An autopsy revealed the solid proliferation and sinusoidal spreading of hepatic angiosarcoma cells. Immunohistochemistry indicated that the tumor was negative for OATP1B3. Gd-EOB-DTPA uptake in the liver tumor in the hepatobiliary phase suggested sinusoidal tumor invasion with residual normal hepatocytes.

Key words: Hepatic angiosarcoma; Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid; Cirrhosis; Hepatocellular carcinoma

Core tip: Hepatic angiosarcoma has various appearances on computed tomography and magnetic resonance (MR) images. In the context of cirrhosis, hepatic angiosarcoma often cannot be readily distinguished from hepatocellular carcinoma. We present contrast uptake in primary hepatic angiosarcoma on gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced MR imaging in the hepatobiliary phase, and contrast uptake suggested sinusoidal tumor invasion with residual normal hepatocytes. This finding may assist physicians in the diagnosis of future cases of hepatic angiosarcoma.

Hayashi M, Kawana S, Sekino H, Abe K, Matsuoka N, Kashiwagi M, Okai K, Kanno Y, Takahashi A, Ito H, Hashimoto Y, Ohira H. Contrast uptake in primary hepatic angiosarcoma on gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging in the hepatobiliary phase. *World J Hepatol* 2018; 10(1): 166-171 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/166.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.166

INTRODUCTION

Primary hepatic angiosarcoma is the most common malignant mesenchymal tumor of the liver but accounts for only 2% of primary hepatic tumors[1-4]. It has a poor prognosis, and most patients die within one year of diagnosis[3]. Although various environmental carcinogens are known causes of hepatic angiosarcoma, other possible major causes of this disease remain unknown[2]. Hepatic angiosarcoma has various appearances on computed tomography (CT) and magnetic resonance (MR) images[5,6]. In the context of cirrhosis, hepatic angiosarcoma often cannot be readily distinguished from hepatocellular carcinoma (HCC)[7]. The usefulness of MR images for detecting HCC is widely known, especially with respect to gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MR imaging in the hepatobiliary phase. Here, we report a case of hepatic angiosarcoma with a characteristic appearance on Gd-EOB-DTPA-enhanced MR imaging in the hepatobiliary phase.

CASE REPORT

A 72-year-old man visited our institution due to the onset of abdominal pain that had begun one month previously. Abdominal ultrasonography revealed a heterogeneous hyperechoic tumor in the left hepatic lobe (Figure 1A), and the patient was admitted to our hospital. He had a history of resection of the right hepatic lobe due to HCC (T2N0M0) with hepatitis B virus-related liver cirrhosis 18 years previously. After this resection, no recurrence was detected on unenhanced CT or ultrasound images until his most recent check-up, which occurred during the previous year. He did not receive antiviral therapy for hepatitis B virus, such as interferon or nucleotide analogues. The patient consumed 360 mL of Japanese sake (containing 40 g of ethanol) per day prior to the hepatic resection and was a non-smoker. He did not have a history of environmental carcinogen exposure. His BMI was 25.9. His abdomen was soft and flat with upper abdominal tenderness. The following blood test results were obtained at admission: white blood cells, 8300/μL; hemoglobin, 10.1 g/dL; platelets, 12.3 × 10^4/μL; albumin, 3.0 g/dL; total bilirubin, 1.5 mg/dL; aspartate aminotransferase, 118 U/L; alanine aminotransferase, 85 U/L; alkaline phosphatase, 553 U/L; γ-glutamyl transpeptidase, 212 U/L; C-reactive protein, 17.08 mg/dL; alpha-fetoprotein, 3.3 ng/mL; des-gamma-carboxy prothrombin, 29 mAU/mL; carcinoembryonic antigen, 4.1 ng/mL; carbohydrate antigen 19–9, 19.2 U/mL; soluble interleukin-2 receptor, 925 U/mL; hepatitis B surface antigen, negative; hepatitis B surface antibody, positive; and hepatitis C virus antibody, negative.

Dynamic contrast-enhanced CT images showed a 16 cm × 10 cm tumor in the left hepatic lobe and multiple nodules (Figure 1B and C). The tumor was not enhanced in the arterial phase. Gd-EOB-DTPA-enhanced MR imaging was then performed (Figure 1D-H). T1-weighted images revealed a dominant tumor with low intensity that contained focal areas of high intensity suggestive of hemorrhage. The dominant tumor had high intensity in T2-weighted images and diffusion-weighted images and did not show enhancement in the arterial phase. In the hepatobiliary phase, the tumor showed slightly elevated intensity, a finding that suggested slight uptake of Gd-EOB-DTPA in the tumor. Based on these findings, we considered the possibility that the tumor was derived from hepatocytes. Given our results, it was difficult to discriminate between HCC and another type of malignant tumor.

One day after admission, a liver tumor biopsy was performed that revealed solid proliferation of spindle cells with enlarged and hyperchromatic nuclei. These spindle cells had an intracytoplasmic lumen with

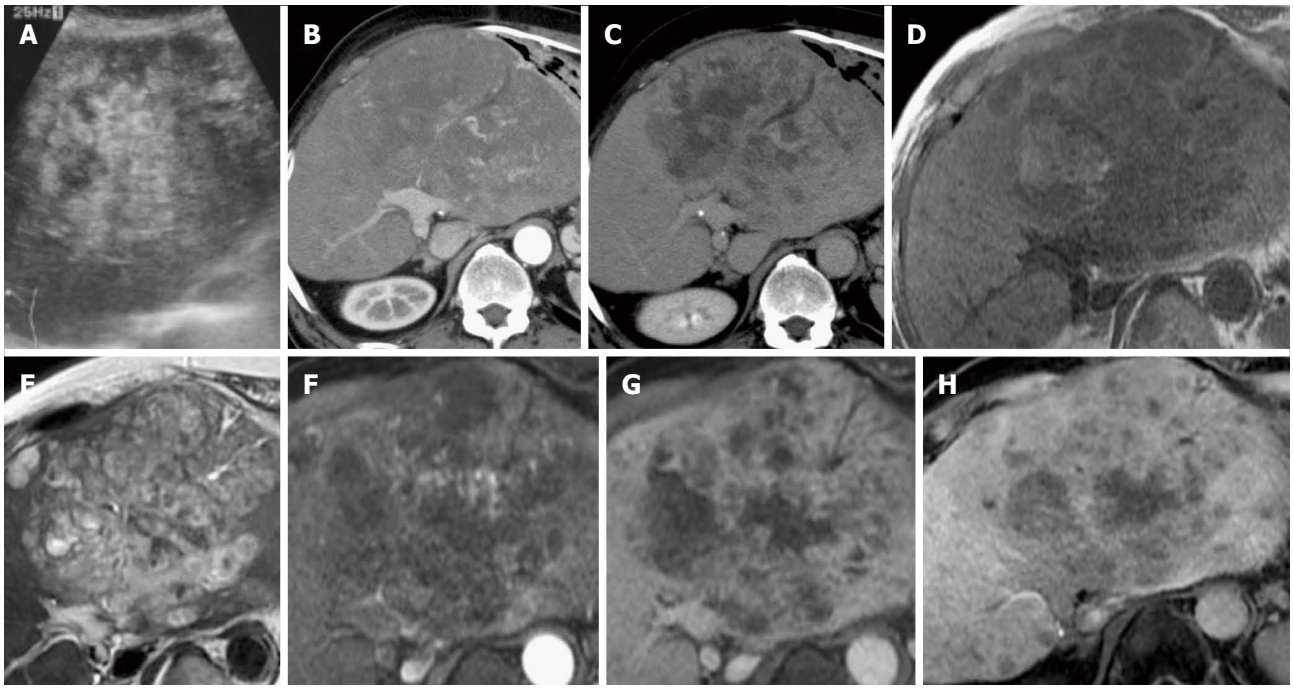

Figure 1 Abdominal ultrasonography. A: B-mode ultrasonography; B: Arterial phase; C: Venous phase images obtained using contrast-enhanced computed tomography; D: T1-weighted phase; E: T2-weighted phase; F: Arterial phase; G: Venous phase; H: Hepatobiliary phase images obtained using gadolinium ethoxybenzyl-diethylenetriamine-enhanced magnetic resonance imaging. In the hepatobiliary phase, the tumor showed slightly elevated intensity, a finding that suggested uptake of gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid in the tumor.

erythrocytes, suggesting endothelial differentiation. With respect to immunohistochemistry, tumor cells were positive for CD34 but negative for CK7 and HepPar 1 (Figure 2A and B). These results were consistent with hepatic angiosarcoma. After admission, the patient experienced worsening liver and renal failure, and his state of consciousness deteriorated. He died from multiple organ failure nine days after admission, and an autopsy was performed.

Macroscopy indicated that the liver was enlarged and weighed 2,440 g. A large tumor and many satellite nodules were observed in the liver (Figure 2C); Figure 2B presents Gd-EOB-DTPA-enhanced MR images in the hepatobiliary phase in almost the same plane as the images in Figure 2C. The boundary between the tumor and surrounding liver tissue was clear. The tumor had a cavernous pattern, and necrosis was present. On microscopy, similarly to the biopsy specimen, the tumor showed the solid proliferation of atypical spindle cells (Figure 2E). Necrosis and hemorrhage were also observed. Spindle cells tended to shift to atypical endothelial cells that had large, irregularly shaped, hyperchromatic nuclei. Atypical endothelial cells had regularly infiltrated into the sinusoid and replaced sinusoidal cells in a broad range of hepatic parenchyma; as a result, hepatic cell cords remained in the tumor (Figure 2F). In addition, in hepatic parenchyma outside of the tumor, atypical endothelial cells had often infiltrated and replaced sinusoidal cells to form ill-defined foci that were difficult to identify *via* macroscopy. With respect to immunohistochemistry, tumor cells were positive for CD34, CD31, and vimentin but negative for Factor VIII, FLI1, HepPar 1, Arginase-1, and Glypican-3 (Figure 2G shows CD34 staining). In addition, tumor cells were negative for OATP1B3, whereas hepatic cells around the spindle cells were positive for OATP1B3 (Figure 2H). A diagnosis of hepatic angiosarcoma was confirmed. Only approximately 30% of the liver tissue remained, and much of this tissue was compressed by the tumor. The remaining liver tissue appeared to be dysfunctional. There were no microvascular thrombi, and no evidence suggested disseminated intravascular coagulation. No recurrent HCC or angiosarcoma metastatic lesions were found. The cause of death was confirmed to be liver failure due to the progression of hepatic angiosarcoma.

DISCUSSION

In this case, the hepatic angiosarcoma showed slightly elevated intensity on Gd-EOB-DTPA-enhanced MR imaging in the hepatobiliary phase. These MR images suggested uptake of Gd-EOB-DTPA in the mass. A comparison of MR imaging results in the hepatobiliary phase with microscopic findings at autopsy indicates that the area of the tumor with Gd-EOB-DTPA uptake exhibited tumor cells spreading in hepatic sinusoids that contained residual normal hepatocytes. In contrast, the area of the tumor with no uptake of Gd-EOB-DTPA showed massive tumor cell proliferation. There were few normal hepatocytes.

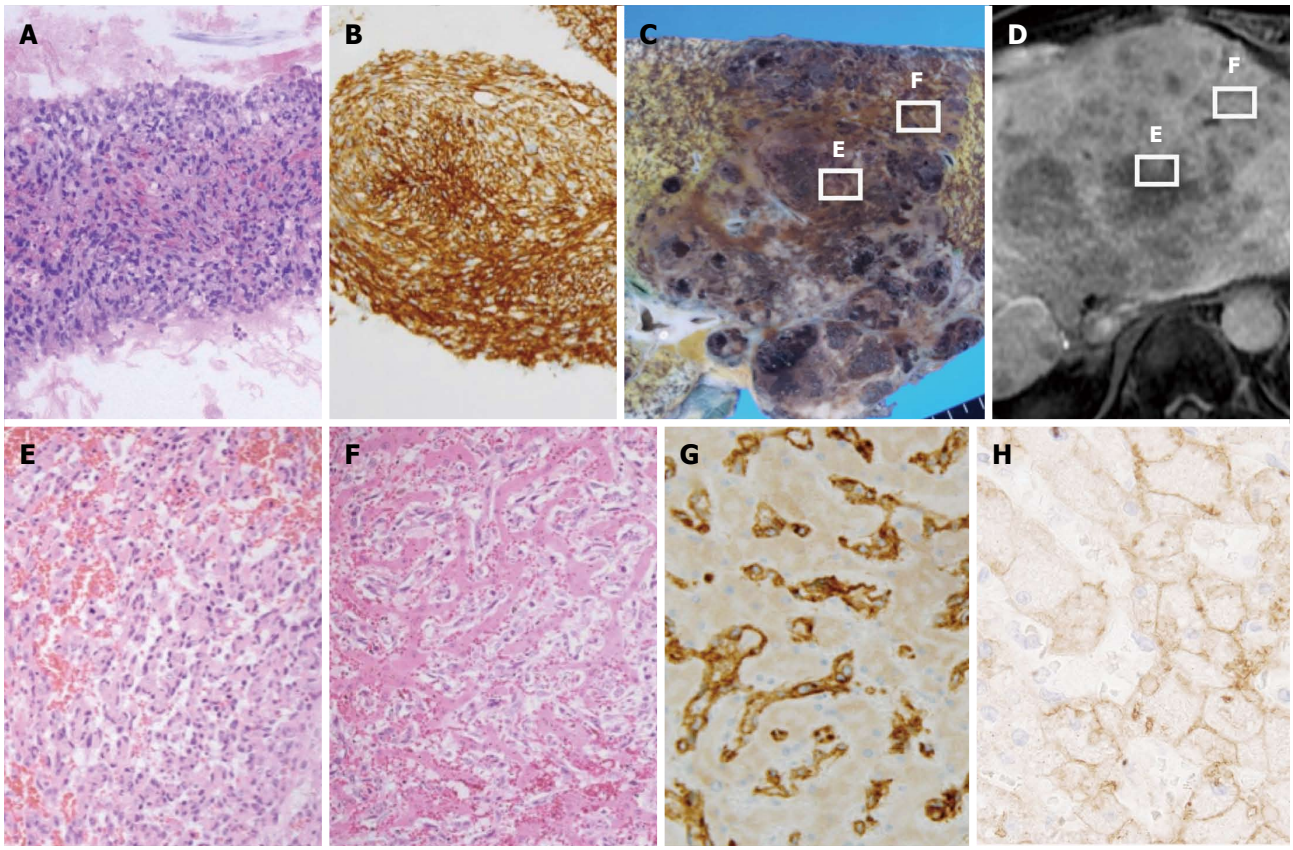

Figure 2 Histological findings from a liver tumor biopsy revealed the solid proliferation of spindle cells. A: Hematoxylin and eosin (HE) staining, × 20; B: CD34 staining, × 20; C: Macroscopically, a large tumor and many satellite nodules were observed in the liver; D: A hepatobiliary phase image obtained using gadolinium-ethoxybenzyl-diethylenetriamine-enhanced magnetic resonance imaging, depict almost the same plane of C; E: The proliferation of atypical spindle cells and hemorrhage were seen (HE staining, × 100); F: Tumor cells had spread to the sinusoids in most of the liver, although normal hepatic cells remained (HE staining, × 100); G: Tumor cells were positive for CD34 (CD34 staining, × 400); H: Tumor cells were negative for OATP1B3, and hepatic cells were positive for OATP1B3 (OATP1B3 staining, × 400).

CT or MR images of hepatic angiosarcoma have shown various appearances. Multiphase contrast-enhanced CT and MR images showed the masses to have heterogeneous and progressive enhancement[7]. On contrast-enhanced CT images, tumor nodules showed hypoattenuating and contained focal areas of enhancement. The attenuation of many foci of enhancement was less than that of the aorta but greater than that of the hepatic parenchyma. The tumor nodules demonstrated heterogeneous enhancement that suggested central necrosis and fibrotic change. On MR T1-weighted images, the nodules were of low intensity but contained focal areas of high intensity, suggesting hemorrhage[7]. In the setting of cirrhosis, lack of tumor washout and vascular invasion argue against multifocal HCC[5]. A previous case report described Gd-EOB-DTPA-enhanced MR imaging of hepatic angiosarcoma[6]. In that report, the hepatic angiosarcoma was entirely hypointense in the hepatobiliary phase. There are many reports describing the radiological findings of HCC. The presence of arterial hypervascularity and washout are considered to be typical imaging features of classical HCC[8]. On the other hand, well-differentiated and poorly differentiated HCC often showed atypical enhancement patterns, such as hypovascularity in the arterial phase[9]. HCC generally can be seen as hypointense in the hepatobiliary phase of Gd-EOB-DTPA-enhanced MR imaging[10]. A minority of HCC tumors showed iso- or hyperintensity because of preserved OATP expression[11]. In the present case, the dominant mass showed hypovascularity in the arterial phase of MR or CT images and slight hyperintensity in the hepatobiliary phase of Gd-EOB-DTPA-enhanced MR imaging. Furthermore, this patient had a history of HCC. Diagnosis was difficult with MRI or CT findings alone.

Gd-EOB-DTPA-enhanced MR imaging has been recognized as a useful imaging technique for diagnosing liver tumors. A prior study found that for HCC, Gd-EOB-DTPA uptake was determined by OATP1B3 expression[12]. The degree of enhancement in Gd-EOB-DTPA-enhanced MR images in the hepatobiliary phase has been positively correlated with OATP1B3 expression levels[13]. A case of pseudolymphoma of the liver with partial uptake of Gd-EOB-DTPA in the hepatobiliary phase has also been reported[14]. In that case, infiltration of lymphoid cells was seen along the hepatic sinusoid, leaving some hepatocytes intact. In our case, the tumor cells microscopically showed a sinusoidal spreading pattern, and numerous viable

hepatic cells remained. Furthermore, staining indicated that tumor cells were negative for OATP1B3 but that hepatic cells were positive for OATP1B3. We speculated that the reason for Gd-EOB-DTPA uptake in the mass was that tumor cells coexisted with hepatic cells. In this case, the findings of slight Gd-EOB-DTPA uptake in the liver tumor in the hepatobiliary phase may suggest the proliferation of malignant tumor cells in the sinusoids and the presence of hepatocytes.

The clinical behavior of hepatic angiosarcoma is extremely aggressive, and this disease has a poor prognosis[3,15]. Although various treatments for patients with hepatic angiosarcoma have been reported, chemotherapy, hepatic resection, and liver transplantation have all been found to have limited effects[16-18]. However, there have been reports of long-term survival after hepatic resection[19,20]. Early diagnosis of hepatic angiosarcoma is an important consideration when recommending surgical treatment for this disease. An association between liver cirrhosis and hepatic angiosarcoma has been shown. Even if patients have a history of treatment for HCC, it is necessary to consider hepatic angiosarcoma as a possible diagnosis.

The described case involved primary hepatic angiosarcoma that developed after the resection of HCC. To the best of our knowledge, there have been no reports describing the occurrence of HCC and hepatic angiosarcoma in the same patient. HCC with sarcomatous change has been observed in patients with a history of treatment for HCC or liver cirrhosis[21]. In the current case, we diagnosed primary hepatic angiosarcoma because HCC components were not observed and because tumor cells expressed neither HepPar 1 nor Arginase-1, which are lineage markers of hepatic cells, in immunohistochemical assessments.

We have reported a case involving primary hepatic angiosarcoma that developed after HCC resection; this tumor had slightly elevated intensity on Gd-EOB-DTPA-enhanced MR imaging. The appearance of uptake of Gd-EOB-DTPA in a liver tumor in the hepatobiliary phase may suggest the presence of a sinusoidal tumor spreading to the remaining hepatic cells. Our findings may assist physicians in the diagnosis of future cases of hepatic angiosarcoma.

ARTICLE HIGHLIGHTS

Case characteristics

A 72-year-old man visited our institution due to the onset of abdominal pain.

Clinical diagnosis

Computed tomography (CT) and magnetic resonance (MR) images revealed a liver tumor.

Differential diagnosis

Hepatocellular carcinoma and another type of malignant tumor of the liver.

Laboratory diagnosis

Laboratory tests demonstrated liver enzyme elevation. Alpha-fetoprotein, des-gamma-carboxy prothrombin, carcinoembryonic antigen and carbohydrate antigen were within normal range.

Imaging diagnosis

Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced (Gd-EOB-DTPA -enhanced) MR imaging revealed a liver tumor that showed slight hyperintensity in the hepatobiliary phase.

Pathological diagnosis

Atypical endothelial cells had regularly infiltrated into the sinusoid and replaced sinusoidal cells in a broad range of hepatic parenchyma; as a result, hepatic cell cords remained in the tumor. Pathological findings were consistent with hepatic angiosarcoma.

Treatment

After admission, the patient experienced worsening liver and renal failure. He died from multiple organ failure nine days after admission.

Related reports

Hepatic angiosarcoma has various appearances on CT and MR images, but contrast uptake in primary hepatic angiosarcoma on Gd-EOB-DTPA-enhanced MR imaging in the hepatobiliary phase has not been reported.

Term explanation

There are no non-standard terms used in this manuscript.

Experiences and lessons

The authors present this case to share important knowledge for hepatic angiosarcoma diagnosis.

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ABOUT COVER

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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Glycogenic hepatopathy: A narrative review

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Abstract

Glycogenic hepatopathy (GH) is a rare complication of the poorly controlled diabetes mellitus characterized by the transient liver dysfunction with elevated liver enzymes and associated hepatomegaly caused by the reversible accumulation of excess glycogen in the hepatocytes. It is predominantly seen in patients with longstanding type 1 diabetes mellitus and rarely reported in association with type 2 diabetes mellitus. Although it was first observed in the pediatric population, since then, it has been reported in adolescents and adults with or without ketoacidosis. The association of GH with hyperglycemia in diabetes has not been well established. One of the essential elements in the pathophysiology of development of GH is the wide fluctuation in both glucose and insulin levels. GH and non-alcoholic fatty liver disease (NAFLD) are clinically indistinguishable, and latter is more prevalent in diabetic patients and can progress to advanced liver disease and cirrhosis. Gradient dual-echo MRI can distinguish GH from NAFLD; however, GH can reliably be diagnosed only by liver biopsy. Adequate glycemic control can result in complete remission of clinical, laboratory and histological abnormalities. There has been a recent report of varying degree of liver fibrosis identified in patients with GH. Future studies are required to understand the biochemical defects underlying GH, noninvasive, rapid diagnostic tests for GH, and to assess the consequence of the fibrosis identified as severe fibrosis may progress to cirrhosis. Awareness of this entity in the medical community including specialists is low. Here we briefly reviewed the English literature on pathogenesis involved, recent progress in the evaluation, differential diagnosis, and management.

Key words: Glycogenic hepatopathy; Diabetes mellitus; Hepatomegaly; Mauriac syndrome; Elevated liver enzymes; Liver biopsy; Gradient dual-echo MRI

Core tip: Glycogenic hepatopathy (GH) is considered as a benign reversible condition. Elevation in transaminases is a common finding in patients with diabetes mellitus, and non-alcoholic fatty liver disease (NAFLD) and GH are the two primary underlying pathologies in most cases. It is essential to distinguish NAFLD from GH as this can progress to advanced liver disease. However, recent studies have identified the varying degree of fibrosis in glycogen hepatopathy as well, further emphasizing the need for future studies. We briefly reviewed the literature on mechanisms involved in the development of GH, evaluation of these patients, recent progress made on diagnostic tests and management.

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INTRODUCTION

GH is a rare clinical condition that develops due to excessive accumulation of glycogen in the hepatocytes predominantly seen in pediatric patients and young adults with poorly controlled type 1 diabetes mellitus (T1DM), and rarely observed in a patient with type 2 diabetes mellitus (T2DM)[1-48] (Tables 1-3). GH is characterized by transient liver dysfunction with elevated liver enzymes and associated hepatomegaly caused by a reversible accumulation of excess glycogen in hepatocytes. The collection of glycogen seen on a liver biopsy is critical for the diagnosis. It is an underrecognized entity, and awareness of this clinical condition by clinicians, including gastroenterologists, is low.

GH was initially described in 1930 by Pierre Mauriac in a pediatric patient with poorly controlled T1DM (brittle DM), who presented with hepatomegaly, cushingoid features, and poor growth and development, a condition known as Mauriac Syndrome[30,45]. Since then, more cases have been reported without the full spectrum of Mauriac Syndrome, both in adults and children with T1DM and rarely in T2DM. Various terms have been used to describe this entity, including hepatic glycogenosis, glycogen hepatopathy, glycogen storage hepatomegaly and hepatic glycogen storage[2,4-7,9,14,47]. In 2006, Torbenson and colleagues proposed the term "Glycogenic Hepatopathy" due to hepatocyte glycogen overload but without showing extrahepatic features of Mauriac Syndrome. This term has been used universally since then to describe this unique pathologic feature[44].

The pathophysiology of GH is incompletely understood, and clinical characteristics have not been fully characterized yet. It is believed to be the consequence of recurrent fluctuations in glucose level with hyperglycemia, hypoglycemia, and hyperinsulinization. In addition to glycogen accumulation, steatosis with varying degree of fibrosis may be evident in liver biopsy[38,45]. Intensive insulin regimens with glycemic control provide full resolution of clinical symptoms, laboratory abnormalities, and histological abnormalities.

GH remains underrecognized in adults, as most clinicians mistake it for NAFLD, a more common hepatic abnormality associated with DM. The clinical or radiological distinction between GH and NAFLD is difficult. It is essential that primary care physicians and other specialists be aware of this entity, as differentiating this condition from NAFLD is vital, given its associated comorbidities such as cardiovascular disease, progressive liver fibrosis, and cirrhosis. The focus of this review is to provide an update on recent development in understanding the pathophysiology and the progress in the noninvasive evaluation of differentiating nonalcoholic fatty liver disease from glycogenic hepatopathy.

LITERATURE SEARCH

A literature search was conducted using PubMed using search terms "glycogen" (MeSH Terms) OR "glycogen" (All Fields) AND ["liver diseases" (MeSH Terms) OR "liver" (All Fields AND) "diseases" (All Fields)] OR "liver diseases" (All Fields) OR "hepatopathy" (All Fields)". A total of fifty-one studies involving adolescents or adults were included for this review.

INCIDENCE

Much of the knowledge on GH that has been accumulated over the decades since GH was first reported in 1930, are from case reports, case series, a retrospective cohort study, or more recently, a case-control study[45,46]. It has been estimated that about 8% of American adults have elevated serum aminotransferases[49]. Following a negative initial serologic evaluation, 90% of patients with chronically elevated abnormal aminotransferases are due to NAFLD[50]. The prevalence of liver disease among people with diabetes is estimated to be 17% to 100%[51], with nonalcoholic fatty liver disease and hepatic glycogenosis being the predominant pathologies.

True incidence and prevalence of GH are unknown. Sixty-two percent of the reported cases are female patients, indicating a slight female predominance. Thirty-eight percent of reported cases are male patients, with most cases occurring in adolescence. Based on published reports in English literature, approximately 98% of GH cases were reported in T1DM, while the remaining 2% is caused by T2DM. The incidence of this syndrome has decreased significantly with the introduction of long-acting insulin and better control of

Table 1 Summary of the major case reports in English (pub med indexed) on Glycogenic hepatopathy in type 1 diabetes mellitus

Ref.	Study design	Age/sex	HbA1C	Keto acidosis	Hepatomegaly	AST/ALT/ALP/T bili	Normalization of LFTs
Ruschhaupt *et al*[1], 1970	Case report	13/M	NA	Yes	Yes	9/14/115/128/NA	5 d
Berman *et al*[2], 1973	Case report	21/F	NA	Yes	Yes	UK	Expired (5 d)
Olsson *et al*[3], 1989	Case report	15/F	NA	Yes	Yes	535/417/570/NA	10 d
		20/F	NA	No	Yes	1117/1235/941/NA	18 mo
		24/F	NA	Yes	No	323/276/429/NA	NA
Munns *et al*[4], 2000	Case reports	13/M	14.1	No	Yes	132/135/170/NA	4 wk
		17/F	13.3	No	Yes	102/147/175/NA	2 wk
		16/F	12.2	No	Yes	567/316/196/NA	2 wk
Fridell *et al*[5], 2007	Case report	18/F	NA	Yes	Yes	Elevated/N	After pancreatic transplant
		21/M	NA	No	Yes	Elevated/N	After pancreatic transplant
Cuthbertson *et al*[6], 2007	Case report	19/F	12.2	Yes	Yes	NA/800/132/N	NA
Sayuk *et al*[7], 2007	Case report	19/F	8.1	Yes	Yes	98/49/147/0.5	NA
		37/M	16.0	Yes	Yes	226/399/326/0.9	NA
Bassett *et al*[8], 2008	Case report	10/F	10.1	No	No	143/147/137/N	NA
Abaci *et al*[9], 2008	Case report	16/M	11.1	No	Yes	66/58/431/0.8	3 mo
Hudacko *et al*[10], 2008	Case report	20/F	13.3	No	Yes	249/383/NA/NA	5 mo
Sweetser *et al*[11], 2010	Case report	27/M	15.0	No	Yes	6720/2549/529/1.7	3 mo
Van Den Brand[12], 2009	Case report	29/F	15.3	No	No	2500/1000/316/NA	NA
Saxena *et al*[13], 2010	Case report	33/F	13.7	No	No	428/404/205/N	12 mo
Bua *et al*[14], 2010	Case report	17/F	12.0	No	Yes	138/164/NA/NA	12 mo
Aljabri *et al*[15], 2011	Case report	13/F	13.0	No	Yes	NA/500/137/N	3 mo
Dantuluri *et al*[16], 2012	Case report	14/F	11.0	No	Yes	Elevated	Few days
Lin *et al*[17], 2012	Case report	10/ F	12.8	Yes	Yes	121/194/185/NA	6 mo
Messeri *et al*[18], 2012	Case report	31/F	10.3	No	Yes	225/258/375/0.54	2 mo
Murata *et al*[19], 2012	Case report	21/M	6.2	Yes	No	119/122/NA/NA	25 d
Cha *et al*[20], 2012	Case report	22/F	13.8	No	Yes	1028/365/346/0.5	8 wk
		26/F	12.9	Yes	Yes	914/307/189/0.5	4 wk
		20/F	13.6	No	No	1310/346/132/0.2	16 wk
Saadi T[21], 2012	Case report	18/M	11.0	No	Yes	144/92/123/1.4	3 mo
Saikusa *et al*[22], 2012	Case report	13/M	12.0	No	Yes	100/191/NA/NA	NA
Imtiaz *et al*[23], 2013	Case report	19/F	14.6	Yes	Yes	NA/199/139/NA	5 mo
Butts *et al*[24], 2014	Case report	13/F	8.8	Yes	Yes	NA/113/NA/NA	NA
Jeong *et al*[25], 2014	Case report	13/F	10.7	Yes	Yes	105/84/NA/NA	3 mo
Martin *et al*[26], 2014	Case report	13/M	10.4	Yes	Yes	3969/1196/NA/NA	16 d
Parmar *et al*[27], 2015	Case report	21/F	NA	Yes	Yes	4202/973/N/N	1 wk
Xu *et al*[28], 2015	Case report	22/M	14.6	No	Yes	331/223/223/0.3	NA
Garcia-Suarez *et al*[29], 2015	Case report	28/F	10.5	No	Yes	1600/534/N/N	NA
Atmaca *et al*[30], 2015	Case report	19/M	NA	Yes	Yes	603/570/921/NA	3 wk
Irani *et al*[31], 2015	Case report	19/M	12.0	Yes	Yes	505/345/145/N	NA
Brouwers *et al*[32], 2015	Case report	19/F	9.5	No	Yes	Elevated	NA
		19/F	13.3	No	Yes	Elevated	NA
		17/M	12.7	No	Yes	Elevated	NA
		20/F	14.7	No	Yes	Elevated	NA
Charndrasekaran *et al*[33], 2015	Case report	5/F	16.1	Yes	Yes	53/20/NA/0.7	NA
Silva *et al*[34], 2016	Case report	21/F	9.0	Yes	Yes	110/120/190/0.74	Few days
		20/F	10.1	Yes	Yes	270/423/179/1	Few days
		29/F	10.9	No	Yes	910/461/172/0.35	3 mo
		22/M	15.7	Yes	Yes	226/109/79/0.64	2 wk
Deemer *et al*[35], 2016	Case report	18/F	11.3	Yes	Yes	NA/36/120/N	NA
Saracho *et al*[36], 2016	Case report	14/M	12.8	Yes	Yes	Elevated	NA
Chandel *et al*[37], 2017	Case report	12/F	10.5	Yes	Yes	690/356/158/0.2	3 mo
Ikarashi *et al*[38], 2017	Case report	21/M	11.7	No	Yes	2723/956/NA/NA	1 mo
		24/F	11.0	Yes	Yes	658/216/NA/NA	Few days
		19/F	13.6	Yes	Yes	737/266/NA/NA	Few days
		29/F	16.5	Yes	Yes	469/243/NA/NA	6 mo
Maharaj *et al*[39], 2017	Case report	23/F	12.3	Yes	Yes	127/199/160/2.7	Few days
Al Sarkhy *et al*[40], 2017	Case report	6/M	11.6	Yes	Yes	367/205/221/N	Few days
Shah *et al*[41], 2017	Case report	31/F	NA	Yes	Yes	627/185/280/0.7	NA
Omer[42], 2017	Case report	14/M	11.0	Yes	Yes	Normal	NA

HbA1C: Hemoglobin A1c; AST: Aspartate transferase; ALT: Alanine transferase; ALP: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available.

Table 2 Summary of the major studies in English (pub med indexed) on Glycogenic hepatopathy in type 1 diabetes mellitus

Ref.	Study design	Age/sex	HbA1C	Keto acidosis	Hepatomegaly	Liver abnormality	Normalization of LFTs
Chatila *et al*[43], 1996	Retrospective Study	Mild Fibrosis (14%)	11patients (8-A and 3-P)				
		41/F	NA	NA	No	20/45/1.4 x ULN/0.35	Few days to weeks
		21/F	NA	NA	No	282/404/0.4 x ULN/0.28	Few days to weeks
		58/F	NA	NA	Yes	35/56/3.2 x ULN/0.40	Few days to weeks
		70/F	NA	NA	Yes	76/NA/NA/1.28	Few days to weeks
		36/F	NA	NA	No	40/86/2.1 x ULN/0.28	Few days to weeks
		46/F	NA	NA	Yes	22/20/1.7 x ULN/N	Few days to weeks
		23/F	NA	NA	Yes	265/410/9.5 x ULN/0.40	Few days to weeks
		19/F	NA	NA	Yes	344/532/2.6 x ULN/0.8	Few days to weeks
		13/M	NA	NA	Yes	940/910/2 x ULN/0.30	Few days to weeks
		13/M	NA	NA	Yes	85/NA/0.3 x ULN/0.48	Few days to weeks
		15/M	NA	NA	Yes	NA/NA/1.9 x ULN/0.08	Few days to weeks
Torbenson *et al*[44], 2006	Retrospective Study	14 patients					
		19/F	NA	Yes	Yes	97/83/80/NA	NA
		1/2M	13.5	Yes	Yes	49/47/182/NA	NA
		22/F	NA	No	Yes	48/77/62/NA	NA
		8/M	NA	No	Yes	Elevated/NA	NA
		15/F	NA	No	NA	Normal	NA
		22/M	16	Yes	Yes	1100/360/251/NA	NA
		25/M	10.8	NA	NA	1629/1128/298/NA	NA
		16/M	NA	Yes	NA	Elevated/NA	NA
		20/M	9.9	No	yes	N/120/147/NA	NA
		18/F	10.8	No	NA	N/57/N/NA	NA
		28/M	NA	No	NA	1099/1544/384/NA	NA
		34/M	10.1	No	Yes	N/NA/N/NA	NA
		16/M	NA	No	Yes	1413/1354/476/NA	NA
		23/F	NA	No	Yes	255/224/307/NA	NA
Fitzpatrick *et al*[45], 2014	Retrospective	31 patients	11(Mean)	Yes-all	76/76/NA/NA(M)	NA	14 (73%) Fibrosis
		16M/15F	12Mild and 2Bridging Fibrosis				
		Median age 15					
Mukewar *et al*[46], 2017	Case control study	20 patients	11.4(Mean)	88.90%	301/308/170/0.5(M)	8 mo	10% Mild fibrosis but no bridging fibrosis
			55%				
		16F/4M					
		24-A, 12-P					

T1DM: Type 1 diabetes mellitus; HbA1C: Hemoglobin A1c; AST: Aspartate transferase; ALT: Alanine transferase; ALP: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available; A: Adults; P: Pediatrics.

blood sugar, but unfortunately, it still exists[52].

PATHOGENESIS

The hallmark of the GH is severe fluctuation in levels of glucose and administration of supraphysiologic levels of insulin to control the hyperglycemia. While first described in association with recurrent hyper- and hypoglycemia with ketoacidosis in T1DM, it has since been reported without ketosis or acidosis in patients with T2DM and with variable insulin requirements[3,4,8-16,42]. The excess glycogen accumulation has also been reported in diabetic patients without previous episodes of hypoglycemia[44,53]. Rapid onset of hyperglycemia causing GH was reported in a case by Murata and colleagues in which the initial Hemoglobin A1C was only 6.2%; however, the patient presented with severe hyperglycemia with ketoacidosis and blood glucose of 1495 mg/dL[19].

Physiologically, the liver takes up glucose after feeding and either utilizes it for fuel or stores it as glycogen. Stored mainly in the liver and skeletal muscles, glycogen is a polymer of glucose that acts as a reservoir for glucose. The hepatic glycogen level is maintained by the balance between glycogenosis and glycogenolysis. High glucose levels cause an influx of glucose into the hepatocytes *via* facilitated diffusion through the glucose transporter 2 (GLUT2), independent of insulin[43]. Once the glucose is present within the hepatocytes, the enzyme glucokinase irreversibly phosphorylates glucose to glucose-6-phosphate, trapping it within the hepatocytes (Figure 1). Subsequent treatment of hyperglycemia with the high dose of insulin enhances further conversion of trapped glucose to polymerize into glycogen[43]. Glucose-6-phosphate is converted to glycogen by the enzyme glycogen synthase, which exists in an active dephosphorylated form and in an inactive

Table 3 Summary of the major case reports in English (PubMed indexed) on Glycogenic hepatopathy in type 2 diabetes mellitus

Ref.	Study design	Age/sex	Type of DM/insulin	A1C	Keto acidosis	Hepatomegaly	AST/ALT/ALP/T bili	normalization fibrosis of LFTs
Olson *et al*[3], 1989	Case report	39/M	Type 2/insulin	NA	Yes	No	429/764/1882/NA	21 d
Tsujimoto *et al*[47], 2006	Case report	41/M	Type 2/insulin	10	No	Yes	1064/1024/202/2.3	17 d
Umpaichitra[48], 2016	Case report	15/M	T2DM/metformin	6.5	No	Yes	245/330/N/N	18 mo

T2DM: Type 2 diabetes mellitus; HbA1C: Hemoglobin A1c; AST; Aspartate transferase; ALT: Alanine transferase; ALP: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available.

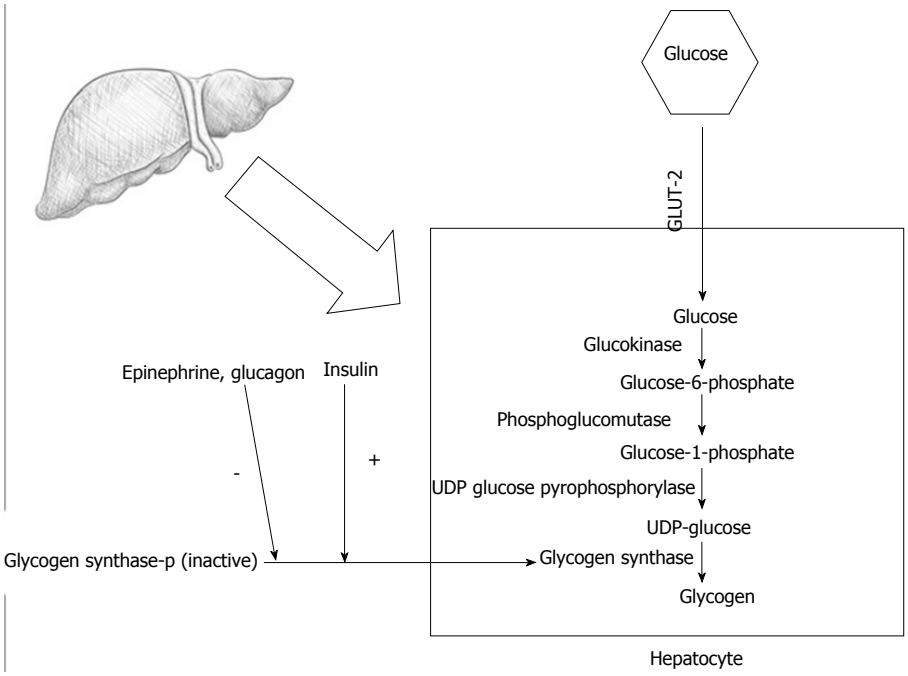

Figure 1 Steps of glycogenesis; effects of hyperglycemia and insulin treatment in glycogenic hepatopathy and formation of glycogen.

phosphorylated form. The active dephosphorylated structure of glycogen synthase is produced by the action of a phosphatase enzyme. The phosphatase enzyme is stimulated by elevated glucose and insulin levels. Glycogen production persists for some time after insulin levels have declined while inhibiting the glycogenolysis[15]. Hepatomegaly can sometimes develop within days to weeks and can improve rapidly once the hyperglycemia is controlled[54].

The reason for excessive accumulation of glycogen in hepatocytes with GH in contrast with the reduced glycogen stores observed in diabetes mellitus (DM) patients is unknown. Even more intriguing is why only a small subset of patients with DM develops GH. GH was first reported after short-acting insulins became available for the treatment of DM. Initially when short-acting insulins were used for the treatment of DM, large doses of insulin were required to control hyperglycemia, causing consequent hypoglycemia. This vicious cycle of excessive doses of short-acting insulin and subsequent administration of glucose to counteract the resulting hypoglycemia could have led to the continued accumulation of glycogen in the liver, a proposed mechanism by various authors[3,19,54]. These findings are infrequently observed today due to widespread use of long-acting insulin and possibly the decreased frequency of hypoglycemic events in this patient cohort.

Another hypothesis is that there is a defect in the genes that code for the proteins which regulate glycogen synthase or glucose 6-phosphatase activity. Although minor abnormalities have been identified in enzymes that control glycogen metabolism, these changes were not considered enough to explain hepatic glycogen storage in GH in the setting of DM[4]. A study by MacDonald and colleagues of an adolescent boy with Mauriac syndrome identified a mutation in PHKG2, is the catalytic subunit of glycogen phosphorylase kinase[55]. Expression of the mutant PHKG2 in a human liver cell line inhibited the enzyme activity of the phosphokinase complex and increased glycogen levels[55]. The mother of the boy with Mauriac syndrome also had the mutant PHKG2 but did not have diabetes or hepatomegaly. These findings do not explain all the cases of GH in adults, as most cases resolved with optimizing the hyperglycemia. Tomihira and colleagues investigated the gene structure of phosphorylase enzyme to define

Table 4 Summary of the published articles in English (PubMed indexed) on Glycogenic hepatopathy without DM

Ref.	Pathology	Study design	Age/sex	Hepatomegaly	AST/ALT/ALP/ T bili	Normalization	Fibrosis of LFTs
Resnick *et al*[56], 2011	Dumping syndrome	Case report	2/M	No	NA/199/NA/NA	16 mo	No fibrosis
Kransdorf *et al*[57], 2015	Anorexia nervosa	Case report	26/F	NA	75/101/108/	NA	No fibrosis
Iancu *et al*[58], 1986	Steroids use	Retrospective	6 mo-14 yr	Yes	Normal	3-5 d	No fibrosis

AST: Aspartate transferase; ALP: Alanine transferase; ALT: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available.

possible mutations; however, they could not determine a probable gene defect that might cause hepatic glycogenosis[53]. Although no direct evidence is available so far, Berman MC hypothesized that the cause of GH is excess glycogen deposits in an intracellular location inaccessible to normal metabolic mechanisms, a phenomenon like that occurring in type Ⅱ glycogenesis in which glycogen deposits within the lysosomes, rendering them unavailable for phosphorylation activity[2]. In patients with a classic presentation of Mauriac syndrome, the wide fluctuations between hyper- and hypoglycemia-a pattern suggestive of under- and over-insulinization with secondary hyperadrenalism-possibly caused the cushingoid features. The pathogenesis of growth retardation in those initial cases reported in children was thought to be multifactorial[52].

The association of ketoacidosis in T1DM and GH not well understood. There could be some mechanisms account for excess deposition of glycogen or failure to mobilize during periods of hypoglycemia. Hormones like adrenaline, cortisol, or growth hormones released due to hypoglycemia, could synergistically act and release large quantities of non-esterified fatty acids from adipose tissue. This high concentration of free fatty acids inhibits glucose oxidation in muscles, and they may have similar effects on liver promoting excess storage of glycogen[2]. Other theory is that extremely low inorganic serum phosphate level in diabetic ketoacidosis may limit recovery from the acute diabetic state by acting as rate limiting factor for hexokinase and phosphokinase reactions. Depletion of intracellular inorganic phosphate for which there is an absolute requirement may be responsible for limitation of phosphorylase activity. Poorly controlled T1DM patients would be expected to have similar recurrent bouts of hypophosphatemia with the consequent entry of large amounts of glucose and inorganic phosphate into the cells[2].

GH in T2DM

The exact mechanism of development of GH in T2DM with insulin resistance is poorly understood and has yet to be clarified. Upon review of previously reported cases of GH, most cases were found in patients who were on an insulin regimen for T1DM, except two instances reported in T2DM[3,47,48] (Table 3). Pathophysiology of GH in T2DM is unclear and could have a different mechanism than that seen in T1DM. Umpaichitra V reported a case of GH in an adolescent male with T2DM whose liver enzymes returned to normal after treatment with metformin[48]. In T2DM, metformin is typically the first-line of treatment. The fact that the patient was in the early stages of diabetes and had not decompensated enough to cause total insulin resistance might explain the preserved effect of insulin on glycogenesis[48]. The mechanism which metformin may have alleviated the glycogenesis, in this case, is also not precise. It was possible that once he was started on metformin, his hepatic gluconeogenesis decreased and glucose uptake in peripheral tissues increased, possibly leading to a paucity of glucose as a substrate for glycogenesis[48].

OTHER DISORDERS ASSOCIATED WITH GH

Excessive hepatic accumulation of glycogen causing GH occurs not only in patients with DM but also in other conditions, including dumping syndrome after gastrectomy, anorexia nervosa, high-dose glucocorticoid use, azathioprine use, and insulin overdose[56-58] (Table 4).

Dumping syndrome after gastric bypass

One of the inherent functions of insulin is to stimulate glycogenesis. Hyperglycemia and hyperinsulinemia are thought to be the etiology for hepatic glycogen deposition in dumping syndrome. Dumping syndrome is a complication of gastric surgery, such as gastric bypass surgery. In patients who had gastric surgery, chyme rapidly "dumped" from the stomach into the small bowel without complete digestion. Resnick *et al*[56] reported a case of a toddler fed *via* gastrostomy tube, who developed GH secondary to Dumping syndrome after Nissen fundoplication. Like GH, it involves fluctuation between hyperglycemia from the rapid nutrition glucose load and hyperinsulinemia. The pathophysiology of Dumping syndrome can resemble that of GH seen in people with diabetes, eventually causing the hepatic accumulation of glycogen.

Anorexia nervos

Anorexia nervos (AN) is characterized by the obsessive fear of gaining weight, distorted body image, and a significantly low body weight. Lisa Kransdorf and colleagues reported a case of anorexia nervosa with glycogen deposition with elevated liver enzymes[57]. It has been reported that 1 in 10 patients with AN will have abnormal liver enzymes. A series of cases of AN with liver failure was reported by Rautou *et al*[59]; about

50% of them presented with hypoglycemia. Again, this lack of glycogen deposits supports the hypothesis that hepatic glycogen accumulation is potentially a protective mechanism. Hepatic glycogenosis is thought to be an adaptive response that protects against potentially fatal hypoglycemia in malnutrition. It is essential to be aware of this association when treating patients with AN.

Short-term high dose steroid therapy

Steroids promote elevation in glucose, gluconeogenesis, and glycogen deposition. Current available evidence regarding GH with steroid use was gathered from pediatric patients. Hepatic glycogen deposition has been described in several experimental models. In a study by Iancu *et al*[58] 141 patients had received steroid therapy: 13% had hepatomegaly, and three patients were noted to have glycogenic hepatopathy. It is unclear whether they had any elevation in liver enzymes. Hepatomegaly resolved soon after discontinuing the steroid therapy in all patients with hepatomegaly. Several mechanisms were involved in the pathogenesis of GH in steroid use. Following hyperglycemia induced by steroids, increased glycogen deposition occurs by the activation of phosphorylase by glucose and the subsequent activation of glycogen synthase. Glycogen synthase is activated by insulin, which increases following steroid-induced hyperglycemia. The knowledge that the hepatomegaly in patients taking steroids could be GH-related will aid in the management of these patients.

Insulin overdose

Excessive insulin levels associated with exogenous insulin administration augments the glycogen deposition. This is evident from a case reported by Tsujimoto *et al*[47] in which a patient with T2DM self-administered a massive dose of long-acting insulin in a suicide attempt. He became hypoglycemic and was later administered a large dose of intravenous glucose to counteract the persistent hypoglycemia. Prior to the suicide attempt, his baseline liver enzymes were initially normal. After the administration of insulin and intravenous glucose the patient developed acute glycogen storage hepatomegaly. His liver enzymes increased significantly to more than 30 times the upper limit of normal.

CLINICOPATHOLOGICAL FEATURES

Clinical manifestations

Clinical presentation of GH varies from asymptomatic patients with elevated liver enzymes to various symptoms associated with hyperglycemia. Patients can present with symptoms of diabetic ketoacidosis (DKA), such as polyuria, polydipsia, marked dehydration, as well as abdominal pain, nausea, or vomiting or may occasionally present with signs of acute hepatitis, jaundice, and pruritus. Pediatric patients may present with extreme hepatomegaly along with growth failure and delayed puberty. The rapid enlargement of the liver causes stretching of the liver capsule, resulting in visceral pain. Although a few reported cases had normal liver sizes on imaging studies, hepatomegaly was observed in more than 90% of the reported cases, with varying degrees of elevation in transaminases and rarely an elevation in alkaline phosphatase (ALP) level. Excessive glycogen with swollen hepatocytes eventually could cause sinusoidal compression and subsequent ascites[34]; therefore, ascites can also be a part of the clinical presentation, albeit rarely[34,44]. Physical examination of patients with GH generally exhibits tender hepatomegaly without splenomegaly.

Biochemical features

Most patients with GH present with hepatocellular abnormality with a predominant elevation in aspartate transaminase (AST) and alanine transaminase (ALT) levels, although a mixed or predominantly cholestatic pattern can rarely occur[3,11,20,30,44]. For patients not experiencing a DKA episode, laboratory studies may show mild elevations in ALT, AST, and ALP. Marked elevations of transaminases and sometimes in the range of more than 100 folds' increase from normal were reported in several reports, mimicking acute hepatitis[26]. However, liver synthetic functions were usually preserved. No histological evidence was identified that suggested the increased enzymes were due to liver necrosis. Elevations in liver enzymes were thought to be due to enzyme leakage from hepatocyte membrane injury, not cell death. However, the exact mechanism is unknown. AST elevations were found to be significantly higher than the ALT elevations. Although ischemic hepatitis is the result of hypotension and hypoperfusion, subjects with inadequate perfusion without the hypotension can also produce a significant elevation in transaminases without liver necrosis[26]. Likewise, patients with DKA will have severe dehydration, which could contribute to these massive elevations in transaminases on top of the GH causing enzyme elevation. In a case-control study by Mukewar *et al*[46] analyzing 36 patients with T1DM, more than half of patients with GH had recurrent episodes of DKA, and these patients had higher levels of HbA1c than patients with TIDM without GH. Patients with GH could have elevated levels of plasma lactate, with or without the presence of a DKA episode, although the mechanism is still poorly understood[35,45]. In a retrospective review by Fitzpatrick of 31 patients with Mauriac syndrome, almost half the patients had elevated lactic acid level despite no signs of DKA[45]. One of the proposed theories is that a reduction in gluconeogenesis in the liver may raise lactate levels in the body. Therefore, lactic acidosis could be secondary to reduced gluconeogenesis and a lack of conversion of pyruvate to glucose[35].

The elevation in amylase and lipase levels less than three times the upper limit of normal without the evidence of acute pancreatitis on imaging has been

seen in some patients with GH[34]. Elevation in these enzymes even in the presence of abdominal pain should not be considered diagnostic of acute pancreatitis, since high levels of amylase and lipase can be seen in patients with DKA without evidence of pancreatitis on CT scan[34]. Hence in patients with GH, elevations of amylase and lipase less than three times the upper limit of normal may be nonspecific yet present. Additionally, antinuclear antibody was positive in some cases, showing a mostly homogeneous speckled pattern. These may be nonspecific as well, as there were no reports of any associated systemic disease[18,26,38,41,48].

DIAGNOSIS

Laboratory evaluation

No single serologic test can diagnose GH. Most patients with GH will have elevated transaminases. It is challenging to diagnose GH solely based on clinical features alone, and liver biopsy is crucial for definitive diagnosis. Typically, the increase in liver enzymes is transient and normalizes in a short period ranging from few days to few weeks[38]. Several laboratory tests may be done initially to rule out other causes of hepatomegaly and chronic hepatitis. Because there is an association between T1DM and autoimmune hepatitis, testing for autoimmune antibodies, such as ANA, anti-smooth muscle antibody and antimitochondrial antibody is essential. The signs and symptoms of Wilson disease often begin during the teenage years and should be ruled out. Glycogen storage diseases (GSDs) are caused by congenital deficiencies in various enzymes and clinically present in the neonatal period or infancy. These patients may already start to present with hypoglycemia and hepatomegaly during infancy[60]. As expressed by Umpaichitra, glucagon stimulation test in children can be tried to rule out GSD[48]. As an up-regulator of glycogenolysis and a down-regulator of glycogenesis, glucagon would facilitate the breakdown of glycogen to allow the release of glucose into the bloodstream. Patients who can demonstrate an increase in serum glucose levels after stimulation by glucagon efficiently rule out a glycogen storage disease[48]. However, the best modality to accurately diagnose a GSD is next-generation genetic sequencing.

Imaging studies

Different imaging studies can be used to support or refute the diagnosis of GH. One of the principal differential diagnoses is NAFLD. In patients with GH, abdominal ultrasound would show hepatomegaly with uniform echogenicity, indicative of glycogen storage, like the fatty change seen in NAFLD. Ultrasound of abdomen is not a useful modality to differentiate NAFLD from GH, as the mildly bright liver can be observed in both. A bright liver compared to the spleen in CT scan imaging can be the clue to diagnosing GH. The liver density on CT scan of the abdomen in patients with GH is increased (hyper dense), compared to a patient with NAFLD in whom it is decreased (hypodense); this subtle difference can give a clue to GH, as reported by Sweetser[11].

Neither CT abdomen nor liver US is a useful test for the definitive diagnosis of GH; however, gradient dual-echo MRI sequence was reported to be able to distinguish fat deposition from an edematous condition such as acute tissue injury; both of which appears as low-density areas on CT. MRI imaging in GH shows low intensities on T2 weighted images. Gradient dual-echo MRI is a powerful tool to distinguish GH from NAFLD. T1 weighted gradient-dual-echo MRI images with in-phase and opposed-phase conditions could efficiently differentiate hepatic glycogen from the fat seen in NAFLD[11,22]. If there is no significant difference in the signal intensities between the two phases then the results are not consistent with intrahepatic fat storage (NAFLD) and are more consistent with GH[19,22] (Figure 2). A few recent reports have shown the presence of varying degrees of fibrosis in patients with GH[45,46]. Presence or absence of liver fibrosis in GH patients can be measured using noninvasive tests such as Fibroscan. Magnetic resonance elastography may have a role in evaluating the degree of fibrosis with GH and needs further studies. Several authors have also demonstrated elevated hepatic glycogen concentration in subjects with glycogen storage disease using 13C MRS (Magnetic Resonance Spectroscopy), which has the advantage of assessing the entire liver while avoiding the risks of liver biopsy[61]. However, these tests may not yet be readily available in most centers.

Liver biopsy and Histological features

A liver biopsy typically shows swollen hepatocytes with an accumulation of glycogen in the cytoplasm. Associated steatosis may be mild to absent in most cases. Although some authors recommended the therapeutic trial of intensive insulin therapy for four weeks preceding any invasive investigations, liver biopsy is the gold standard for diagnosing GH. A hematoxylin and eosin (HE) stain of the biopsy specimen in a patient with GH would show pale and enlarged hepatocytes with prominent plasma membranes, increased cytoplasmic volume, and numerous glycogenated nuclei, which are empty nuclei with ring-like chromatin elements (Figures 3 and 4). Sinusoidal compression by the swollen hepatocytes can produce a paved appearance to the liver parenchyma. The architectural structure of the liver parenchyma likewise remains intact. Furthermore, an addition of diastase to the Periodic-Acid Schiff (PAS) stained specimen would cause enzymatic breakdown of glycogen in the hepatocytes, causing these hepatocytes to turn into "ghost cells"[41] (Figure 4). Histological examination of hepatocytes from patients with GH normally does not exhibit significant portal inflammation, steatosis, or significant fibrosis. In fact, only a minority of cases reported had fibrosis, which was minimal except two cases reported by Fitzpatrick and colleague showed bridging fibrosis[44-46]. In a review

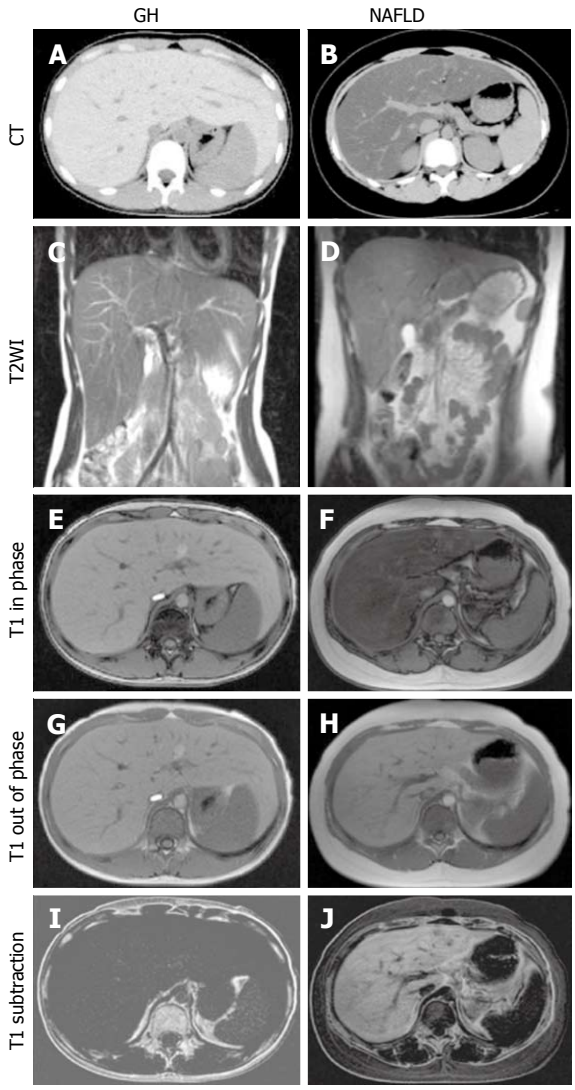

Figure 2 Computed tomography (CT) and magnetic resonance imaging (MRI) of (A, C, E, G, I) glycogenic hepatopathy (GH) in a 13-year-old girl and (B, D, F, H, J) non-alcoholic fatty liver disease (NAFLD) in a 13-year-old boy. On CT, GH was (A) high density, but NAFLD was typically (B) low density; On T2-weighted imaging (T2WI), both enlarged livers were (C) 19.1 cm and (D) 16.8 cm along the right midclavicular line; On gradient dual-echo MRI, the GH liver was iso-intense between the (E) in-phase and (G) out-of-phase images, namely; I: Low intensity on subtraction. The NAFLD liver, however, had low intensity on the (F) in-phase image, and high intensity on the (H) out-of-phase image, namely; J: High intensity on subtraction. With permission from Saikusa *et al*[22], John Wiley and Sons publications.

of cases of GH by Torbenson *et al*[44], mild steatosis was noted in 14% of cases, mild steatohepatitis in 7% of cases, and mild fibrosis in 14% of cases with GH. Histopathology of a case reported by Shah *et al*[41]. also showed GH with focal portal tract fibrosis. Future studies warranted to further assess for development of fibrosis in GH as it can progress to cirrhosis.

DIFFERENTIAL DIAGNOSIS

For patients with DM who present with hepatomegaly and elevated transaminase levels, there are several differential diagnoses apart from glycogenic hepatopathy, including NAFLD. Among T2DM patients, hepatic enlargement with elevated transaminases is usually the result of NAFLD, while GH is the most likely pathology in patients with T1DM[25]. Other potential causes of liver damage in people with diabetes include celiac disease and autoimmune hepatitis. Hepatitis A, Hepatitis B, Hepatitis C, hemochromatosis, and Wilson's disease should all be considered in the list of differential diagnoses, depending on extent and pattern of liver abnormality on presentation (Table 5).

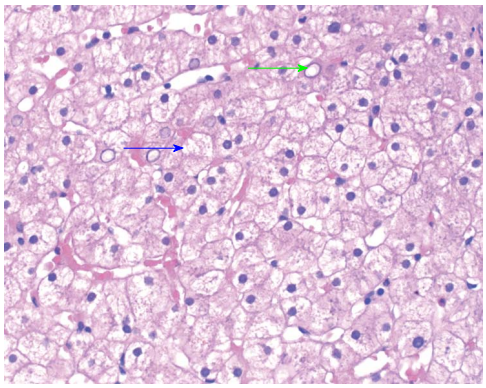

Figure 3 Percutaneous liver biopsy section of a patient with glycogenic hepatopathy. HE stain showing enlarged hepatocytes with cytoplasmic pallor with reddish pink globules consistent with glycogen accumulation (blue arrow), and prominent glycogenated nuclei (green arrow).

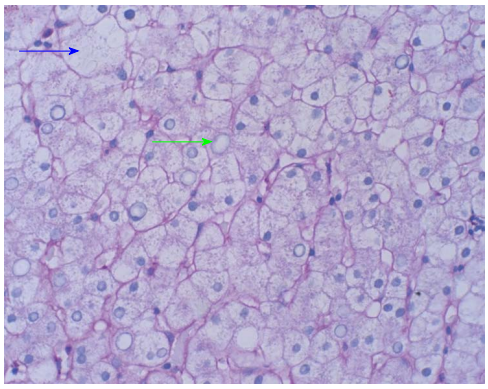

Figure 4 D-Periodic-Acid Schiff stain remove glycogen leaving empty looking cytoplasm (blue arrow) and nuclei (green arrow).

NAFLD

The most common etiology for elevated live enzymes in general population including patients with DM is NAFLD. NAFLD is more typically found in obese adults with T2DM secondary to insulin resistance, while GH is more common in patients with lower body mass index and pediatric patients. Upon assessment of laboratory studies from a patient with NAFLD, steatohepatitis would show mild elevations in AST, ALT, and ALP, just like in most cases of GH. CT scan imaging of a patient with NAFLD would show hepatomegaly with decreased liver density, while the gradient-dual-echo MRI would show low intensity in phase and high

Table 5 Summery of the main differential diagnosis for glycogenic hepatopathy

	Main etiology	Clinical presentation	Imaging characteristics	Key, liver biopsy pathological features	Diagnosis	Management	Cirrhosis
GH	Acquired excessive glycogen deposition in the liver mostly seen in patients with T1DM	Hyperglycemia with hyperglycemic symptoms; could be asymptomatic. Liver enzyme elevation is mild to extreme range in some case	US and CT shows increased echogenicity. Dual-Echo MRI; iso-intense between the in-phase and out-of-phase images, and low intensity on subtraction	Glycogen deposition in the cytoplasm with swollen hepatocytes, with or without mild steatosis and fibrosis. Diastase digestion of glycogen cause hepatocytes to turn into "ghost cells"	Radiologic and liver biopsy	Optimal control of DM	May have mild fibrosis, severe fibrosis is very rare and seen in only a few reported cases
GSD	Inborn errors of glucose and glycogen metabolism results in abnormal deposition of glycogen	Presentation varies depend on types of GSDs They will have manifestations of a liver, kidney, and skeletal muscle involvement with hypoglycemia, hepatomegaly, muscle cramps, and weakness, *etc*	Like GH	Findings vary in different type of GSDs; Nonspecific histologic findings to PAS positive glycogen deposition which could be diastase sensitive or resistant	Biochemical tests and molecular testing	Symptomatic treatment to dietary changes to maintain the blood glucose level and pharmacologic therapy in different types of GSDs. May need liver transplantation in selected cases	Some GSD can progress to cirrhosis
NAFLD	Hepatic steatosis	Most patients asymptomatic and some may have minor symptoms, Liver enzymes elevation usually < 5 times upper limit of normal	US and CT shows increased echogenicity. Dual-Echo MRI; low intensity on the in-phase image, and high intensity on the out-of-phase image and high intensity on subtraction.	Steatosis with or without lobular inflammation and hepatocyte ballooning. May have varying degrees of fibrosis	Radiologic and liver biopsy	Lifestyle modification and pharmacologic therapies. May need liver transplant in advanced cirrhosis	Can progress to cirrhosis
Hepatosclerosis	Is a hepatic manifestation of microangiopathic disease seen in long -standing DM	Often clinically silent, Serum aminotransferases are normal or minimally elevated. ALP, Total bilirubin may be elevated	No specific imaging characteristics	Extensive dense perisinusoidal fibrosis	Liver biopsy	Unknown	Unknown
AIH	Chronic Hepatitis of unknown etiology	Spectrum of clinical manifestations ranges from asymptomatic patients to those with considerable symptoms, and rarely presents with acute liver failure	No characteristic imaging features, may show cirrhotic liver in advance case	Interface hepatitis and portal lymphoplasmacytic infiltrate with varying degree of fibrosis	Characteristic biochemical tests and liver biopsy	Glucocorticoid monotherapy or in combination with immunomodulators. Rarely may require liver transplantation	Can progress to cirrhosis
Hemochromatosis	Autosomal recessive disorder. Mutations cause increased iron absorption and excessive deposition in the liver, heart, pancreas, and pituitary	Asymptomatic or chronic liver disease with elevated transaminases, skin pigmentation, DM, arthropathy, impotence and cardiac enlargement, *etc*	MRI is most sensitive and can estimate iron concentration in the liver. Dual-Echo MRI; demonstrates decreased signal intensity in the affected tissues on the in-phase images compared with the out of- phase images (opposite of steatosis)	A liver biopsy will reveal iron overload. Presence of cirrhosis can be determined	Biochemical tests including genetic testing, radiologic, and liver biopsy	Phlebotomy or Chelation therapy if unable to tolerate phlebotomy	Can progress to cirrhosis

Wilson disease	Autosomal recessive disorder with impaired cellular copper transport and impaired biliary copper excretion results in accumulation of copper most notably the liver, brain, and cornea.	Predominantly hepatic, neurologic, and psychiatric manifestations. Elevated transaminases mild to moderate and ALP may be markedly subnormal	US, CT, may show signs of cirrhosis and normal caudate lobe which is contrary to other types cirrhosis	Vary largely from fatty changes to cirrhosis and occasionally fulminant hepatic necrosis. Can be stained for copper	Biochemical tests and slit lamp examination with or without genetic testing and liver biopsy	Treatment with a chelating agent. Some cases may require liver transplantation	Can progress to cirrhosis
Acute viral hepatitis A, B, C, D and E. Rarely, HSV, VZ, EBV and CMV	Hepatitis A and E are transmitted by feco- oral route; Rest of the viruses spread either by sexual contact, contact with body fluids or blood or from birth from an infected mother	Many of the symptoms are nonspecific; May have marked elevation in transaminases often > 15 times the normal	US or CT findings are nonspecific; Could be used to rule out other causes	Liver biopsy shows hepatocyte necrosis with a portal, periportal and lobular lymphocytic infiltration; Plasma cells present during resolving phase	Diagnosis by biochemical tests	Treatment conservative or antiviral therapy	Acute infection may progress to chronic, and that may progress to cirrhosis

PAS: Periodic-acid Schiff; T1DM: Type 1 diabetes mellitus; US: Ultrasound; CT: Computed tomography; GH: Glycogenic hepatopathy; GSD: Glycogen storage disease; NAFLD: Non-alcoholic fatty liver disease; AIH: Auto immune hepatitis; DM: Diabetes mellitus; ALP: Alkaline phosphatase; HSV: Herpes simplex virus; VZ: Varicella zoster virus; CMV; Cytomegalovirus.

intensity out of phase[22]. Histological examination of a liver biopsy specimen from a patient with NAFLD could likewise show macro vesicular steatosis, mild lobular and portal inflammation, and varying degrees of fibrosis. Evidence of hepatocellular injury and fibrosis would indicate increased severity and progression to actual steatohepatitis. NAFLD is likewise more likely to progress to fibrosis, cirrhosis and or hepatocellular carcinoma. Because of the potential for progressive liver disease and liver failure in NAFLD, it is crucial to distinguish NAFLD from GH, generally considered a more benign and reversible condition.

Hepatosclerosis

Hepatosclerosis is an underrecognized form of hepatic diabetic microangiopathy that needs to be differentiated from GH. It occurs in patients with T1DM more often than in T2DM and is associated with severe microvascular disease in other organs. Hepatosclerosis is characterized by an indolent course and is seen predominantly in female diabetic patients, although actual prevalence of this remains unknown. Generally, ALP is elevated with or without an elevation in total bilirubin, and serum aminotransferase levels may be normal or minimally elevated[62]. Liver biopsy may show extensive, dense, perisinusoidal fibrosis, and immunostaining reveals basement membrane components in a perisinusoidally distribution[62]. It is unclear whether hepatosclerosis plays any role in pathogenesis of NAFLD or cirrhosis in patients with DM. Long-term follow-up and future studies are required to examine the natural history and to explore treatment options for this form of microvascular complication involving the liver.

GSD

GSD is a group of inherited disorders in which excess glycogen accumulates in different tissues including liver, skeletal muscles, or both due to the deficiency of enzymes that regulate glycogenolysis or gluconeogenesis. Glycogen accumulation is not only caused by mutations in enzymes directly involved with glycogen catabolism or glucose metabolism, but it can also be caused by mutations in proteins that have an indirect impact on glycogen metabolism. It is vital to differentiate GH and GSDs with genetic testing, as management would vary vastly between the two pathologies. The hepatocytes in both conditions are markedly swollen and filled with glycogen, while the subtle difference can be the presence of higher cytoplasmic clumping of glycogen in GSD[44]. A clinical parameter such as the response to diabetic control in patients with poorly controlled DM is an important component to distinguish GH from GSD[44]. Management for GSDs revolves around management of complex carbohydrate intake and surveillance for complications, whereas management for GH centers on proper glycemic control with insulin and prevention of hyperglycemic episodes.

Drug induced liver injury and GH

Should a diabetic patient be on additional medications and the course of management be complicated by continued elevations in transaminases despite fluid resuscitation and treatment with insulin, it would be wise to drug induced liver injury (DILI) along with associated GH. Maharaj *et al*[39] published the case of a patient who initially presented with DKA, hepatomegaly, and elevated transaminases but was later diagnosed

with GH accompanied by DILI. He had a past medical history of T1DM and bipolar disorder that was being treated with the antipsychotic drugs paliperidone and asenapine. The author recommended DILI as a possibility whenever GH is suspected with concomitant use of hepatotoxic medications.

MANAGEMENT, PROGNOSIS AND FOLLOW UP

Once the diagnosis is made, improved glycemic control is the mainstay of management. Although there has not yet been any research in support of any pharmacological treatment strictly targeting or preventing glycogen deposits in the liver, it is regarded that resolution of the hyperglycemia would cause a decrease in transaminases level back to normal. GH can resolve with both clinical and biochemical resolution as quickly as it develops within days to weeks with good glycemic control[40]. Due to the autoimmune destruction of beta-islet cells in the pancreas, type 1 diabetics are unable to secrete endogenous insulin inherently. As such, exogenous insulin therapy is necessary to maintain their serum glucose levels. The mainstay of treatment for GH is strict control of glucose levels, close supervision of Hemoglobin A1C and prevention of recurrence of episodes of DKA. Prognosis with improved glycemic control is excellent. Reversal of GH has also been reported following pancreatic transplantation in people with diabetes, further consolidating the fact that it is entirely reversible by treating the diabetes[18].

Pathology results of majority of the cases reported in the literature did not show any significant fibrosis of the liver; however, further long-term studies are required to assess for the consequence of the mild fibrosis identified in some reports, and few cases of bridging fibrosis as severe fibrosis may further progress to cirrhosis. Despite showing a benign clinical course with strict glycemic control, GH could recur and relapse with uncontrolled glycemic levels[38]. Therefore, patients with a history of GH may still need to be followed up for any relapse of symptoms if persistent control of hyperglycemia is not maintained.

CONCLUSION

In conclusion, GH should be one of the differential diagnoses upon examination of a patient with uncontrolled DM presenting with elevated transaminases and hepatomegaly. GH should not be overlooked as a differential diagnosis solely due to its underrepresentation in diabetic patients. Compared to other liver diseases associated with DM, GH is a favorable diagnosis due to its benign nature and good prognosis.

Future progress is required in understanding the biochemical defects underlying GH and development of fibrosis. Further research is needed for an ideal noninvasive, rapid diagnostic test to avoid the extensive workup and associated costs in evaluating suspected cases of GH. For now, a more aggressive pursuit of liver biopsy in the evaluation of elevated transaminases could identify additional cases of GH, allowing for continued elucidation of prevalence and natural history of this entity. Clinicians should also continue to pool patient data from case studies of patients with GH, to better understand the underlying risk factors and characteristics of this disease.

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REVIEW

Hepatitis C virus: Morphogenesis, infection and therapy

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Abstract

Hepatitis C virus (HCV) is a major cause of liver diseases including liver cirrhosis and hepatocellular carcinoma. Approximately 3% of the world population is infected with HCV. Thus, HCV infection is considered a public healthy challenge. It is worth mentioning, that the HCV prevalence is dependent on the countries with infection rates around 20% in high endemic countries. The review summarizes recent data on HCV molecular biology, the physiopathology of infection (immune-mediated liver damage, liver fibrosis and lipid metabolism), virus diagnostic and treatment. In addition, currently available *in vitro*, *ex vivo* and animal models to study the virus life cycle, virus pathogenesis and therapy are described. Understanding of both host and viral factors may in the future lead to creation of new approaches in generation of an efficient therapeutic vaccine.

Key words: Hepatitis C virus; Transmission; Molecular biology; Pathogenesis; *In vitro* and *ex vivo* models of hepatitis C virus infection; Treatment

Core tip: Brief overviews on epidemiology of hepatitis C virus (HCV), virus morphology and the virus life cycle are presented. A special attention was focused on *in vitro* and *in vivo* models that are currently used to study the HCV infection. In fact, extensive use of existing models and creating a new ones is a way to reveal important events in the virus-cell interaction. In particular, the models might shed light on the mechanisms behind virus induced pathogenesis and chronicity, and by that contribute to the development of new drugs and prophylactic vaccine. Recently, multiple therapies with a pan-genotypic activity appeared on the market. The new agents (third generation) and new inhibitors (entry inhibitors, release inhibitors) being studied, should allow to cure most of the patients in the mid-term, if they will have equal access to the therapy.

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INTRODUCTION

The hepatitis C virus (HCV) is a major blood borne human pathogen. There are approximately 120-130 million or 3% of the total world population that are HCV infected (Figure 1). According to World Health Organization (WHO), annually there are about 3-4 million new cases of infection[1,2]. HCV is considered a major public health issue, since the virus is the etiological factor of chronic hepatitis that frequently progress to a cirrhosis and hepatocellular carcinoma (HCC). In developed countries, the most important route of HCV transmission is intravenous drug abuse, whereas in resource-poor countries invasive procedures or injection-based therapies with contaminated instruments are the predominant source of new infections[3].

Without treatment, most of the acute infections progress to chronic ones, followed by liver disease, such as cirrhosis and HCC. Alcohol abuse and the metabolic syndrome are the main cofactors influencing the progression to advanced liver disease and HCC[4]. Each year, about a third of liver transplantations are performed on patients with complications associated with the HCV infection, with decompensated cirrhosis or HCC[5]. In the next decade, an increase in the burden of hepatitis C is expected because of aging of the currently infected population[6,7]. During that period, the number of HCV-related cirrhosis cases is estimated to increase by 31% and HCC by approximately 50%[6] with an additive effect due to the occurrence of the metabolic syndrome[8,9]. Thus, HCV infection represents a major public health issue that should be addressed with strong policy interventions to effectively identify and treat HCV infected patients.

There are seven genotypes (gt 1-7) and numerous subtypes of HCV. The rate of infection and subtype prevalence are country depend. The difference between the infection rate in low and high endemic countries is about 20% (Figure 1)[10,11]. For example, North America, Western Europe and North and Australia's have the lowest HCV prevalence. Conversely, in Asian and African countries the virus prevalence is high. The highest virus prevalence was registered in Egypt, where 22% of population is infected[12]. It has been postulated that the epidemic has been caused by extensive iatrogenic transmission during the era of parenteral-antischistosomal-therapy mass-treatment campaigns before 1985[13].

MOLECULAR BIOLOGY OF HCV

HCV morphology and parameters

HCV is a small, enveloped, positive single-stranded RNA virus that belongs to the *Flaviviridae* family, genus *Hepacivirus*. Analysis of viruses from plasma and from cell culture supernatant indicated that enveloped particles are icosahedral and 56-65 nm[14,15] in diameter, while and the viral core is about 45 nm[14,15]. Viral spikes on the membrane of the virion are about 6 nm[14] and they are formed by heterodimers of E1 and E2 glycoproteins. In fact, the population of the extracellular HCV particles is heterogeneous. Particles are pleomorphic and size, buoyant density, and infectivity might differ significantly[14,15]. A large majority of particles is non-infectious. Interestingly, the buoyant densities of infectious particles isolated from serum and from cell culture medium are different. A significant amount of the particles are associated with cellular lipoproteins making that a hallmark mark of HCV[16,17]. The pattern of virus-associated lipoproteins might differ and several of those are associated with HCV most frequently: low density lipoproteins (LDL), very low-density lipoproteins (VLDL) and apolipoproteins (Apo) A1, B, C and E (Figure 2)[18]. The viral particles associated with lipoproteins are called "lipoviral particles (LVP)". More details on LVP are given in "Virus assembly and release" section. Detailed update on HCV-associated lipoproteins is given in the review by Grassi *et al*[19].

Viral genome

The genome of HCV is approximately 9600 nucleotides long (Figure 3A). It contains two highly conserved untranslated regions (UTR) 5'-UTR and 3'-UTR that are flanking a single open reading frame (ORF). Dependent on the genotype, the ORF might contain from 9030 to 9099 nucleotides and it is coding for a single polyprotein precursor of 3010 to 3033 amino acids (aa), respectively (Figure 3B and C)[20,21]. Translation occurs in the endoplasmic reticulum and it is initiated by IRES at the 5' UTR[22].

5'-untranslated region

The 5'-UTR is highly conserved including special site to control the HCV genome replication and the viral polyprotein translation. The region is 341 nucleotides long and it contains four distinct domains (Ⅰ-Ⅳ). The first 125 nucleotides of 5'UTR spanning the domains Ⅰ and Ⅱ have been shown to be essential for the viral RNA replication. The domains Ⅲ-Ⅳ composes an internal ribosomal entry site (IRES) involved in ribosome binding which can initiate viral polyprotein translation in a cap-independent manner[20]. The initiation of HCV protein synthesis requires the ordered assembly of ribosomal pre-initiation complexes, beginning with the association of the small (40S) ribosomal subunit with a messenger RNA (mRNA). The cap-independent translation begins with the 40S ribosome binding and the scanning to the initiation codon which is followed by association with the 60S ribosomal subunit to form an active 80S ribosome[21,22]. The HCV IRES is folded into highly structured domains which are called Ⅱ to Ⅳ. The mutational analysis of the IRES domains suggested that a structural integrity was necessary for an efficient protein synthesis both *in vitro* and *in vivo*[23-25].

Table 1 Overview of the size of hepatitis C virus proteins

Protein	No. of aa	aa position n ref. seq.	MW of protein, kDa
Core immature	191	1-191	23
Core mature	174	1-174	21
F protein of ARF protein	126-161		Approximately 16-17
E1	192	192-383	35
E2	363	384-746	70
p7	63	747-809	7
NS2	217	810-1026	21
NS3	631	1027-1657	70
NS4A	54	1658-1711	4
NS4B	261	1712-1972	27
NS5A	448	1973-2420	56
NS5B	591	2421-3011	66

No. of aa: Number of amino acid; MW: Molecular weight; kDa: KiloDalton; ref.seq.: Reference sequence (HCV strain H77 ; accession number NC_0041).

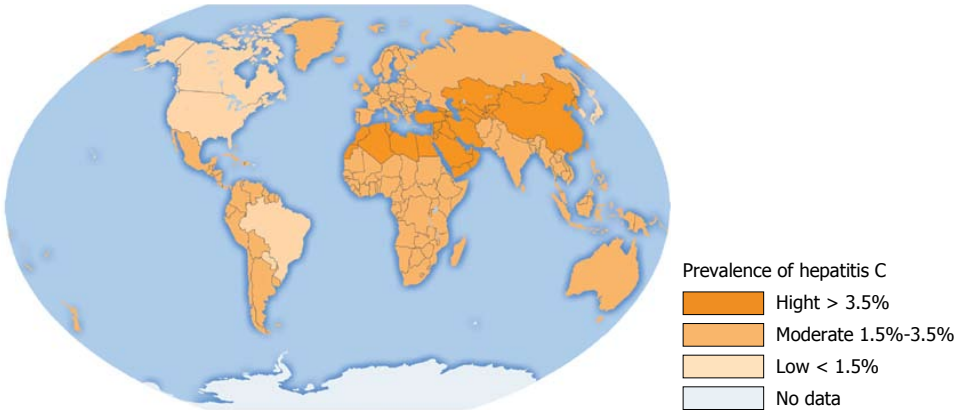

Figure 1 Hepatitis C virus infection in the World. Analysis of seroprevalence[2].

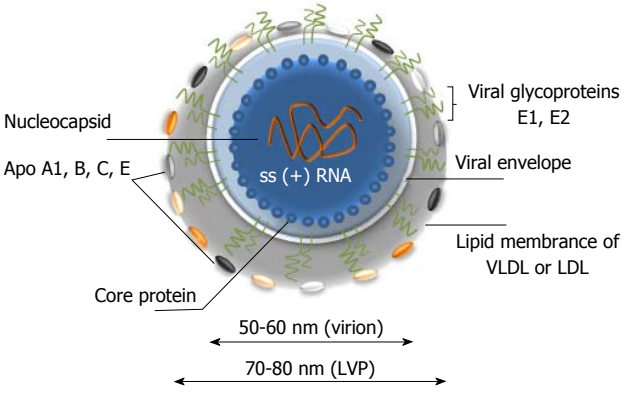

Figure 2 A model of hepatitis C virus lipoviral particle. Lipid membrane formed by low density lipoproteins (LDL) and very low-density lipoproteins (VLDL) on the surface of the virion (given in grey). Viral core is given in blue and viral RNA is shown in orange. Heterodimers of glycoproteins E1 and E2 are partially embedded in the lipid bilayer and are forming 6 nm long spikes (projections) on the surface of the virion[14-19]. As a result of association with LDL and VLDL, the morphology of the virion is not icosahedral. Depending on the viral source, the shape and size of the particles might vary.

3'-untranslated region

The 400 nucleotides long 3'-UTR region is thought to playing a crucial role in the HCV replication. The region is highly conserved and it is divided into three functionally parts: A variable sequence of 40 nucleotides, a variable internal poly (U/UC) rich tract of 30 to 80 nucleotides (depending on the HCV strains), which is followed by a highly conserved 98-nucleotide X-tail containing three stable stem loop (SL) structures called: 3'SL1, 3'SL2, 3'SL3[25].

Viral proteins

The translation occurs in the endoplasmic reticulum and it is initiated by IRES at the 5'UTR[25]. A single polyprotein precursor is processed by cellular and viral proteases into ten proteins (Figure 3B). Three structural proteins (core, E1, E2) are located at the amino-terminal part of the polyprotein and are essential components of the virions. Seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) are located in the remaining part of the polyprotein and these proteins are involved in particle morphogenesis, RNA replication and in regulation of cell functions (Table 1). It is worth mentioning, that the structural and non-structural proteins of HCV are multifunctional. A brief characterization of the protein is given below.

Core protein (p22)

The core protein of HCV is translated as an immature protein of 22 kDa, that it is composed of 191 amino acids (aa). It is excised from the polyprotein in the

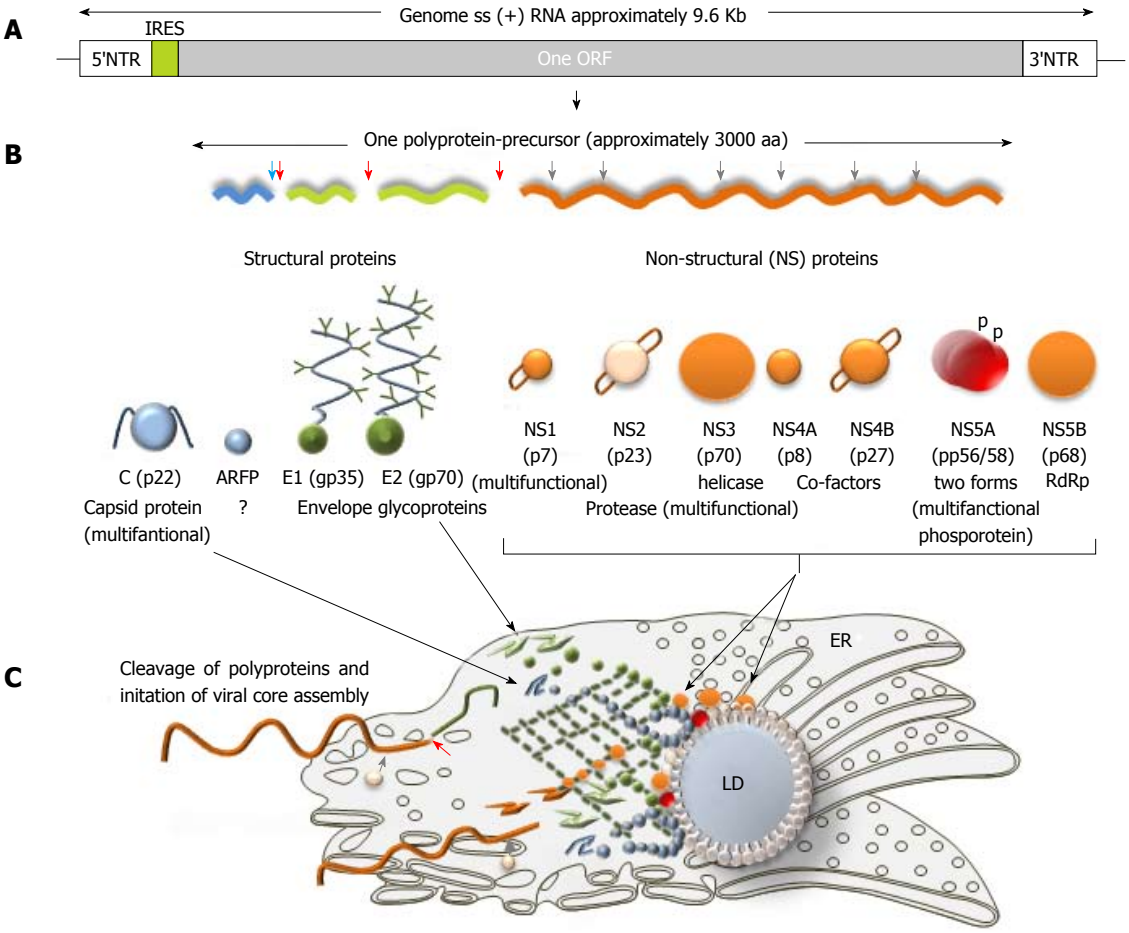

Figure 3 Hepatitis C virus genome, polyprotein precursor and the initial steps of core assembly in endoplasmic reticulum. A: Being structurally identical, the genomes of seven hepatitis C virus (HCV) genotypes demonstrated approximately 30% of sequence diversity[140]. Two non-translated regions (5'- and 3'-NTR) are flanking a single open reading frame (ORF) shown in grey; B: Polyprotein precursor composed of about 3000 amino acids translated from a single ORF. Three structural proteins - a core protein (shown in blue) and two envelope proteins (shown in green) and seven non-structural (NS) proteins NS1, NS3, NS4A, NS4B, NS5A, and NS5B (shown in orange) that are involved in cleavage, assembly, transcription and some other functions are shown. Alternative reading frame protein (ARFP) that overlaps with the core protein sequence is given in blue. Serine protease NS2 is given in pale yellow. Transmembrane fragments in non-structural proteins are shown as staples; p- phosphoprotein; C: A model of initial steps of the virion core assembly in the endoplasmic reticulum (membranous web is given in dark green) on lipid droplet (LD). Polyprotein precursor is cleaved by cellular C-terminal signal peptidase (red arrow) and cellular signal peptidase (blue arrow) to release capsid protein. NS3-NS4A serine protease cleaves the remaining proteins, while NS2-NS3 (grey arrow) protease cleaves itself. Pre-assembled cores are transported to the LD, where the final steps of assembly take place[183].

endoplasmic reticulum (ER) by a cellular signal peptidase (SP)[26]. An additional cleavage of the immature protein results in the mature 21 kDa core protein. The core protein contains three domains. The first domain spans the N-terminal region of 117 aa (aa 1-117). It contains mostly basic residues and there are two short hydrophobic regions. This domain is involved in binding to the viral RNA. The second domain between the aa 118 and 174 is more hydrophobic and less basic. This domain is engaged in the creation of links with the lipid droplet (LD). LDs are intracellular structures that are used for the lipid storage (Figure 3C)[18] . The third domain is localized between the aa 175 and aa 191 and it contains the signal sequence for the ER membrane translocation of E1 ectodomain[27]. *In vivo*, the mature core proteins are believed to form homo-multimers that are accumulated mainly at the ER membrane and can be self-assembly into the HCV-like particles[28]. During the viral capsid assembly, the protein also interacts with the HCV RNA. Besides that, the core protein poses regulatory functions during the RNA translation. Analyses of HCV core protein expression indicate that additionally it may be involved in several processes in cells such as apoptosis, lipid metabolism [HCV-related (mainly gt 3) fatty liver] and the development of HCC[29]. Interestingly, the core protein can induce the redistribution of LDs by the regeneration and the regression of these organelles in specific intracellular domains[30]. This phenomenon is also linked to the assembly of HCV particles. LDs are associated with the HCV core protein at the second domain and the interaction seems to trigger steatosis[30]. It has been shown, that if the core protein is mutated, the association with LDs might be disrupted. A decrease of this association has a negative effect on the HCVcc production. The HCV core protein was also shown to be involved in the Ca^{2+} regulation[31]. Thus, it is not only a

basic structural element of HCV, but it is an active player in a significant amount of additional processes, including signaling pathways regulation[17,30,31].

Envelope proteins E1 (gp35) and E2 (gp70)

Glycoproteins E1 (gp35 kDa, 192 aa long) and E2 (gp70 kDa, 363 aa long) are type 1 transmembrane proteins that are forming the envelope of the virus particle (Figures 2 and 3B). E1 an E2 are cleaved from the precursor in ER by the cellular SP. The glycoproteins contained large hydrophilic ectodomains and 30 aa long transmembrane domains (TMD). The TMD is responsible for an anchoring of the envelope proteins in the membrane of the ER and their ER retention[32]. The ectodomains of both glycoproteins are heavily glycosylated. The E1 has 4-5 and the E2 - 11 putative N-glycosylation sites, respectively. Interestingly, the glycosylation sites are rather conserved in each genotype[33], but the number of glycosylation sites varies between the genotypes. It's worth mentioning, that some of the glycan's are engaged in folding and formation of the E1-E2 heterodimer complexes on the surface of the virion. These complexes are essential for the interaction with cellular receptors and helps to promote the virus-to-cell fusion[34].

The HCV E2 contains two hypervariable regions HVR 1 and HVR2 which are the most mutable parts of the HCV genome. The first 27 amino acids of E2 are attributed to the HVR1 sequence. It is suggested that a prominent heterogeneity of the region could help the virus to evade from the immune system pressure and to develop a chronic infection[35]. It is worth mentioning, that an infectious clone lacking the HVR1 was still capable to infect chimpanzee, but with a largely reduced efficiency, thus supporting the HVR1 engagement in the cell infection[36]. The HVR2 includes seven aa (91-97) and this region was shown to contribute to the HCV E2 receptor binding[37]. The high variability of the HVRs reflects the exposure of these domains to HCV-specific antibodies. In fact, the E2-HVR1 is the most frequent target for neutralizing antibodies[38,39]. However, the combination of a viral mutation with the selective pressure of the humoral immune response leads to the viral escape *via* epitope alterations[40]. Moreover, the association of virions with lipoproteins and the presence of a glycan shield (possessed by the E1-E2 heterodimers), reduce the antibody access to the "sensitive" neutralizing epitopes[24,41,42]. This protection is representing a serious obstacle on the way to obtain broadly neutralizing antibodies.

Non-structural protein NS1 (p7)

The NS1 (also called "p7") is a small 63 aa long protein. It is located between E2 and NS2 proteins and it is linked to both structural and non-structural proteins. Its cleavage is mediated by the cellular SP. Interestingly, the E2-p7-NS2 precursor can also be detected[43,44]. It is suggested, that such a precursor may regulate the kinetic of the HCV infection; however, up to now the precise role of this protein in the HCV life cycle remains obscure. NS1 has two transmembrane domains (TMDS) embedded in the ER membrane and the C-terminal TMD of NS1 can act as a signal sequence to promote the translocation of NS2 to the ER lumen[45]. The protein can also form the ion channel which has an important role in the HCV infection. This channel can be blocked by the antiviral drug[45]. The recent studies demonstrate that NS1 acts to prevent the acidification which is required for the production of HCV particles[46]. In addition, the protein appears to be essential during assembly and release of infectious particles as shown on different genotypes[47]. Based on that, NS1 may be considered as a potential target for new antivirals[48,49].

Non-structural protein NS2 (p23)

The NS2 is a hydrophobic transmembrane protein of 23 kDa composed of 217 aa. It is a cysteine protease which is required for the HCV infectivity[50]. The N-terminal residues of NS2 can form 3 or 4 transmembrane helices which are inserted in the ER membrane. The crystal structure has been determined. The C-terminal residues of NS2 with the 181 aa long N-terminal domain of NS3 play an important role in the function of the NS2/3 cysteine protease[51]. The NS2/3 protease is spanning from the amino acid 810 to amino acid 1206. It has been shown that the NS3 zinc-binding domain could stimulate the activity of the NS2 protease[52]. The well-known function of NS2 is the auto-cleavage at the NS2/3 site[53]. Oem *et al*[53] suggested that the expression of HCV NS2 results in the up-regulation of the fatty acid synthase transcription. In fact, this may implicate the role of NS2 in the prompting HCV induced steatosis[54]. There data are in favor of the protein engagement in the viral assembly and release[54].

Non-structural protein NS3 (p70)

The NS3 is a 70 kDa protein composed of 631 aa. It is cleaved at its N-terminus by the viral NS2/NS3 autoprotease. The C-terminal part of NS3 (442 aa) has an ATPase/helicase activity, and it catalyses the binding and unwinding of the viral RNA genome during the viral replication[55,56]. The N-terminal part (189 aa) of the NS3 protein has both serine protease and NTPase activities[56]. The NS3 along with the non-structural protein NS4 is involved in cleavage of the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions[57,58]. A direct interaction between NS3 and NS5B is mediated through the protease domain of NS3. It is suggested, that these proteins may act together during the HCV replication[59]. The HCV protease NS3/4A can also cleave some cellular targets involved in the innate immunity, such as MAVS (an antiviral signaling protein), which can indirectly activate the nuclear factor-kappa B (NF-κB) and the IFN regulatory factor 3 to induce type Ⅰ interferon[60,61]. The protein NS3/NS4A can interfere with the adaptive immunity. In addition, the NS3/NS4A protease is

essential for the viral infectivity, thus, it is a promising target for antivirals[61-64]. In 2011, two potent NS3/NS4A inhibitors, boceprevir[61] and telaprevir[62] were approved by FDA and EMA and were used in combination with IFN α and ribavirin. However, several resistance-associated mutations within the NS3/NS4A coding region have been observed.

Non-structural protein NS4A (p8)

The NS4A is an 8 kDa proteins that is composed of 54 aa. As indicated above, it is a co-factor required for the NS3 protease activity. The N-amino-terminal domain of NS4A is hydrophobic and the deletion analysis shows that NS4A was required for the ER targeting of NS3[65-68]. In addition the NS4A interaction with NS5A is essential for the phosphorylation of NS5A[68]. Besides its important role in HCV replication, NS4A can also contribute to the viral pathogenesis by influencing some cellular function[66]. Interestingly, NS4A was detected not only on the ER, but also on the mitochondria either alone or together with NS3 in the form of NS3/4A polyprotein. It has been shown, that the NS4 expression altered the distribution of mitochondria and caused damage which leads to the host cell apoptosis[66].

Non-structural protein NS4B (p27)

The NS4B is a 27 kDa protein of 217 aa. It has four membrane spanning domains which are important in recruitment of the other non-structural viral proteins[68]. By electron microscopy, it has been shown, that NS4B induced a tight structure termed "membranous web". All viral proteins were found to be associated with this membranous web that formed a viral replication complex ("factory") in HCV infected cells[67]. The polymerization activity and lipid modification of NS4B are important for the induction of the specialized membrane structure involved in the viral RNA replication[68,69]. NS4B is likely not engaged in the virus replication, but may contribute to the assembly and the release of the virus particles[69].

Non-structural protein NS5A (p56/p58)

The NS5A is a 56 kDa phosphoprotein of 485 aa (Figure 3). In HCV infected cells, the protein is present in two differently phosphorylated forms: phosphorylated (56 kDa) and hyper phosphorylated (58 kDa). NS5A is multifunctional and it contributes to the HCV replication, virus pathogenesis, modulation of cell signaling pathways, virus propagation and the interferon response[70,71]. The N-terminal amphipathic helix of the NS5A protein is conserved in different genotypes and it is necessary for the membrane localization[72-76]. Mutations in NS5A enhanced the capacity of the sub genomic HCV RNA replication in cell culture systems[77]. It was shown that the N-terminal part of NS5A contained a new zinc-coordination motif which affected the HCV replication[70]. NS5A has a potential role in mediating the IFN response. It contains a so-called "interferon-α sensitivity-determining region" (ISDR, aa 237-276)[78]. ISDR can be used to predict the resistance and the sensitivity of HCV to the IFN treatment. NS5A was also showed to be associated with a variety of cellular signaling pathways including apoptosis[70]. NS5A is already used as a target for the direct acting antivirals.

NS5B protein (p66-68)

The NS5B is a 591 aa long protein and it is located at the C-terminus of the precursor. NS5B is a RNA-dependent RNA polymerase containing the GDD motif in its active site[79]. The NS5B initiates synthesis of the HCV negative-strand RNA. The crystal structure of NS5B showed a typical 'right hand' polymerase shape with finger, palm and thumb sub domain. Since NS5B lacks the "proof-reading'' function[79], numerous mutants might be generated during transcription. Because of its key role in the virus replication, the NS5B protein is considered as a potential target for the antiviral drug[80].

F protein, ARFP

In addition to ten proteins described above, the frameshift (F) or alternate reading frame protein (ARFP), or "core+1" protein has been reported[81-83]. The ARFP is the result of a -2/+1 ribosomal frameshift between codons 8 and 14 of the adenosine-rich region encoding the core protein. ARFP ends have different stop codons depending on the genotype. Thus, its length may vary from 126 to 161 amino acids. In the case of genotype 1a, the protein contains 161 amino acids. However, the situation with ARFP is more complicated, since alternative forms of this protein such as ARFP/DF (double-frame shift) in genotype 1b, and ARFP/S (short form) were recently described[83]. ARFP is a short-living protein located in the cytoplasm[84] in associated with the endoplasmic reticulum[85]. Detection of anti-ARFP antibodies in sera of HCV-positive subjects indicates that the protein is expressed during infection[86], but is likely not involved in the virus replication. Some findings suggested engagement of ARFP in the modulation of dendritic cells function and stimulation of the T cell responses[87]. The implication of ARFP in the viral life cycle remain to be elucidated.

HCV TRANSMISSION ROUTES

The HCV in blood and blood products is the main source of infection. However, the transmission routes of HCV might be different and ountry dependent. The iatrogenic transmissions are: the blood transfusion of unscreened products[88], the transfusion of clotting factors or other blood products[89,90], the organ transplantation, the reuse of medical instruments used in invasive settings (*e.g.*, needles, infusion sets, syringes, catheters) in Egypt before 1985, hemodialysis, endoscopy, intravenous drug use[91-95]. The sexual way of transmission is controversial. Nevertheless, the risk may increase when favoring conditions such as sexually transmitted infections, the frequencies and the type of sexual activity are taking

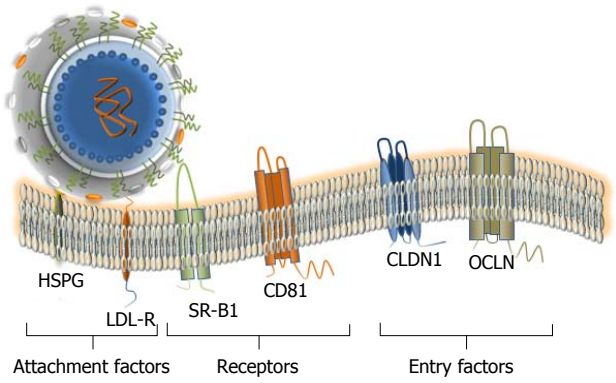

Figure 4 Attachment factors, receptor and entry factors utilized by hepatitis C virus. At least six membrane proteins are essential for the virus attachment and entry. Heparan sulfate proteoglycan (HSPG) - a type of glycosaminoglycan (GAG) and low density lipoproteins (LDL)-receptor that is promoting the LDL endocytosis are considered as binding factors for hepatitis C virus (HCV). Scavenger Receptor class B member 1 (SR-B1) and CD81 that are ubiquitously expressed on the cell surface, are considered as true receptors. The receptor–viral cargo complex is then moving to the cell-cell contacts (not shown), where the interaction with the tight-junction proteins Claudin 1 (CLDN1) and Occludin (OCLN) takes place. Both proteins are required at the late stage of the entry. Additional interaction partners that are likely engaged in the HCV entry are described in the text. The docking of the LVP to the cellular membrane is shown.

place[94-101].

The rate of HCV mother-to-child transmission is about 4.3%, but it is much higher, 22.1% among the human immunodeficiency virus (HIV) co-infected mothers[102,103]. The mother-to-child transmission generally occurs at delivery, but also *in utero*, if associated with high risk factors, such as high maternal HCV RNA levels and/or HIV co-infection. Interestingly, vaginal *vs* cesarean section delivery, amniocentesis and breast-feeding did seem to increase the transmission risk[102-108]. Because of a passive transfer of anti-HCV antibodies at, or after 18 mo of ages, perinatal transmission diagnosis as serum HCV RNA and/or anti-HCV antibodies should be performed twice in infants (6 mo and 1 year of age)[109] during this period, as recommended by the European pediatric HCV network[110]. It should be emphasized, that the INFL3 CC (IL28B) genotype is likely associated with a spontaneous clearance of the HCV genotype 1(gt1) infection[104].

According to the World Health Organization, thousands of new cases of the HCV infection (as a result of an occupational exposure *via* skin injury), are registered annually[111]. Most of these cases occurred during surgery in emergency departments, and routine medical procedures[112]. Seroconversion rates in infected individuals are ranging from 0% to 10.3% (+/- 0.75%)[113,114]. For instance, after accidental needle stick, seroconversion rate was reported as 1.8%[115]. During a 5-year period (2008-2012), a total of 16 HCV outbreaks resulting in 160 outbreak-associated cases and more than 90000 at-risk persons notified for screening were reported by the Centers for Disease Control (CDC) and Prevention[115]. Among dentists[116] and surgeons[117], an eye protection should be reinforced because of possible HCV transmission by splashes of blood and other body fluids[117]. Cosmetic procedures and/or acupuncture, as well as circumcision are extensively associated with HCV transmission, but the risk seems to be small[118,119]. Lacking of sterile techniques and/or nonprofessionally performed tattoos or piercings, especially before the mid-1980s, raised the HCV transmission rate significantly[120-126].

HCV GENOTYPES/SUBTYPES DEPENDENT TRANSMISSION

The HCV genome demonstrated a prominent genetic diversity. Seven genotypes (gt 1-7) and 67 subtypes (a, b, c, *etc*.) have been described. The genotypes are characterized by a distinct geographic distribution and clinical manifestations[127,128]. For example, the genotype 1 is prevalent in Americas, Japan and Europe[129]. Indeed, the HCV genotype prevalence also differs according to the transmission route and the age of infected individuals. For instance, the HCV gt 3a and 1a are highly represented among intravenous drug users and the HCV gt 1b is frequent among patients who received blood transfusions[130-134]. In Japan, the HCV gt 1b is the most prevalent[1] while, the infected population is generally older than in the United States[2], and an iatrogenic transmission is a predominant risk factor for the HCV acquisition.

HCV LIFE CYCLE

Binding and entry

The HCV entry initiates the viral replication cycle. Virus that is a complex and multi-step process that is not completely understood. However, the main principal steps and the principal cell surface interaction partners are known. At least, four cellular entry factors and two specific receptors are required for a successful virus entry (Figure 4). Since many interactions should take place, the binding to the cell surface is a relatively slow process which should be well coordinated[135-141]. The initiation of HCV entry seems to be similar to that of many other viruses, that utilize for attachment the glycosaminoglycans (GAGs)[141]. Earlier, it was proposed that E1 and E2 are involved in these interactions[141]. However, the HCV particle interacts with lipoproteins and in this regard, it is more likely that the apolipoprotein E is responsible for the interaction with GAGs[141]. Furthermore, due to the nature of the viral lipoprotein particle, it was proposed that the LDL receptor is required in the initial stage of attachment. However, this interaction leads the particle to a degradation pathway[141]. After the completion of docking, the viral particle interacts with specific cellular receptors. It was shown that the envelope glycoprotein E2 interacts with two receptors a scavenger receptor B1 (SR-B1), and with CD81 (tetraspanin family protein -TSPAN28)[142-144]. It is likely, that interaction of HCV with SR-B1 comes first[142]. In this regard, it remains to be determined whether SR-B1

and CD81 form a complex that facilitates the entry. It is noteworthy that the interaction with CD81 alone can activate signaling pathways that may be important for the viral infectious cycle[141] and the interaction between CD81 and the E2 glycoprotein appears to be essential for initiating the adsorption[141]. Next, the receptor complex with attached virion is moving to the tight junction, where the interaction with the proteins claudin-1 (Cldn1) and occludin (OCLN) is likely taking place. However, there is no experimental evidence indicating a direct interaction between the viral particle and the tight junction proteins. Thus, these proteins cannot be considered as classical receptors. Finally, other cellular factors such as epidermal growth factor receptor[145] and the Niemann-Pick C1-like 1 cholesterol uptake receptor[146] are likely involved in the HCV entry. It has been shown that CD81 and Cldn1 proteins may interact with the surface of hepatocytes[147], suggesting that CD81-Cldn1 form a complex that is implicated in the HCV entry. Although the tight junction proteins are involved in the HCV entry, the role of cell polarization in viral entry remains controversial. Interestingly, a live imaging of the fluorescent HCV particle in Huh7.5 cells showed that the particle was first attached on filopodia and then migrated towards the main body of the cell using presumably an actin-dependent transport mechanism[148]. It is important to note that the hepatocyte is a polarized cell with multiple apical and basolateral poles presenting a unique organization that is observed only in the liver. Another characteristic of HCV is its ability to spread by cell-to-cell contacts[149]. The essential role of the E1, E2 transmembrane domains in the viral entry[150] and the involvement of different regions of the ectodomain of E2 in the viral assembly were demonstrated[151]. It has been shown that glycans on the envelope proteins may be involved in different stages of the viral infectious cycle[42,152,153]. Furthermore, the N-linked glycan shield of the viral glycoproteins is essential to protect against the neutralization of the virus by antibodies[154].

An additional interaction partner of the CD81 receptor, called "EWI-2wint" was identified[155]. It was demonstrated that EWI-2wint is a natural inhibitor of the HCV entry, which by an interaction with the CD81 receptor can prevent CD81-E2 interaction. Later on, the regions involved in the interaction between CD81 and EWI-2wint were identified[156] and studies in living cells at a single molecule level proved that EWI-2wint reduces the mobility of CD81 at the plasma membrane[157,158]. Finally, it was reported that the enrichment of the plasma membrane in ceramides has an inhibitory effect on the viral entry[159].

Post-entry events

It was shown by a traffic monitoring that after binding to the cells surface, the virus particles enter into the cells using clathrin dependent endocytic pathway[160,161]. The viral particles are transported to the early endosomes expressing RAB5A where the merger took place. This process requires an acidification of the compartment[162]. The virus capsid is then released and destroyed, while the viral RNA is released to the cytoplasm. The RNA is used for both processes the replication and the polyprotein translation (Figure 5). The RNA translation occurs in the endoplasmic reticulum (ER) and it is initiated by a binding of the 5'UTR IRES to the ribosome. The primary translation product is approximately 3000 amino acid long polyprotein precursor which contains structural and non-structural proteins of the HCV. Then, the polyprotein is cleaved by the host and viral proteases into structural and non-structural proteins.

Several studies evidenced that the HCV infection results in ER stress and autophagy responses and that HCV can regulate the autophagy pathway. In fact, the autophagy machinery is required to initiate the HCV replication and suppression of autophagy inhibits this process[163-171]. Interestingly, it has been demonstrated that HCV induces autophagosomes *via* a Class Ⅲ PI3K-independent pathway and uses autophagosomal membranes as sites for its own RNA replication[171]. For the HCV RNA replication, polarized positive HCV RNA genome synthesizes a negative strand by the NS5B RNA-dependent RNA polymerase. The newly synthesized negative RNA strand may further act as a template to synthesize the positive strand of the viral RNA[172,173].

Viral assembly and release

A assembly of the virion is another multi-step process and certain steps of it remains obscure. It is known that the particle assembly occurs within the ER in a close proximity or directly on the surface to LD[170] (Figure 5). After the proteolytic cleavage of the polyprotein precursor, NS5A initiates the early phase of the viral particle formation by the interaction with the core protein and its C-terminal serine cluster determines the NS5A-core protein interaction[174].

The viral RNA released from the viral core is recruited to the replication complex within the membranous web. The HCV RNA-dependent RNA polymerase (NS5B) has no proofreading mechanism to correct errors during the strand synthesis. This propensity for error during replication results in an accumulation of the HCV quasispecies, those are closely related, but genetically somehow distinct. The newly synthesized positive-sense viral RNA is transported to the site of the core assembly and is encapsulated. However, the mechanism that engaged in RNA delivery to the capsid during assembly is not known. The nucleocapsids with RNA are presumably enveloped by budding into the lumen of the ER. So, after the nucleocapsid formation, it is associated with the envelope protein forming an immature particle and secreted from cell through the cytoplasmic membrane[173,174]. However, it remains unclear how the coupling with lipoproteins and apolipoproteins is regulated, since the pattern of lipoproteins on the virus particle might differ significantly. Next, the virions are

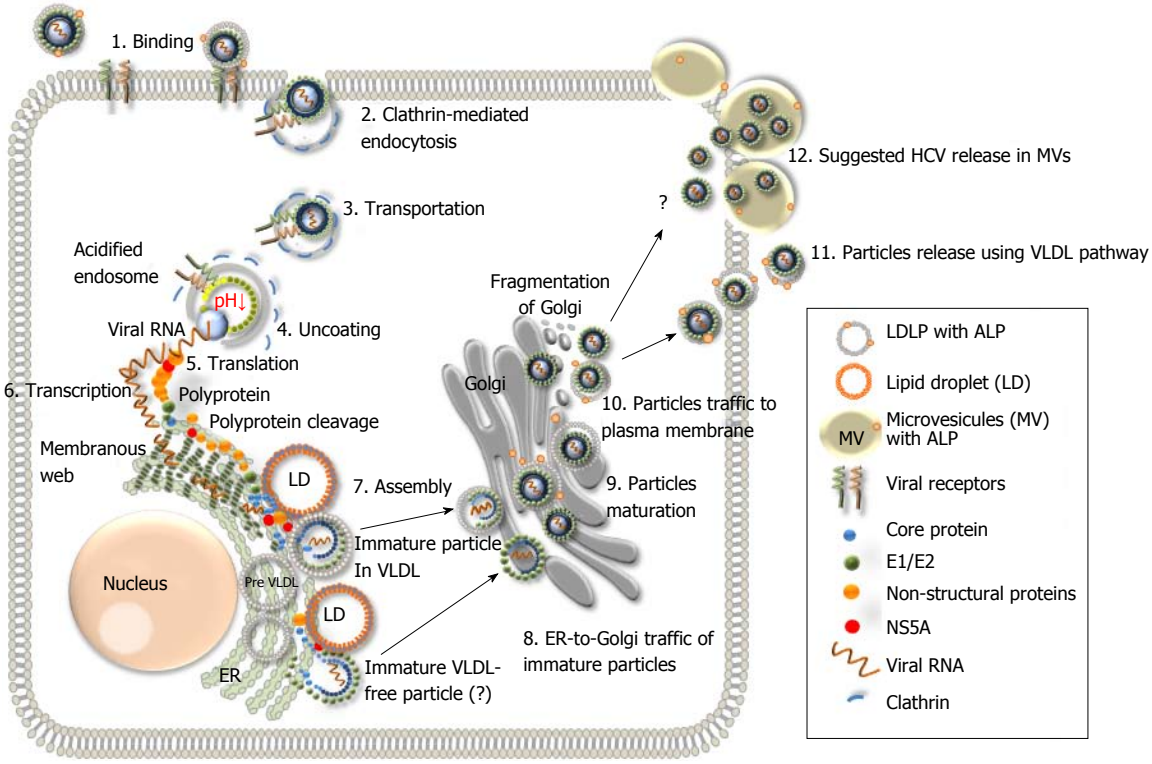

Figure 5 Schematic representation of the hepatitis C virus life cycle. (1) The lipoviral particles (LVP) binds to entry factors and receptors on the surface of hepatocyte; (2) The virus enters into the cell by a clathrin-mediated endocytosis; (3) Transportation of the virus in endosome; (3) Acidification of endosome, uncoating of the virion and dissociation of the viral core; (4) Release of the viral RNA; (5 and 6) Translation and replication of the viral RNA in the ER in the convoluted membrane structure called the membranous web (shown in dark green); (7) Cleavage of the protein precursor by cellular and viral proteases, assembly of the core on the surface of the lipid droplet (LD) and recruitment of the newly synthesized viral RNA to the viral core during the formation. The mechanism of the viral RNA recruitment to the site of the assembly is not known. It is also unclear whether the core maturation is finalized at this stage; (8) The viral nucleocapsid egresses into the lumen side of the ER, likely interacted with very low-density lipoproteins (VLDL) and translocated to Golgi. It is not clear if some VLDL-free particles can also be produced (?). Details of traffic machinery are not well defined; (9) Final maturation of viruses in Golgi and virus induced partial fragmentation of Golgi; (10) The hepatitis C virus (HCV) complex with VLDL is directed to the plasma membrane using the VLDL secretory pathway; (11) Release of particles from the cell (adapted from Ref.[16,19,135]); and (12) The HCV release in MV cannot be excluded as another pathway for the virus release during active replication.

transported through the Golgi compartment to finalize the maturation. Recently, it has been shown, that HCV can induce the fragmentation of Golgi[175]. Thus, it might be another important indication of the virus-induced pathogenesis. However, if the fragmentation of Golgi is significant, it may cause not only destructions of Golgi, but also provoke the cell death. Definitely, it might be not advantageous for the virus. It cannot be excluded, that this phenomenon might take place predominantly during the active replication of the virus. In this regard, it might be useful to investigate the HCV-induced Golgi destructions, as a possible trigger of liver necrosis.

Virus release and extracellular particles

The detail mechanism of the HCV release requires more comprehensive investigation. It is well established that secretion of the HCV particle depends on both the VLDL and the apolipoprotein (apoB) presence[176-187]. So, when the maturation is finished, the virions are transported to the plasma membrane using the VLDL pathway[145]. Particles isolated from cell-cultures and from patient sera are pleomorphic, immature and most of them are non-infectious[170]. In this regard, in the virus preparations from human plasma examined by electronic microscopy (EM), it was difficult to reveal "perfect" mature particle[170]. The association with fragments of cellular membranes (or other cellular debris) and/or an irregular lipoprotein content might be the reasons of the problem. Anyhow, it is evident, that a classical "assembly-maturation-release" processing line of HCV is facing problems. A diverse buoyant density of infectious particles is reflecting the problems. The analysis of LVP from plasma by sucrose gradient centrifugation demonstrated, that the buoyant density of the virus-contained fractions is diverse (1.10 g/mL to 1.16 g/mL)[170]. Positioning of virus-containing fraction on the gradient depends on the initial material that was used for the virus isolation. The infectious particles isolated by isopicnic centrifugation from cell-culture were mostly present in the fraction with a density 1.14 g/mL and examined by cryo EM, when were about 60 nm in diameter[170]. Significantly more virus-associated cellular impurities including lipoproteins, are associated with viruses isolated from patient sera and that influence the virus density and the particles are significantly less dense[177,178]. It is considered that the observed density

fluctuation is a result of irregular content of lipoproteins tightly associated with virion. In fact, this diversity might have an additional reason. It cannot be excluded that *in vivo* the virus particles might in addition be transported by extracellular vesicles. In fact, the size of putative transporters might be large enough to carry not a single, but numerous viral particles. For example, membrane derived microvesicles (MVs) can be 500-1000 nm in diameter, so as large as lipid droplets[184,185]. MVs are present in the fractions of different densities, indicating a diversity of diameter and/or cargo[186].

Analysis of cell supernatant by gradient density centrifugation demonstrated that the MVs subpopulations are different, depending on the cell line. It is known, that MVs are intensively released by primary hepatocytes and immortalized cells of hepatic origin[186]. The release from hepatocytes might be increased by an undergoing lipotoxicity[187]. Thus, MVs are actively used by the hepatocytes as delivery system and the amounts of MVs might be increased in response to lipotoxicity and likely other complications. In this regard, we speculate that during acute phase of infection (or high rate of replication) the virus can trigger the MV release and that might be an additional pathway for the virus. How MVs containing virus particles can influence HCV distribution along the density gradient? In fact, MV containing different amount of viruses may contribute to observed diversity of buoyant densities of HCV, especially when plasma (or serum) is investigated.

It is known, that the diffusion of spherical particle through a viscous liquid is dependent on the particle diameter. It is described by Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\eta r}$$

D = diffusion constant, k = Boltzmann constant, T = temperature (K), η = solvent viscosity, r = radius of spherical particle.

Another parameter that influences the diffusion is the mass of spherical particle and the equation counting these parameters looks as follows:

$$nD = \frac{6\pi\eta\alpha}{M}$$

where: D = diffusion; α = radius of the spherical particle; M = mass of the particle; η = viscosity of the medium.

So, the diffusion of a spherical particle is decreased with the increase of diameter and mass. Thus, if MVs besides the regular cargo (mRNA, miRNA, modulators, peptides, proteins) have in addition a different particle load, it might explain why MVs of the same sizes might be recovered in fractions with different densities, and vice versa. Thus, when supernatants (or plasma) are examined by density gradient centrifugation, MVs of different size and different virus loaded, can increase the amount of virus-containing fractions. The expected distribution of lipoproteins, LVP from human plasma in a sucrose gradient and the suggested positioning of MVs with different virus load are given below (Figure 6, adapted from[170] with modifications).

What might be the advantage, for the virus to use the MVs secretory pathway? Since the MV are released by budding from the plasma membrane, it allows the virus to acquire additional protection from the "rough environment". Second, if MV is loaded with numerous particles, it might be the way to increase the probability of a successful infection of distant cells by creating a high "local MOI" after MV distruction. That effect might be even more prominent, if the MV loaded with viruses would be endocytosed by the cell. We suggest, that the MV pathway might be used during active virus replication of the virus. The possible implication of MVs for the virus delivery might be interesting to investigate using *in vitro* models.

MODELS TO STUDY HCV

The progress in HCV research is completely dependent on model systems[188]. The most frequently used models (Figure 7) for investigation of HCV are described below.

HCV replicon systems

The HCV genome was identified in 1989 by cloning it from infected chimpanzee, while in humans the amounts were too low for detection[189]. The first complete full-length HCV cDNA clone was constructed from the HCV strain H77 (genotype 1a). The HCV RNA transcribed from this clone was found to be infectious after intrahepatic injection in a chimpanzee. The HCV viremia was detected at week 1 and increased from 1×10^2 genomes/mL to 1×10^6 genomes/mL at week 8[190,191]. Then, several full-length HCV RNAs were synthesized and were shown to be infectious in chimpanzees[190-193]. However, these HCV clones were found to replicate inefficiently *in vitro*. This limitation was resolved by the group of Prof. Ralf Bartenschlager, when subgenomic HCV replicon, cloned from the HCV genome was constructed[73]. After transfection of this subgenomic clone into the Huh7 cells, it was found that in drug-resistant cells a high-level of the HCV RNA replication occurred. The replication of the HCV subgenomic replicon was confirmed in several cell lines, but the hepatocarcinoma cell line Huh7 was the most permissive. The interferon treatment of the replicon inoculated Huh7 cell clones, made them more permissive to support both subgenomic and full-length HCV replication, (these so-called cured cell lines are Huh7.5 or Huh 7.5.1 cells)[15,194]. Afterwards, several studies demonstrated that the virus and host factors were important for the HCV replication in cells. Some mutations in the wide-type (wt) consensus sequence efficiently enhanced the HCV replicon replication, some mutations in the non-structural protein efficiently contributed to the replication and the adaptation to the host cells[195-198]. The mechanism of improved replication caused by adaptive mutations is still unknown. Although

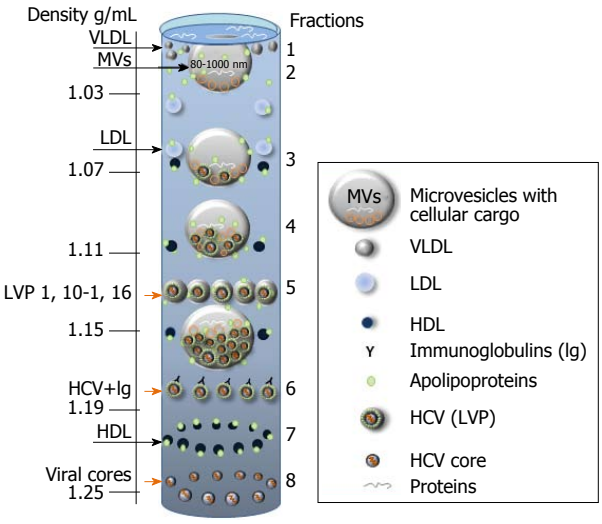

Figure 6 Putative distribution of lipoproteins (LDL, VLDL, HDL), lipoviral particles and viral cores after density gradient centrifugation of plasma from infected individual. The picture is based on the known buoyant densities of analyzed elements[15,170,184-187]. The density values (g/mL) are given on the left. The initial material that has been used for the virus isolation, contributes greatly to the associated lipid content of the virions and, as a consequence, it influences the buoyant density[177,183-187]. Because of an irregular protein to cholesterol amounts, high density lipoproteins (HDL) of 5-15 nm in diameter can be detected in the different fractions (1.06-1.21 g/mL). The family of HDL is given as dark blue spheres. The buoyant density of microvesicles (MVs) may vary from 1.02 g/mL to 1.16 g/mL, depending on MVs diameter and cargo (miRNA, tRNA, fragments of mRNA and proteins). If hepatitis C virus (HCV) is released in MVs with similar diffusion parameters (size and mass), the buoyant density of vesicle should be viral cargo-dependent. Fractions 1: Single cellular or viral proteins "floating" on the surface, very low-density lipoproteins (VLDL, diameter 50-75 nm, density 0.95-1.006 g/mL); Fraction 2: Low density lipoproteins (LDL, diameter 18-25 nm, density 1.019-1.06 g/mL) and virus-free MVs with a low cellular cargo content; Fraction 3: MVs that carry few viral particles, possible overlap with LDL and HDL; Fraction 4: MVs with a higher viral load, possible overlaps with HDL and with densely loaded virus-free MVs; Fraction 5: Lipoviral particles (LVP, diameter 60-80 nm, density 1.10-1.16 g/mL) and MVs with a significant viral and/or mixed load. Overlaps with HDL, virus-loaded MVs and "dense" virus-free MVs are possible; Fraction 6: Lipid-free virions with attached immunoglobulins, possible overlap with HDL; Fractions 7: HDL with a high protein load; Fraction 8: Non-enveloped viral cores that might overlap with dense HDL. Small grey spheres - VLDL. Light blue spheres - LDL. Orange arrows indicated fractions of the gradient that are most likely to contain viruses and viral cores.

these replicons with adaptive mutations could replicate with a high efficiency, they were not able to produce infectious particles *in vitro*.

This problem was solved with development of the genotype 2a infectious clone JFH-1. A selectable HCV replicon was constructed containing the full-length HCV cDNA and showed to produced infectious particles *in vitro* and *in vivo*[199]. Based on this infectious clone, several different genotypes chimeric clones (genotype 1a, 1b, 2a and 3b) where the non-structural genes have been replaced by those of JFH-1 were constructed and were shown to be infectious in Huh-7 cells. The most efficient construct is the genotype 2a/2a clone which consists of J6CF and JFH-1 derived sequence[200]. The HCV replicon is remarkably valuable for studying the HCV replication and for testing of new antiviral drugs. The HCV subgenomic replicons containing reporter genes (luciferase, secreted alkaline phosphatase and chloramphenicol transferase) facilitated the study of the HCV infection. This high-throughput screening assay allowed the visualization and tracking of the HCV replication complex in living host cells without affecting the HCV replication[47,201].

HCV pseudotype virus particles

The HCV pseudotyped particles were constructed with chimeric genes expressing HCV (genotype 1a) envelope E1 and E2 proteins (HCVpp) and the transmembrane and cytoplasmic tail of vesicular stomatitis virus G protein. The pseudotyped particles allowed a detailed study of the role of HCV receptors in the early steps of HCV infection (adsorption, and viral entry) in Huh7 cells and primary human hepatocytes[202-204]. The system is useful for testing of new antiviral drugs[203].

The HCV subgenomic replicon and the HCV pseudotyped particles (HCVpp) have markedly improved studies on the HCV infection. However, the high replication rate in cells was not correlated with amount of released infectious virions. However, the main disadvantage of these *in vitro* systems is that the viruses released from these cells are not infectious. The major reason of that might be that the adaptive mutations enhancing HCV replication rates are deleterious for HCV particles assembly and release. The problem was solved when a genotype 2a subgenomic replicon was established in the Huh-7 cell lines. It contained a full-length genotype 2a clone JFH-1 derived from serum of a Japanese patient with fulminant hepatitis. The replication rate was about 20 times greater than Con1 (gt1b) when transfected into Huh-7 cells. In addition, this replicon could replicate efficiently without amino acid mutations[47]. In 2005, Wakita *et al*[200] continued to transfect this JFH-1 clone into Huh-7 cell lines and found high levels of intracellular HCV RNA replication and protein expression. By means of passages in infected cells, they demonstrated that JFH-1 transfected cells had continuous HCV replication. In this study, it was also shown, that the HCV viral particles (HCVcc) secreted in the culture supernatant had a density of approximately 1.15-1.17 g/mL. The released particles have a spherical morphology and a diameter of about 55 nm. The JFH-1 infectivity could be neutralized by anti-CD81 antibody, suggesting the important role of CD81 in HCV entry. In addition it was found that the secreted particles could also infect chimpanzee[191]. Later, several groups observed that JFH-1 could efficiently infect and replicate without adaptive gene mutation in different cell types and HCV infectious particles could be produced in the culture supernatant[25,199,205-209]. Zhong *et al*[192] established a robust highly infectious *in vitro* system with JFH-1 and Huh-7.5.1 cells. The advantage of this model, compared to that established by the group of Prof. Wakita, was the possibility of HCV to undergo serial

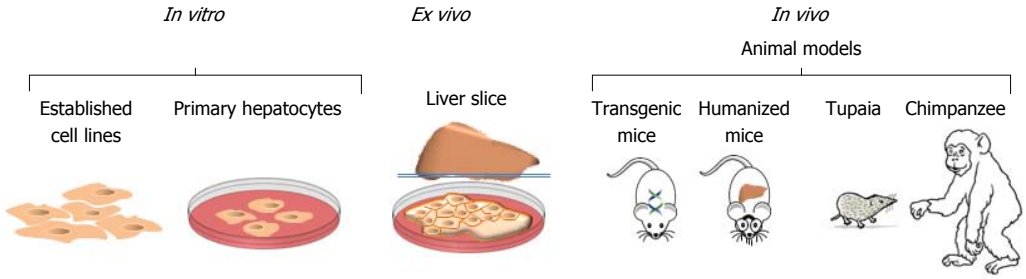

Figure 7 ***In vitro*, *ex vivo* and *in vivo* models to study hepatitis C virus.**

passages without losing of infectivity. Although JFH-1 *in vitro* system provides a powerful tool to study antiviral drugs and vaccines, it has some limitations: HCV JFH-1 derived from an exceptional case of HCV-related fulminant hepatitis belongs to genotype 2a which is not the dominant genotype worldwide. That is why since 2005, different chimeric JFH-1 clones were constructed to transfect Huh-7.5 cells, based on intergenotypic genotype replicons such as J6/JFH-1 (genotype 2a/2a), S52/JFH1 (genotype 3a/2a) and sa13/JFH-1 (genotype 5a/2a)[209,210], but they are less infectious than HCV JFH-1. The reason why JFH-1 is more permissive to Huh-7.5 cell lines than other genotypes is still not fully understood. In summary, the HCVcc model allowed to understand the entire life cycle of HCV and that serves as a background for development of numerous antivirals[211].

Animal models

Chimpanzee could be a good model to study the HCV infection[194,212]. However, these animals are rare, difficult to handle, very costly and limited in their use by ethical issues[212]. Few data obtained using chimpanzee model indicated a specific immune response at the acute phase of infection, resulting in inconsistent specific neutralization and viral eradication.

Tupaia (Treeshrew, *Anathana ellioti*) is potentially a good model to study the HCV infection, but the instability of the infection and its low level limit the use of these animals.

The immunotolerized rat model supports the HCV replication. Histological and biochemical evidence of infection were present, but viremia was relatively low as compared with viral load in humans[188].

Mouse models compared with other animal models, have some advantages, such as producing animals in a short time (short gestation period), lower breeding cost and their small size making them easy to manipulate[188,213-217]. Heterotopic liver graft mouse model seemed to be suitable to evaluate the putative effect of anti-HCV drugs, but the short and low viremia and loss of the liver graft were limiting factors. Hepatic repopulation mouse model as the chimeric urokinase-type plasminogen activator/severe combined immunodeficient disorder mouse model has many similarities to human systems, and so is the most successful small animal model for HCV infection. The main limitation of the model is the absence of a normal immune system, which may be overcome by combining the model with a human hemato-lymphoid system, the value and reproducibility of which has yet to be established[218].

A mouse combined human immune system/human liver chimeric Rag2-g(c) model was developed in order to study immune system and liver pathogens. This 'Hu-HEP' model was used to study hepatocytes derived from human induced pluripotent stem cells. The main limitations of these models are the low number of animals that can be generated and the high cost[219-221].

Recent study demonstrated that hepatocyte-like cells differentiated from human embryonic stem cells and patient-derived induced pluripotent stem cells could be engrafted in the liver parenchyma of immune-deficient transgenic mice carrying the urokinase-type plasminogen activator gene driven by the major urinary protein promoter. This efficient engraftment and *in vivo* HCV infection of human stem cell-derived hepatocytes provide a model to study chronic HCV infection in patient-derived hepatocytes, action of antiviral therapies, and the biology of HCV infection[222-225].

In vitro models

Established human hepatocarcinoma cell line Huh-7 and its derivatives support the HCV replication. However, as every transformed cell line, they resemble the primary cells only partially. Thus, results of experiments performed on these cells might not be always appropriated[222].

The primary human hepatocytes and human fetal hepatocytes are more clinically and physiologically relevant. Thus, they are used to test the susceptibility of HCV to drugs and drug-metabolizing enzymes. Another *in vitro* model, the micropatterned co-cultures of primary human hepatocytes surrounded by a supportive stroma that expressed all known HCV entry factors. The cells could be infected by HCV pseudotyped particles and HCVcc, albeit with low viral titers. Using this method in combination with the highly sensitive luminescence-based and fluorescence reporter systems, the efficiency of anti-HCV therapeutics has been evaluated[222].

Recent studies show that both embryonic[223] and induced pluripotent[224,225] stem cells can be differentiated into hepatocytes, that are phenotypically similar to

human fetal liver. Study of genetic defects that impact the HCV infection could be performed in a human iPS-derived hepatocyte-like cell-based model[225]. Induced human liver-like cells supported the entire life cycle of HCV genotype 2a reporter virus. Produced infectious particles were able to infect HuH-7.5 cells and secretion of TNF-α, and IL-28B/IL-29 was detected in cell culture supernatants. Thus, the evaluation of antiviral drugs using these cells was possible[222].

Ex vivo model

Analysis of precision-cutting adult human liver slices from infected or non-infected individuals represents another promising model. In fact, this model allowes to maintain the tridimensional structure of liver and analyse gene and protein expression.It is worth mentioning, that using this model, it was demonstrated for the first time, the ability of primary isolates (as well as JFH-1, H77/C3, Con1/C3) to undergo *de novo* viral replication with the production of high titer infectious virus. Thus, this approach allowed to validate the efficiency of the new antiviral drugs[226,227].

PATHOGENESIS

Immune-mediated liver damage

The HCV causes damage to the liver cells, but the exact mechanism of this phenomenon is unknown. It is believed, that the damage is largely mediated by the host immune response. In immunocompetent and immunocompromised patients with little, or no intra-hepatic damage, including inflammation, high levels of the HCV replication have been reported[133,228,229]. In about 30% of HCV liver transplanted patients, despite the high levels of HCV replication, a recurrent hepatitis is developed one year after transplantation [230]. However, high levels of an intrahepatic HCV replication are usually tolerated by the host immune system. A lympho-mononuclear infiltrate represented mainly by $CD8^+$ T cells expected to play a major role in the viral containment, though other subsets, such as $CD4^+$ T and natural killer (NK) cells, and regulatory T cells (Treg) are considered[231]. The intrahepatic $CD4^+$ and $CD8^+$ T cells can recognize HCV structural and nonstructural antigens[232,233]. However, why in most patients the immune response cannot resolve the infection remains obscure. In fact, cytotoxic $CD8^+$ T cell-mediated killing could be blunt by a predominant Treg response[234].

The liver fibrosis is caused by inflammatory cells of the intrahepatic infiltrate secreting cytokines and chemokines to activate hepatic stellate cells (HSC) to secrete collagen[235]. HSCs may exist as several different phenotypes with distinct molecular and cellular functions and features, each of which contributes significantly to the liver homeostasis and the disease. The quiescent stellate cells are critical to the normal metabolic functioning of the liver. The liver injury provokes the transdifferentiation of quiescent stellate cells to their activated phenotype, leading to a metabolic reprogramming. That increases the autophagy (to fuel the metabolic demands), the amplification of parenchymal injury and the development of 'classic' phenotypic features of activated HSCs/myofibroblasts. Through these changes, the activated stellate cells drive the fibrotic response to injury and the development of cirrhosis. As liver injury subsides, the activated stellate cells can be eliminated by one of three pathways: Apoptosis, senescence or reversion to an inactivated phenotype. The senescent stellate cells are more likely to be cleared by the NK cell-mediated cell death while the inactivated stellate cells remain 'primed' to respond to further liver injury. Reduction in the number of activated stellate cells contributes to the regression of fibrosis or cirrhosis and the liver repair in most, but not all patients. The relative inputs of these three pathways on the fibrosis regression are not clearly defined[233]. The HCV-specific $CD8^+$ T cells expressing PD-1, a marker for exhaustion, were found at the time of the acute phase[236]. The acquisition of a memory phenotype and the recovery of an efficient $CD8^+$ T cell function declined with HCV infections PD-1 expression, whereas, when HCV persisted and the HCV-specific $CD8^+$ cells remained dysfunctional, high levels of PD-1 were maintained, the PD-1 ligation likely provides an overall inhibitory signal to Tregs cells shown in a study of PD-1 expression on Tregs in humans during the chronic HCV infection. In response to HCV antigens, the PD-1 blockade enhanced an interleukin-2 (IL-2)–dependent proliferation of intrahepatic Tregs and enhanced the overall ability of Tregs to inhibit T-effector cells. An increase in Treg proliferation with PD-1 blockade was linked to this effect[237]. A typical model of the wound-healing response to a persistent liver injury is the hepatic fibrosis occurring in the chronic hepatitis C[238]. Cytokines and chemokines capable of activating hepatic stellate cells to secrete collagen are secreted by the inflammatory cells of the intrahepatic infiltrate[239,240]. Thus, the fibrogenesis seems to be linked to the HCV expression through indirect mechanisms, mediated by a virally driven inflammation, but the direct role of viral factors in the disease progression should be investigated in more details. The cell injury, such as an oxidative stress and a steatosis, may be induced specifically by several viral proteins alone which could directly activate the hepatic stellate cells[241,242]. These observations may explain why some patients with chronic hepatitis C with normal liver enzymes and a minimal/mild inflammation may present a significant liver fibrosis as shown by the histology of liver biopsies. Overall, up to 70% of patients with a HCV cirrhosis will demonstrate a reversibility on follow-up biopsies[243-248]. Moreover, a reduced portal pressure and a decreased all-cause mortality are improved when the reversal occurs[245]. It is to point out that approximately 10% of HCV patients present a persistent or even progressive fibrosis following SVR, which might reflect other concurrent underlying liver diseases, especially a

nonalcoholic fatty liver disease (NAFLD)[248]. The stellate cells can respond to cytokines and growth factors after priming stimuli. Then the proliferation, contractility, fibrogenesis, matrix degradation and proinflammatory signaling are enhanced. Now, it is clear that there are disease-specific pathways of fibrosis, without all activated cytokine pathways[249,250]. This is especially relevant to NAFLD, where there are many convergent pathogenic routes[248-250]. Importantly, different families of inflammatory cell types and their subsets may either promote or inhibit fibrosis[249-252].

Alterations of lipid metabolism

Lipids are required for the HCV replication and particles assembly. As mentioned above, HCV can modify the host serum lipid profile and this(ese) modification(s) can provoke the steatosis[253]. The steatosis is more frequent and more severe in patients with HCV gt 3 and it is correlated with a high HCV RNA levels. On one hand, in HCV-infected patients, the steatosis can be considered as a marker of the liver disease progression[254] and, on the other hand, as an indication of the reduced response to therapy[254]. However, if it is not metabolic or alcoholic steatosis, an efficient antiviral therapy is capable to reduce it[255,256].

Extrahepatic replication of HCV

The apparent presence of the HCV genomes in extra-hepatic sites of patients infected with HCV has been shown. Using sensitive PCR assays, the HCV was revealed in leukocytes and there are evidences that these cells may represent a reservoir of the virus after treatment[257,258]. Interestingly, the pool of the HCV quasi-species differs between the plasma and peripheral blood monocytes, suggesting an independent spread of HCV within different cell types[259,260]. The infection of B-cells from non-Hodgkin's lymphoma by HCV has also been demonstrated. A cell line established from transformed lymphocytes supported the HCV replication and also enable production of infectious viral particles capable to infect peripheral blood B cells[261]. The significance of these extrahepatic HCV reservoirs is not well understood, although one could speculate that the leukocyte compartments might represent an additional route by which HCV can directly manipulate the immune system and also another means by which the virus avoids the eradication.

Extrahepatic manifestations associated with the HCV infection

At least, one clinically significant extrahepatic manifestation occurs in 38% to 76% of HCV infected patients with chronic HCV[262,263]. The most frequent associated pathology is a mixed cryoglobulinemia. It is detected in 19% to 50% of HCV infected patients, while only 15% of them are symptomatic. The cryoglobulins are immunoglobulins which precipitate at a temperature below 37 ℃. They are produced by HCV activated B cells. The cryoglobulins deposed in small and medium vessels are the cause of systemic vasculitis which can manifest in level joint, skin, renal or peripheral nerves[262]. Other observed extrahepatic manifestations are the following: lymphoma, thyroid disorders, diabetes, xerostomia and xerophthalmia[263-265]. The HCV infection cure leads to a gradual decrease of the cryoglobulin level in serum, followed by the remission of cryoglobulin-related symptoms and pathologic lesions[265]. Interestingly, as a result of treatment the incidence of type 2 diabetes is also reduced by approximately two thirds[264,265].

CLINICAL MANIFESTATION OF HCV INFECTION

Acute hepatitis

The acute hepatitis C is asymptomatic in 90% of infected people[266]. In some cases, asthenia, fever and muscle, and joint pain can appear. While, signs of jaundice are not frequent. Acute hepatitis C is characterized by a transient increase in the rate of serum transaminases. The first detectable virus marker is viral RNA that appears one to two weeks after exposure. Then seven-eight weeks later, the anti-HCV IgG response can be detected[266-268]. In 20% of cases, hepatitis C is resolved spontaneously through the innate and adaptive immunity[269]. The viral RNA becomes undetectable within three to four months after infection. Various factors could promote the viral clearance. Similarly, hepatitis acute symptoms would reflect a significant immune response of the host. The gene polymorphism of interleukin (IL) 28B also influences the host immune response[268,269]. The fulminant hepatitis C is exceptional[268].

Chronic infection

In about 80% of cases[268], the immune system is not capable to eradicate the HCV during the acute phase of infection. When the viral replication persists for more than six months after acute infection, the hepatitis is considered chronic. At the stage of chronic hepatitis, most patients are asymptomatic and may have no non-specific symptoms such as fatigue, arthralgia or myalgia. The transaminase levels may be moderately increased or even normal[270]. The long-term evolution of chronic infection is variable. The factors that accelerate the disease progression are the following: acquisition of more than 40 years, male gender, co-infection by HIV, higher body mass index, fatty liver and alcohol consumption[269]. After 10 to 30 years, about 20 to 30% of patients develop a cirrhosis. The cirrhosis may be associated with a liver failure, as a decompensation following a portal hypertension (ascites, gastrointestinal bleeding, *etc*.). In cirrhotic patients, the risk of death from complications is 4% per year, and their risk of developing an hepatocellular carcinoma (HCC) is 1 to 5% per year. Thirty-three percent of patients with HCC die within one year after diagnosis[269,270]. Overall, among patients with cirrhosis, the 5-year survival rate is 50%.

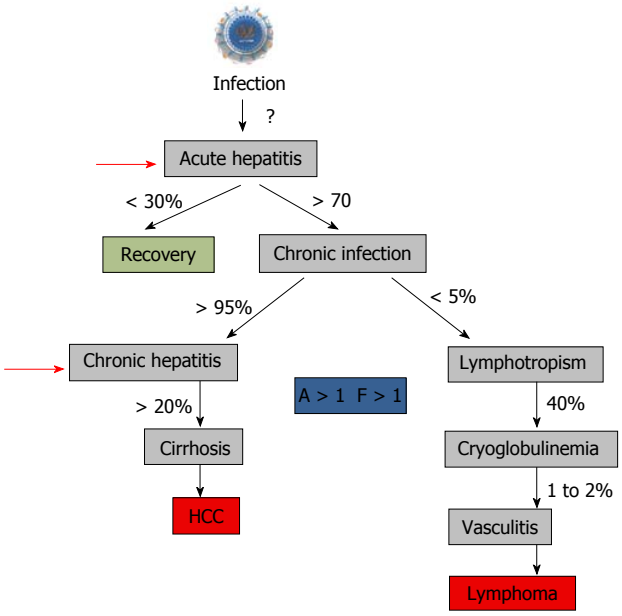

Figure 8 Natural history of hepatitis C virus infection and start to treat. The recommendation to treat chronic hepatitis is usually a "significant" fibrosis as defined by a Child–Pugh score (A to C) and a fibrosis grade (F) greater than 1 by the Metavir scoring system, with usually a significant necrotic-inflammation as defined by an activity stage greater than 1 by the Metavir scoring system[288,289]. The Child–Pugh score employs five clinical measures of liver disease: Total bilirubin, Serum albumin, Prothrombin time, Ascites, Hepatic encephalopathy. The letter F refers to the scars of the liver caused by the aggression. It is classified from F0 to F4: F1, F2 are minimal to moderate fibrosis, F3 corresponds to a pre-cirrhotic stage and F4 corresponds to cirrhosis. Red arrows indicated the time to start treatment[290].

In fact, the decompensated cirrhosis is the leading cause of liver transplantation[268,269] (Figure 8).

DIAGNOSTIC

For HCV diagnosis both serologic and nucleic acid-based tests were developed[270,271]. Serologic tests are sufficient when chronic hepatitis C is expected, with a sensitivity of more than 99% if using the 3rd generation assays. Positive serologic results require additional HCV RNA or (with slightly reduced sensitivity) HCV core antigen measurements in order to differentiate between chronic hepatitis C and resolved HCV infection from the past. When an acute hepatitis C is considered, a serologic screening alone is insufficient, because mature anti-HCV antibodies are developed late after transmission of the virus.

Morphological methods like immunohistochemistry, *in situ* hybridization or PCR from liver specimens play no relevant role in the diagnosis of hepatitis C because of their low sensitivity, poor specificity and low efficacy compared to serologic and nucleic acid-based approaches.

HCV core antigen assay

Recently, a new quantitative HCV core antigen assay (Architect HCV Ag, Abbott Diagnostics) was approved by the EMA. This assay comprises 5 different antibodies targeted the HCV core. The test is highly specific (99.8%), equally effective for different HCV genotypes, and shows a relatively high sensitivity for the determination of chronic hepatitis C (corresponding to 600-1000 IU/mL HCV RNA). However, HCV core antigen correlated well, but not fully linearly, with HCV RNA serum levels, and false-negative results might be obtained in patients with an impaired immunity[271-273]. Another study has shown that the HCV core antigen quantification could be an alternative to the HCV RNA quantification for on-treatment antiviral response monitoring[274]. Here, a HCV core antigen below the limit of quantification at treatment 1 wk was strongly predictive of RVR, whereas patients with a less than 1 log10 decline in HCV core antigen at treatment 12 wk had a high probability of achieving nonresponse. The new HCV core antigen assay could be a cheaper, though somewhat less sensitive, alternative for nucleic acid testing.

Nucleic acid testing for HCV

Since the HCV RNA is detectable within a few days of infection; the nucleic acid-based tests are efficient in an early diagnostic of acute hepatitis C and should be considered as mandatory. The HCV RNA measurement is furthermore important in determination of the HCV genotype, selection of treatment strategy, therapy duration and evaluation of the treatment success[274]. For a number of antiviral combination therapies, the HCV RNA follow-up studies are essential to define the outcome of the treatment and further therapeutic strategies, if necessary. Traditionally, the tests should be repeated 24 wk after treatment completion to assess whether a sustained virologic response (SVR) has been achieved. However, as the probability of a virologic relapse is similar after 12 and 24 wk, the new time point for assessment of final virological treatment outcome is 12 wk after the end-of-treatment[275,276]. Both qualitative and quantitative PCR-based detection assays are available. Qualitative PCR tests are sensitive and are used for initial diagnostic of hepatitis C, for screening of blood and organ donations and for confirming SVR after treatment completion (Table 2). Quantitative reverse transcriptase (RT) real-time PCR-based assays can detect and quantify the HCV RNA over a very wide range, from approximately 10 IU/mL to 10 million IU/mL. The measurements are essential in the treatment monitoring when the virus load is gradually reducing.

HCV genotyping

HCV genotyping is mandatory for every patient who considers antiviral therapy[277]. For DAA-based therapies, the determination of HCV genotypes and even subtypes is important because of significantly distinct barriers to resistance on the HCV subtype level. However, the importance for the HCV genotyping may decline with the availability of highly and broadly effective all oral combination therapies in the future. Both direct sequence

Table 2 Commercially available hepatitis C virus RNA detection assays

Assay	Distributor	Technology	Detection limits (IU/mL)	Genotypes	Approval status
Qualitative HCV RNA detection assays					
Amplicor™ HCV 2.0	Roche molecular Systems	PCR	50	All	FDA, CE
Versant™ HCV	Siemens Medical Solutions Diagnostics	TMA	5-10	All	FDA, CE
Quantitative HCV RNA detection					
Amplicor™ HCV Monitor 2.0	Roche molecular Systems	PCR	500 to approximately 5.10^5	All	CE
HCV SuperQuant™	National Genetics institute	PCR	30 to 10^7	All	U.S only
Versant® HCV RNA 3.0	Siemens Medical Solutions Diagnostics	bDNA	615 to 8.10^6	All	FDA, CE
Cobas® AmpliPrep/High pure system/Cobas® TaqMan®	Roche molecular Systems	Real-time PCR	15 to 10^7	All	FDA, CE
Abbot RealTime™ HCV	Abbot Diagnostics	Real-time PCR	10 to 10^7	All	FDA, CE
Artus HCV QS-RGQ assay	Qiagen	Real-time PCR	34 to 10^8	All	CE
Versant® HCV 1.0 kPCR assay	Siemens	Real-time PCR	10 to approximately 10^7	-	CE

HCV: hepatitis C virus; TMA: Transcription-mediated amplification of RNA; bDNA: Branched DNA hybridization assay.

analysis and reverse hybridization technology allow the HCV genotyping. Initial assays were designed to analyze exclusively the 5'UTR, which was burdened with a high rate of misclassification especially on the subtype level. Current assays were improved by additionally analyzing the coding regions, in particular the genes encoding core protein and the NS5B, both of which provide non-overlapping sequence differences between the genotypes and subtypes[278,279].

TREATMENT

All people with confirmed chronic HCV infection should be offered a high-quality care as soon as possible. Simultaneously, the screening and management of alcohol use is essential to prevent the progression to cirrhosis[278,280]. The modelling suggests that the treatment early in the course of HCV disease for all or specific populations could prevent the disease progression and onward transmission[281-283]. In fact, not all people with chronic HCV infection will progress to fibrosis[284]. So, medical authorities of a country to should decide when to start anti-HCV treatment. It should be emphasized that the transmission has been documented among people recently cured who lacked access to prevention services[285]. More research is needed to determine cost-effective eligibility criteria for both key and other populations that maximize reductions in the HCV-related morbidity, mortality, and transmission in different epidemiological contexts.

The WHO guidelines recommend the prioritizing treatment among people with an advanced fibrosis or cirrhosis in order to prevent a liver cancer[286]. The HCV co-infection in people living with HIV increases mortality while HIV has been shown to accelerate the progression of HCV disease[287,288]. Therefore, people with HIV/HCV co-infection may also warrant treatment prioritization. The stage of HCV disease could be estimated through liver biopsy (METAVIR system) identifying individuals needing an immediate treatment[288]. However, the liver biopsy test is expensive and can lead to complications such as infections, excessive bleeding, pain, or accidental injury to other organs. The use of liver function tests and platelet counts to determine the degree of liver fibrosis, such as the aminotransferase-to-platelet ratio index and the Fibrosis-4 score could be a non-invasive alternative and a more useful approach for gauging treatment eligibility across different tiers of health systems[289,290]. Monitoring systems should be put in place for people who do not initiate treatment immediately.

In the course of the last two decades, treatment of the HCV infection has significantly improved. For nearly 15 years, the combination of pegylated interferon alfa and ribavirin (PR) allowed a moderate sustain virologic response (SVR). Cure of the infection was achieved in 45% of genotype 1 and 65% of genotype 3 and around 85% of genotype 2 infected patients[291]. Development of the direct-acting antiviral drugs (DAAs) targeting viral proteins (NS3/4A protease, NS5B polymerase with nucleotide and non-nucleotide inhibitors, NS5A viral replication complex) was achieved due to the better understanding of the HCV life cycle. It revolutionized the treatment of chronic hepatitis C[292,293]. The classes of oral anti-HCV inhibitors and their characteristics are shown (Table 3). The combination of PR with the first-generation protease inhibitors demonstrated a high antiviral effectiveness (75% of SVR, but restricted to genotypes 1) with substantial adverse effects for the first-generation protease inhibitors. In 2011, telaprevir and boceprevir obtained a market approval. In 2012, the recommendations were approved for their use in HCV mono- and in 2013 for HCV/HIV co-infected infected patients. Then, SVR rates increased from 75% to 90% while reducing treatment duration, adverse effects, and the number of pills, thanks to the combination of PR with second-generation protease inhibitors[63,294,295]. From 2015 the standard of care is a combination of DAAs. Sofosbuvir, daclatasvir and the sofosbuvir/ledipasvir combination are part of the preferred regimens in the WHO guidelines, and can achieve cure rates above 95%[63]. These medicines are much more effective, safer and better-tolerated than the older therapies. In the first studies, excellent results were obtained on a group of so-called "easy-to-treat patients" and in

Table 3 Selected directly acting antiviral agents and host targeting agents in the pipeline[63]

Drugs name	Company	Target/active site	Phase
NS3/4A protease inhibitors			
Vaniprevir (MK-7009)	Merck	Active site/macrocyclic	Ⅲ
Voxilaprevir GS-9857	Gilead	Active site	Ⅲ
Glecaprevir (ABT-493)	Abbvie	Active site	Ⅲ
IDX21437	Idenix	Active site	Ⅱ
Sovaprevir (ACH-1625)	Achillion	Active site/macrocyclic?	Ⅱ
Nucleoside analog NS5B polymerase inhibitors (NI)			
MK-3682 (formerly IDX20963)	Merck	Active site	Ⅱ
ACH-3422	Achillion/Janssen	Active site	Ⅱ
Non- Nucleoside analog NS5B polymerase inhibitors (NNI)			
Beclabuvir (BMS-791325)	Bristol-Myers Squib	NNI site 1/Thumb 1	Ⅲ
Setrobuvir (ANA598)	Anadys/Roche	NNI site 4?/palm 1	Ⅱ
NS5A inhibitors			
BMS-824393	Bristol-Myers Squibb	NS5A protein	Ⅱ
PPI-461	Presidio	NS5A protein	Ⅱ
PPI-668	Presidio	NS5A protein	Ⅱ
Pibrentasvir (ABT-530)	Abbvie	NS5A protein	Ⅲ
ACH-2928	Achillion	NS5A protein	Ⅰ
Ruzasvir (MK-8408)	Merck	NS5A protein	Ⅱ
Host targeting agents			
SCY-635	Scynexis	Cyclophilin inhibitor	Ⅱ
Miravirsen	Santaris	miRNA122 antisense NA	Ⅱ
RG-101-	Regulus	miRNA122 antisense NA	Ⅱ
TT-0034	Tacere Therapeutics	RNA interference with HCV	Ⅱ

Table 4 Selected directly acting antiviral agents and host targeting agents whose development has been stopped or temporarily halted[63]

Drugs name	Company	Target/Active site
NS3 /4A protease inhibitors		
Ciluprevir BILN 2061	Boehringer Ingelheim	Active site/macrocyclic
Narlaprevir (SCH900518)	Schering-Plough	Active site/linear
PHX1766	Pheromix	Active site
Danoprevir (R7227)	Roche/InterMune	Active site/macrocyclic
Faldaprevir (BI201335)	Boehringer Ingelheim	Active site/linear
Telaprevir (VX-950)	Vertex	Withdrawn
Boceprevir (SCH503034)	Merck	Withdrawn
Nucleoside analogue NS5B polymerase inhibitors (NI)		
Valopicitabine (NM283)	Idenix/Novartis	Active site
R1626	Roche	Active site
Mericitabine (R7128)	Roche/Pharmasset	Active site
GS-938	Gilead	Active site
IDX184	Idenix	Active site
Non-nucleoside NS5B polymerase inhibitors (NNI)		
BILB 1941	Boehringer Ingelheim	NNI site 1/thumb
MK-3281	Merck	NNI site 1/thumb 1
VX-759	Vertex	NNI site 2/thumb 2
VX-222	Vertex	NNI site 2/thumb 2
VX-916	Vertex	NNI site 2/thumb 2
ABT-072	AbbVie	NNI site 3/palm 1
HCV-796	ViroPharma/Wyeth	NNI site 4/palm 2
Filibuvir (PF-00868554)	Pfizer	NNI site 2/thumb 2
IDX375	Idenix	NNI site 4/palm 2
Tegobuvir (GS-9190)	Gilead	NNI site 4/palm 2
GS-9669	Gilead	NNI site 3/palm 1
Deleobuvir	Böhringer	NNI site 3/palm 1
Host targeting agents		
NIM811	Novartis	Cyclophilin inhibitor
Alisporivir (Debio-025)	Novartis	Cyclophilin inhibitor

small groups of patients have been confirmed in the phase Ⅲ studies and in "difficult-to-treat patients" [such as in check of previous regimens (including protease inhibitors), cirrhotic patients, liver or kidney transplanted patients, HIV infected patients or multi-drug treated patients, at an increased risk of the drug interaction][63]. Nevertheless, viral variants resistant to DAAs can emerge during the antiviral therapy[295-298]

(Table 4). The estimation of a putative virus resistance profile prior to an antiviral therapy can help to select the optimal treatment regimen for individual patients[295,296].

CONCLUSION

A prominent genetic diversity of HCV in combination with non-trivial replication cycle poses serious problems in virus research and therapy of virus-associated diseases. However, during the last several years a significant progress in understanding of the HCV molecular biology and pathogenesis has been achieved. In many aspects that allows to making a great step forward towards antivirals design, that result in DAA therapy. However, even with the new drugs the HCV-associated problems, of both fundamental and applied origin, are not solved. In fact, the resolution of these problems is going hand in hand.

First, in the absence of direct cytopathic effect, the mechanism of lipid deregulation, that results in liver pathologies, is not completely understood. Second, the system of the HCV control during latency and putative triggers of virus active replication were poorly investigated. Third, design of prophylactic vaccine is still in its infancy. Finally, the access to drugs and therapies for the people leaving in the third world is very restricted. Even if SVR rates > 90% are reached, nearly 10 million people will be living without treatment options[63]. Indeed, an easy access to diagnosis and treatment is still missing to drug users, people in difficult socio-economic situations, migrants, prisoners, because of themselves or health policy.

Therefore, fundamental studies on virus-cell interactions and studies directing towards development of the prophylactic vaccine should be intensified.

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REVIEW

Cell fusion in the liver, revisited

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Abstract

There is wide agreement that cell fusion is a physiological process in cells in mammalian bone, muscle and placenta. In other organs, such as the cerebellum, cell fusion is controversial. The liver contains a considerable number of polyploid cells: They are commonly believed to originate by genome endoreplication, although the contribution of cell fusion to polyploidization has not been excluded. Here, we address the topic of cell fusion in the liver from a historical point of view. We discuss experimental evidence clearly supporting the hypothesis that cell fusion occurs in the liver, specifically when bone marrow cells were injected into mice and shown to rescue genetic hepatic degenerative defects. Those experiments-carried out in the latter half of the last century-were initially interpreted to show "transdifferentiation", but are now believed to demonstrate fusion between donor macrophages and host hepatocytes, raising the possibility that physiologically polyploid cells, such as hepatocytes, could originate, at least partially, through homotypic cell fusion. In support of the homotypic cell fusion hypothesis, we present new data generated using a chimera-based model, a much simpler model than those previously used. Cell fusion as a road to polyploidization in the liver has not been extensively investigated, and its contribution to a variety of conditions, such as viral

infections, carcinogenesis and aging, remains unclear.

Key words: Cell fusion; Hepatocytes; TdTomato; Lineage tracing; Chimeras; Extracellular vesicles

Core tip: About 70% of hepatocytes are polyploid, arising either from genome duplication without division (endoreplication) or from cell fusion. Experiments with chimeric mice containing two cell populations each bearing a different genetic marker had shown that some liver cells express markers of both genomes, suggesting that cell fusion occurred. Here, we review the data in the literature and describe new experiments using a chimeric model that confirms that cell fusion contributes to liver polyploidy. We argue that the role of cell fusion in pathological conditions, such as viral hepatitis and neoplastic transformation, is worth further study.

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INTRODUCTION

Mammalian cells are usually diploid, with the exception of mature gametes, which are haploid. Interestingly, a few tissues contain polyploid cells, such as muscle cells, osteoclasts, hepatocytes, megakaryocytes and trophoblasts[1]. A common feature in all these tissues is the presence of a diploid progenitor cell that at some point during the differentiation/maturation process becomes polyploid. Polyploidization can be explained by two main mechanisms: endoreplication and cell fusion. Endoreplication occurs when the genome is duplicated without cell division, whereas cell fusion occurs between two different cells, either of the same or of a different identity. In the latter case, the genomes from two different cell types-which can come even from different species-fuse within the same membrane and, therefore, coexist within the same cell[2]. For both mechanisms, the outcome is a polyploid cell.

POLYPLOIDY IN NORMAL CELLS

It is generally recognized that polyploidization in normal organisms is adaptive since it helps specialized cells acquire the ability to perform new, specific functions[3]. For example, osteoclasts are large multinucleated cells that perform the difficult task of resorbing bone matrix. This specialized function cannot be accomplished, for example, by mononucleated TRAP$^+$ osteoclasts as this leads to osteopetrosis, a disease in which bone is not degraded[4]. Among the other types of polyploid cells, there is agreement that muscle cells and trophoblasts are products of cell fusion. In contrast, megakaryocytes are polyploid cells generated by genome duplication followed by aborted cytokinesis[5].

Polyploid liver cells are usually considered to be formed by endoreplication of the genome[6,7]. This conclusion was originally based on the seminal work of Mintz and colleagues, who pioneered the use of chimeric mice to study gene expression[8]. In the chimeric mouse, cells with two different genomes coexist in a single organism. If a cell fuses with another having a different genome, the resultant cell will contain markers of both. In their studies, Mintz and colleagues concluded that fusion occurred in muscle but not in the liver[8-10]. However, they had to rely mainly on the analysis of isoforms expressed by tissue-specific enzymes because single-cell markers were not yet available. Contrary to osteoclasts, trophoblasts and muscle cells, whose nuclei maintain their individuality, polyploid liver cells can be bi- or mono-nuclear.

Specific cell cycle genes, such as Cdk1, are involved in polyploidy formation and maintenance in liver[11]. Interestingly, after partial hepatectomy, quiescent hepatocytes can start proliferating again: deletion of cyclin-dependent kinase 1 (Cdk1), 2 (Cdk2), cyclin E1 or E2 individual genes does not limit liver regeneration, but concomitant ablation of Cdk2 and Cyclin E1 reduces liver regeneration, suggesting partial overlapping function of some cell cycle genes[11,12].

TRANSDIFFERENTIATION AND FUSION: A DEBATE NOT YET SETTLED

The study of cell fusion in the liver began while investigating the existence of lineage transdifferentiation. The possibility of cell transdifferentiation was raised at the end of the last century in a paper published in Science[13] in which the authors, after having transplanted neural stem cells transgenic for the beta-galactosidase (βgal) gene into wild-type mice, detected βgal$^+$ cells in peripheral blood. Their interpretation was that neural cells had transdifferentiated into cells of the hematological lineage. This plasticity was surprising to many but, because the report was published just after the birth of Dolly the sheep, looked plausible[14]. We must emphasize here that that work had nothing to do with the reprogramming approach reported several years later by Yamanaka, who, in contrast, obtained reprogramming by forced expression of intracellular transcription factors[15]. The plasticity of neuronal stem cells was claimed by Bjornson *et al*[13] to occur by simple exposure to endogenous factors present *in vivo*.

Essentially, studies on plasticity were performed by transplanting cells from a mouse transgenic for an easily detectable marker gene (*e.g.*, βgal) into a non-

transgenic animal. The appearance of βgal-marked cells in an organ different from the tissue of origin was interpreted to be the result of transdifferentiation. The Science paper in which cells of the nervous system were suggested to acquire a hematological fate, was rapidly followed by other examples of transdifferentiation involving cells of several other lineages, including blood, brain, muscle, kidney and heart[16-25].

Due to the high potential for translation to the clinic, bone marrow cells (BMCs) escalated to center stage. BMCs are easily obtained, extensively investigated, routinely transplanted and well-characterized in humans. If simple transplantation protocols allowed the rescue of degenerative defects in organs such as brain, kidney or liver, we would have a sort of panacea in hand. Unfortunately, although many clinics-mostly in the United States-still advertise these kinds of treatments[26], transdifferentiation as originally proposed in the Science paper has not been confirmed by subsequent, more controlled studies[27-32]. Indeed, although a limited transdifferentiation capacity of some cells cannot be completely ruled out, more-recent studies have shown that transdifferentiation is often an experimental artifact. As stated above, transdifferentiation was claimed to occur if, after a given lineage (for example, hematopoietic cells) expressing a reporter gene was transplanted into a wild type mouse, cells of other lineages (for example, brain) were found to coexpress the reporter gene with accepted markers of their lineage. While βgal was initially used as a marker, most subsequent papers exploited fluorescent reporter genes that could be easily traced *in vivo*. It was assumed that all fluorescent cells found in a normal, non-transgenic, mouse had to be the progeny of transgenic donor cells: Hence, if they were found in other organs, they must have derived from original cells that had acquired a new fate by transdifferentiation.

In addition to trivial technical artifacts, cell fusion was raised to explain some of these results: in the experimental design discussed above, fusion between any cells of the host with transplanted donor cells could have provided the former with the reporter gene. It is difficult to discriminate between the two possibilities-transdifferentiation and cell fusion-with simple marker analysis.

As mentioned above, transdifferentiation of BMCs would be an attractive approach for regenerative medicine. Heart, brain and liver are heavily affected by degenerative genetic diseases that have a huge impact on human health; they would all greatly benefit from cell fusion-based therapies using exogenous cells, if that mechanism indeed occurs *in vivo*. Certainly, exogenous cells could provide defective endogenous ones with the missing genetic component while maintaining the differentiation status of the mature cell.

With regard to the liver, several reports in which BMCs were transplanted into recipient mice in the hope of inducing hepatocyte transdifferentiation showed that cells bearing donor-derived cellular markers could be found in host livers[18-21]. These "transdifferentiated" cells increased in number when the host livers were either injured (partial hepatectomy) or affected by a chronic degenerative genetic defect. However, the results were challenged by scientists who were unable to reproduce the transdifferentiation of hematological cells into non-hematological ones[33-36]. Cell fusion was shown to occur *in vivo*, so several reports investigated fusion events in a variety of other models. In the liver, the most spectacular experiments were performed by Grompe's group on the classical model of fumarylacetoacetate hydrolase (Fah) deficiency[37]. Mice recessive for a Fah mutation are models for tyrosinemia type Ⅰ, a severe genetic disease leading to liver failure in humans. Grompe and coworkers showed that bone marrow transplants in these mice led to the generation of liver cells bearing the donor marker, and demonstrated that this event was not due to transdifferentiation of hematological into hepatic lineage cells. Instead, these marker-carrier liver cells originated from cell fusion between donor bone marrow and resident hepatocytes, leading to polyploid cells that were not easily distinguishable from true hepatocytes in that the latter could also be polyploid. Due to the growth advantage shown by normal hepatocytes over diseased ones, the approach was so efficient that several mice were essentially cured. Results were confirmed by further studies[38-40], which also pointed to macrophages as the hematological cell responsible for fusion[41,42]. These results are in agreement with macrophages being physiologically prone to cell fusion[43]. In a review of 77 published studies on the generation of hepatocytes by hematopoietic cells transplanted in liver, the authors concluded that cell fusion was the mechanism involved[44]. Cell fusion is enhanced by the presence of liver injury or chronic disease, such as in the Fah model, since in a well-controlled study in which BMCs were injected into normal recipients, only 7 out of 470000 liver cells examined bore donor markers as a result of cell fusion[34]. In addition to BMCs, other types of cells, such as mesenchymal or amniotic stem cells and cells differentiated from pluripotent stem cells, can fuse with cells in injured livers, even when injected into a different species[45]. Human umbilical cord blood cells have also been reported to fuse with hepatocytes of immunocompromised mice[46], although no evidence of cell fusion was reported in other studies[47-49]. Moreover, cell fusion and transdifferentiation have been claimed to coexist[50].

The cell fusion-based explanation was found to hold also in other similar experimental settings[34,37,39,51,52] (reviewed in[27,53,54]). However, the possibility that at least in some cases, especially when an injury is applied to the recipient organ, bone marrow donor cells could be directed toward a different fate has not been completely ruled out, since several reports of well controlled differentiation have been published[55-64].

CELL FUSION IN THE NORMAL LIVER

The discovery that cell fusion can cure a degenerative disease of the liver prompted Grompe's group to investigate whether cell fusion occurs also in the disease-free state. The experimental plan to address this was as follows: they transplanted 1 × 10^5 wild-type ($Fah^{+/+}$) hepatocytes into each of four $Fah^{-/-}$/βgal+ recipients. After more than 80% of the liver was repopulated, 1 × 10^5 hepatocytes were serially transplanted into each of two $Fah^{-/-}$ recipients and the liver was again repopulated to a donor contribution of more than 80%. Then they analyzed 3 × 10^7 Fah^+ hepatocytes, but were unable to find a single $Fah^+/\beta gal^+$ cell. They concluded that the frequency of cell fusion, if any, was very low[41].

It must be taken into considerations that the protocol involved damaged livers and injections of adult cells. However, although complex, the approach looks suitable to address the question of cell fusion in the disease-free liver. The only caveat is that, if the originally transplanted wild-type cells were mature hepatocytes (the age of the mice used was not specified), then it is possible that they represent polyploid cells that were already fully differentiated and functional and, therefore, less prone to fuse. This is because at this stage they have already achieved the benefits of being large cells with multiple genomes.

Apart from the original studies by Mintz and colleagues already cited, other studies investigating whether cell fusion occurs in the normal liver are lacking. Cell fusion has occasionally been reported to occur in hepatocytes or in hepatic tumor lines cultured *in vitro*[65-67]. Yet, this does not prove that the mechanism is physiologically relevant *in vivo*. For these reasons, Faggioli and coworkers devised and implemented a relatively simple but straightforward protocol based on chimeric mice, as originally proposed by Mintz's group[8-10]. Embryo-derived mouse chimeras are mice born from embryonal cells carrying different genomes[68]. They can be created either by morula aggregation or by injection of embryonic stem cells (ESCs) into blastocysts, and they can be exploited for the study of cell fusion. If each of the two aggregated morulae contains a different reporter gene, then cells positive for both reporters will definitively be fused cells.

Faggioli *et al*[71] reasoned that by aggregating morulae from two different strains of transgenic mice expressing either green fluorescent protein (GFP)[69], or the βgal protein (Rosa 26 mouse[70]), the outcome would be animals that display two genetically distinct liver cell populations, each bearing a single marker (either GFP or βgal); any cell displaying both markers must be the result of cell fusion. With the appropriate controls, they identified three populations: $GFP^+/\beta gal^-$; $GFP^-/\beta gal^+$; and $GFP^+/\beta gal^+$. The percentage of double-positive cells in the chimeric samples was estimated to be about 25%.

The authors confirmed their results with two other independent strategies. Briefly, they performed PCR amplification on single hepatocytes with primers specific for each reporter gene, finding cells displaying both markers only in chimeric mice, in a percentage close to 10% of cells bearing at least one marker. In addition, the authors used fluorescent *in situ* hybridization (FISH) to investigate the sex chromosome content of hepatocytes in XY◀═▶XX chimeric mice. They reasoned that, if fusion occurred between a female and a male cell, some binucleated cells containing Y chromosome(s) only in one of the two nuclei would be detected. Similarly, if mononucleated polyploid hepatocytes were analyzed, they should contain only X chromosomes in various numbers in the case they derived from the XX component of the chimeric mouse, or as many X as Y chromosomes if derived from an XY cell. In contrast, if the mononucleated polyploid hepatocytes were products of a cell fusion event between a female and a male cell, an unbalanced complement of X and Y chromosomes would be found. In the end, sex chromosome patterns were detected that were clearly indicative of cell fusion in binucleated as well as mononucleated hepatocytes in about 5%-10% of cells[71].

These results are at odds with those presented by Willenbring *et al*[41]. This discrepancy could be explained by the different approaches used, since that of Faggioli *et al*[71] mimics normal liver development, while the one used by Willenbring *et al*[41] involves the injection of exogenous hepatocytes into damaged liver and complex transplantation experiments. As mentioned before, this could ultimately lead to underestimation of fusion events in the latter study.

We are not aware of recent studies investigating cell fusion in normal liver, although replication of the chimera studies would not be too time consuming. Apparently, fusion is neither considered to occur frequently nor to be of physiological relevance. For this reason, while performing a study on the role of cell fusion in cancer[72], we addressed cell fusion with an even simpler approach based on the production of chimeric Cre: tdTomato mice. Morulae derived from mice transgenic for Cre recombinase under the control of a constitutive promoter were fused to morulae from mice transgenic for an inactive floxable tdTomato gene that is activated only if Cre recombinase is expressed in the same cell (Figure 1A). Cells from the two morulae will develop independently and no cell will be tdTomato-positive unless fusion with a Cre-containing cell has occurred (see the schematic representation in Figure 1B).

This approach-which has been widely used for lineage and transplantation studies-has the advantages of having an undetectable background if cell fusion does not occur, no interference between the two fluorescent reporter genes, and simple assessment in liver sections with well-validated tdTomato-specific antibodies. In addition, leakiness of the promoter, which sometimes occurs in Cre-based conditional mice, does not affect this model.

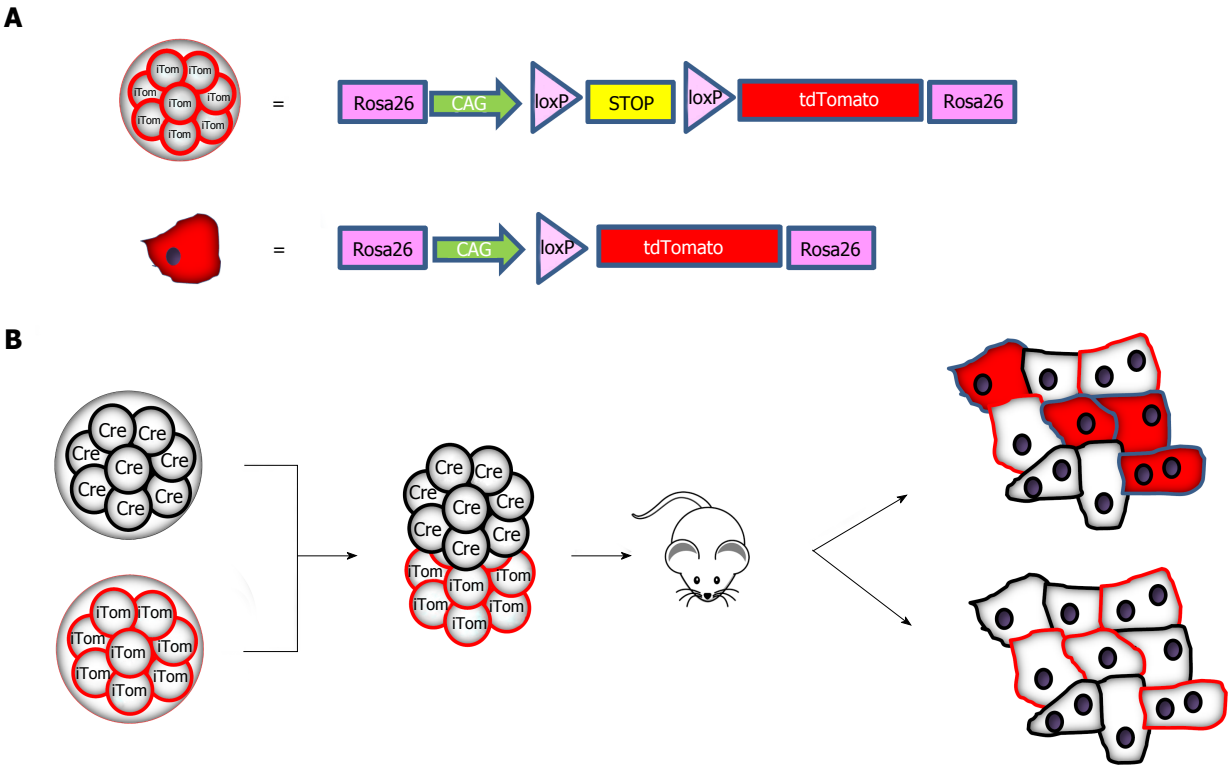

Figure 1 Schematic representation of the Cre: TdTomato approach here described. A: Depiction of the transgene carried by the tdTomato mouse (JAX 007905). The transgene is inserted in the Rosa26 locus and contains a Tomato gene (tdTomato) under the control of a constitutive promoter. Between the promoter and the gene there is a stop cassette flanked by two loxP sequences in the same orientation that prevents tdTomato transcription in this configuration. If the stop cassette is removed by exposure to the Cre recombinase, the tdTomato gene becomes expressed and its expression is maintained through the cell life; B: The generation of mouse chimeras by aggregation of one Cre+ morula (Cre, black-circled cells) derived from CMV-Cre mice (JAX 006054) with a tdTomato one (iTom, red-circled cells) is shown. The aggregated morulae are transferred to females and the progeny analyzed at three months of age. tdTomato expression should occur only if a Cre+ cell fuses with a tdTomato one (full red hepatocyte), whereas if no fusion occurs only tdTomato-negative cells are seen.

Analysis performed to date on two chimeric mice has clearly identified the presence of tdTomato-positive cells in the liver (Figure 2). Positivity was not detected in wild-type mice or in inactive tdTomato mice. As expected, the progeny of tdTomato-Cre mice crosses were positive in all tissues.

Fused cells are distributed all over the liver parenchyma, but are often found in clusters. This is in keeping with cell fusion occurring in cells maintaining their proliferative capacity, giving rise to a progeny that expands but remains in close proximity to their original location. This is in agreement with other studies showing hepatocytes originating form clonally derived clusters in postnatal liver[73,74].

However, the devil is in the detail and we are always at risk of artifacts[75]. In the chimeric experimental design, coexpression in the same cell of two reporter genes originally expressed independently by two distinct cells is commonly accepted as proof of a fusion event. This assumption was used in our original work on cell fusion[71]. However, the detection of fluorescence is prone to artifacts caused by endogenous background fluorescence, a phenomenon especially marked in liver; in the case of βgal, endogenous enzymatic activity can also lead to misinterpretation. In addition, it has become increasingly appreciated over the last ten years that transfer of materials-including RNA and proteins-between cells *via* extracellular vesicles is a frequent phenomenon[76-78]. Therefore, it cannot be excluded that in the Cre-tdTomato approach aforementioned, RNA encoding Cre recombinase or tdTomato could have been transferred from the Cre^+ cell to the tdTomato one, and thus activating the reporter locus leading to expression of the reporter protein. Even the transfer of a few RNA or protein molecules over a very short period of time can activate the tdTomato gene, which then would become permanently expressed. However, the Cre-Lox and GFP systems have been widely used, in general giving consistent results for expression and expected specificity. Unfortunately, with the technologies available to date there is no way of discriminating fusion events from vesicle-mediated transfer *in vivo* while maintaining physiological conditions. In this regard, it is worth mentioning that several recent papers analyzing the fate of GFP^+ cells transplanted into mouse retina have reported the detection of GFP^+ cells that did not originate from the donor[79-81]. This suggests that GFP activity was leaked into the intracellular space and absorbed by endogenous cells or was transferred to them by extracellular vesicles–fusion can be excluded since retinal cells were normal in size and not polyploid. This is troubling if true, and some lineage

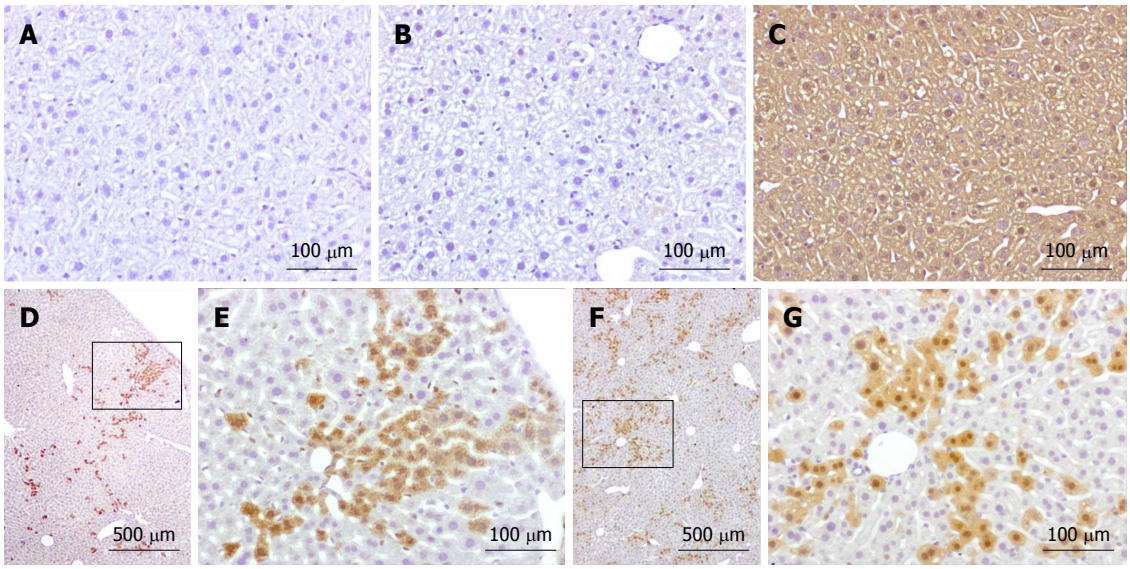

Figure 2 Immunohistochemical analysis of tdTomato expression in livers of chimeras and controls. For tdTomato detection, 3 μm slices were stained with anti-RFP antibody (ab124754: Abcam, Cambridge, United Kingdom) diluted 1:100 in PBS containing 0.05% Tween 20. Detection was performed using Mach 1 HRP-polymer (Biocare Medical, Concord, CA, United States) incubation followed by the revelation with Betazoid DAB (Biocare Medical). A: C57BL/6J wild-type mouse; B: Inactive tdTomato mouse; C: Cre+/tdTomato+ double transgenic mouse; D-G: Cre:tdTomato chimera 1 and 2, respectively. Wild type and Inactive Tomato mice are completely negative; Cre+/tdTomato+ are completely positive due to activation of the tdTomato gene in all cells. In the chimeras, many cells are negative, but a fraction shows clear expression of the tdTomato gene, which has been activated by the co-expression of Cre in fused cells. E and G: higher magnification views of the boxed area in D and F respectively.

or transplantation studies based on the detection of reporter genes should be carefully re-examined.

Techniques based on *in situ* hybridization with probes specific for sex chromosomes can be used to demonstrate cell fusion[71], since the presence of an XY nucleus as well as an XX one in a binucleated cell should definitively be due to cell fusion. This technique-which does not allow the analysis of live cells-has been used in studies on the ploidy of hepatocytes, with the caveat that the analysis might be complicated by the aneuploidy shown by some normal human and murine liver cells[82-85]. In any case, it will be difficult to investigate cell fusion in man: in theory, transplantation of male hepatocytes in female hosts performed for regenerative liver diseases could detect cell fusion, but this is a very rare occurrence and would require biopsies or post-mortem examination.

CONCLUSION

Cell fusion in the liver is still controversial. Thus, replication of previous studies with appropriate mouse chimeras is welcomed. Endoreplication and cell fusion are not mutually exclusive, as suggested by Gentric and Desdouets[86]. We strongly believe that fusion in the liver should be studied in order to confirm and explain this phenomenon. If established, this will open several new lines of investigation. For example, is cell fusion or endoreplication preferred in different contexts, or are they interchangeable? What is the fusion potential of hepatocytes with a DNA content higher than 4n? Are there hepatocytes with unbalanced or uneven-n chromosome numbers, and are there fusion products between one diploid and one tetraploid cell? Does cell fusion occur in species other than rodents, and particularly in man? Can fused cells participate in the ploidy reduction occurring after partial hepatectomy? Are HBV or HCV infections, which are themselves fusogenic viruses, able to change hepatocyte ploidy and binuclearity[87], or do other metabolic stresses[88] affect endoreplication or fusion? Does cell fusion play a role in HCV-mediated liver carcinogenesis[89]?

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REVIEW

Management of bacterial infection in the liver transplant candidate

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Abstract

Bacterial infection (BI) is a common cause of impairment of liver function in patients with cirrhosis, especially in the liver transplant candidates. These patients share an immunocompromised state and increased susceptibility to develop community and hospital-acquired infections. The changing epidemiology of BI, with an increase of multidrug resistant strains, especially in healthcare-associated settings, represents a critical issue both in the waiting list and in the post-operative management. This review focused on the role played by BI in patients awaiting liver transplantation, evaluating the risk of drop-out from the waiting list, the possibility to undergo liver transplantation after recovery from infection or during a controlled infection.

Key words: Cirrhosis; Portal hypertension; Bacterial infection; Liver transplantation

Core tip: Bacterial infection (BI) is a common cause of impairment of liver function in patients with cirrhosis, especially in the liver transplant candidates. BI may play a detrimental role in patients awaiting liver transplantation, increasing the risk of drop-out from the waiting list.

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INTRODUCTION

The liver is actively involved in inflammatory response against bacteria, and plays a central role in the regulation of immune defense, bacterial clearance, acute-phase protein, cytokine production and metabolic adaptation to inflammation[1]. Conversely, sepsis-induced hypoxic hepatitis and cholestasis make hepatic dysfunction an independent predictor of mortality during bacterial infection (BI)[2,3].

Cirrhosis is *per se* an immunocompromised state which predisposes to the development of BI, and sepsis-related death[4]. It's characterized by an immunodeficient state due to an impaired response to pathogens at different levels of the immune system, involving innate and adaptive cell dysfunction[5]; this condition coexists with a persistent stimulation of immune system, with enhanced serum levels of pro-inflammatory cytokines[6,7]. The severity of this inflammatory state correlates with severity of liver dysfunction[8,9]. Moreover, other superimposed conditions - such as impaired gut microbiota and intestinal barrier dysfunction - further increase the risk of BI[10]. In a study by Rasaratnam *et al*[11], when patients were treated with selective intestinal decontamination, their hepatic venous pressure gradient decreased by a mean of 2.43 mmHg, further strengthening the hypothesis that bacteria contribute to the hyperdynamic circulation and portal hypertension in cirrhosis.

Sepsis-related organ damage in cirrhosis is characterized by both an excessive inflammatory response and a decrease in the hepatic capacity of tolerance[12]. This further increases circulatory dysfunction, with splanchnic vasodilation and organ hypo-perfusion[13,14], leading to worsening of portal hypertension (*via* activation of neurohumoral pathways) and fluid retention[15]. Development of BI is a common trigger of extra-hepatic organ failures, in particular acute kidney injury[16], hepatic encephalopathy[17], coagulopathy[18], adrenal insufficiency[19] and respiratory failure[20].

CHANGING EPIDEMIOLOGY OF BACTERIAL INFECTION IN DECOMPENSATED CIRRHOSIS

In the past decades, there have been several improvements in the management of cirrhosis and its complications, such as hepatocellular carcinoma and portal hypertension. However, a significant proportion of patients with decompensated cirrhosis still need liver transplantation (LT), which represents the only effective therapeutic option.

In this setting, development of BI could significantly impair the natural history of the liver transplant candidate[21,22]. Preventive and therapeutic strategies for most of the complications of cirrhosis are well-defined; nevertheless, even if risk factors for the onset of BI in decompensated patients awaiting LT are well-known, they remain poor preventable.

The protean epidemiology of BI in cirrhosis depends on several factors, such as site of infection, setting of BI development, and local epidemiology.

Spontaneous bacterial peritonitis (SBP) and urinary tract infections are the most frequent BI in cirrhosis, followed by pneumonia, skin and soft tissue infections, bloodstream infections (BSI)[12,23]. SBP is mainly due to bacterial translocation - especially gram-negative strains[24], however epidemiology is rapidly changing. A multicenter study from Portugal[25], evaluating patients with severe liver dysfunction (median Child-Pugh class C-10; MELD score 19) recently showed an increase in gram-positive bacteria (GPB, 42%) at diagnosis of SBP; notably, one out of three SBP episodes occurred during hospitalization. This has determined the adoption of new antibiotic strategies: Piano *et al*[26] demonstrated that the combination of broad-spectrum antibiotics, meropenem plus daptomycin, was significantly more effective than conventional therapy (ceftazidime) in the treatment of nosocomial SBP (86.7% *vs* 25%; $P < 0.001$).

BSI represent another common cause of BI in cirrhosis[12], mainly due to gram-positive strains[27], because of the high number of invasive procedures and quinolone prophylaxis. However, there's an increasing prevalence of gram-negative bacteria (GNB) as the cause of BSI; Bartoletti *et al*[28] showed in a multicenter observational study on 312 cirrhotic patients in Italy, an equal distribution between GNB and GPB (53% *vs* 47%) at diagnosis of BSI.

In the last decades, clinical practice in Hepatology has dramatically changed as a consequence of the implementation of the liver transplant programs. Cirrhotic patients are nowadays frequently admitted to the ICU and undergo many diagnostic and therapeutic invasive procedures. This is associated with a higher risk of secondary infections caused by nonclassical pathogens. Fernandez *et al*[29] included 572 BI, 39% of which were nosocomial, reporting an increase in the rate of GPB infections associated with the increasing use of invasive procedures during hospitalization and in the ICU. More recently, Merli *et al*[30], collected 173 episodes of BI requiring hospitalization or occurred during hospitalization in 424 patients with cirrhosis; BI episodes were further divided into three classes (community acquired, hospital acquired, health-care acquired). GNB were more frequent in community acquired and health-care acquired infections, while GPB in hospital-acquired ones. *Enterobacteriaceae* (44.3%) (particularly *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*) and *Enterococci* (*Enterococcus faecium* and *Enterococcus faecalis*) (19.7%), were the pathogens most frequently responsible for infection.

Table 1 Risk factors of bacterial infection in cirrhosis

Risk factors for bacterial infection in cirrhosis
Impairment of liver function
Child-Pugh score[36-38]
MELD score ⩾ 15[40]
Low serum albumin[39]
Alcohol related disease[45,51]
Total ascitic fluid protein concentration < 15 g/L[84]
ICU admission[39,85]
Variceal bleeding[41,86]
Blood transfusion requirements
Mean arterial pressure
Severity of bleeding
Malnutrition[40]
Invasive procedures[29]
ERCP in PSC patients or with incomplete drainage[87]
Hospitalization[29,40,43,44]

MELD: Model for end stage liver disease; ICU: Intensive care unit; ERCP: Endoscopic retrograde cholangiopancreatography; PSC: Primary sclerosing cholangitis.

The spreading of multidrug resistant (MDR) bacteria-related infection is alarming worldwide[31,32]. In the past, patients with cirrhosis remained largely unaffected by this phenomenon because they were rarely admitted to the ICU. Nowadays, frequent hospitalizations, development of LT programs and antibiotic prophylaxis made cirrhosis at high risk of MDR infections[33]. A multicenter study in Italy[34] reported a 27% (83/395) prevalence of MDR infections, mainly due to GNB (extended-spectrum β lactamase *E. Coli* and Carbapenems resistant *K. Pneumoniae*). Another study from Greece[35] reported 19% MDR infection rate amongst patients with SBP, being MDR bacteria associated with healthcare-acquired infections and with patients at higher MELD score (28 *vs* 19, $P = 0.012$). Also in the above-mentioned study by Merli *et al*[30], MDR infections occurred more frequently during hospitalization (56% in hospital acquired/ healthcare-associated infections *vs* 22% in community acquired infections, $P = 0.008$).

RISK FACTORS FOR BACTERIAL INFECTION IN END STAGE LIVER DISEASE

Patients with end stage liver disease, listed for LT, have the highest risk for BI development. This might be due to frequent hospitalizations, severity of liver dysfunction and multiorgan failure (Table 1). Correlation between the development of BI and severity of liver disease has been widely demonstrated[36]. In a Japanese group of patients with cirrhosis and hepatocellular carcinoma[37], incidence of BI rose from 2.3% in Child-Pugh class A patients to 25.6% in Child-Pugh class C patients. In a further study[38], even if patients with cirrhosis were younger and had less cardiopulmonary comorbidities than patients without cirrhosis, they had higher rates of septic shock at hospital admission, and equal mortality. Moreover, bacteremia and mortality increased with the severity of liver disease (5.6%, 20.5%, 33.3% and 0%, 8.5%, 38.9% in Child-Pugh classes A, B, and C, respectively; $P = 0.038$).

Furthermore, low serum albumin, ICU admission, and GI bleeding, are other independent predictors of BI development, according to Deschenes *et al*[39]. Merli *et al*[40] analyzing 54 BI episodes occurred in 50 patients, showed that, at multivariate analysis, a MELD score ⩾ 15 (OR: 2.8, 95%CI: 1.3-6.1), history of previous infection within 12 mo (OR: 4.7, 95%CI: 2.2-10.6), and a diagnosis of malnutrition (OR: 4; 95%CI: 1.5-10) were independent predictors for infections and sepsis.

Variceal bleeding is another predictor of BI onset; Tandon *et al*[41], reviewing the available literature on patients admitted for GI bleeding without receiving antibiotic prophylaxis, showed that 242 out of 552 (44%) developed an episode of BI. Severity of GI bleeding, according to blood transfusion requirements (HR: 1.22; 95%CI: 1.01-1.47), mean arterial pressure (HR: 0.96; 95%CI: 0.93-0.99) were independent predictors for BI onset[42]. In another Spanish study[29], 126 patients underwent at least one invasive procedure, comprising variceal sclerotherapy or banding, surgical intervention, trans-jugular intrahepatic portosystemic shunt, having a higher probability of developing BI due to GPB.

Sinclair *et al*[43] showed that 43% of LT candidates required at least one hospitalization within 1 year; moreover, a significant proportion of hospitalized patients (> 45%) required repetitive hospitalisations. Patients with cirrhosis have 4 to 5-fold higher probability to develop a BI episode during hospitalization than non cirrhotic population[42,44].

The role of etiology of underlying liver disease as a risk factor for BI development is debated[45]. Alcohol abuse is associated with increased intestinal permeability, dysbiosis and increased bacterial translocation[46]. Furthermore, Legionella and Mycobacterium tuberculosis infections are significantly more prevalent in patients with alcohol abuse[47,48]. In the setting of cirrhosis, several studies reported a higher rate of BI in patients with alcoholic etiology when compared with non-alcoholic[49,50]. Sargenti *et al*[51] evaluating characteristics of 398 BI in 633 cirrhotics (363 alcoholic, 270 nonalcoholic), reported a similar occurrence of BI between groups, but pointed out that alcohol related disease was significantly associated with bacterial pneumonia and GPB.

IMPACT OF BACTERIAL INFECTIONS IN LIVER FUNCTION

Worsening of liver function is frequently observed in patients with infection, especially in those with sepsis, being itself a trigger for multiorgan failure, and development of Acute on Chronic Liver Failure (ACLF)[52-54].

In the above-mentioned study by Merli *et al*[40], Child–Pugh and MELD scores worsened in 62% of patients after infection; moreover, onset of ascites, hepatic encephalopathy, hyponatremia, hepatorenal

syndrome, were more frequent in patients with infection as compared with those who were not infected.

Prognosis of BI significantly correlates with the severity of liver disease and with the severity of extra-hepatic organ involvement[54,55]. A systematic review[21] considering 11987 patients with an episode of BI from 178 different studies, reported 1-, 3-, and 12-mo mortality of 30.3%, 44%, and 63%, respectively, and almost half of patients surviving at 1 mo died within a year. Renal failure, stage of cirrhosis (according to Child-Pugh score), age and severe sepsis were the factors independently associated with death. Several studies confirmed the critical role of renal failure in patients with cirrhosis and BI[56,57]. Mortality increased with the occurrence and severity of acute kidney injury and with the outcome of renal failure (15% 90-d mortality after complete recovery, 40% after partial renal recovery, and 80% in patients without renal recovery or progression). According to the study by Cazzaniga *et al*[58], systemic inflammation and fulfillment of SIRS criteria, are other factors significantly associated with mortality, since in-hospital mortality of these decompensated patients with MELD score > 18 rose from 12% to 43%. Dionigi *et al*[22] retrospectively evaluated prognosis of patients who were hospitalized in a tertiary center in the United Kingdom; they demonstrated that in-hospital mortality rate was higher in those patients who had infection at admission and/or developed infection during hospitalization (HR: 5.02; 95%CI: 2.75–9.16; P < 0.001).

IMPACT OF BACTERIAL INFECTION IN THE LIVER TRANSPLANT CANDIDATE

The onset of BI usually determines a further worsening of liver function and multiorgan failure, with high probability of death or drop-out from the WL[59,60]. Reddy *et al*[61] prospectively evaluated the outcome of 136 patients after an episode of BI developed while awaiting LT: 42% were delisted or died, 35% underwent transplantation, and only 24% achieved transplant-free survival within 6 mo. As expected, those who remained in the waiting list had a lower MELD score compared to those who either received a transplant or died/delisted; furthermore, those patients who underwent LT after BI recovery had a significant higher survival than those without LT (95% *vs* 5%; P < 0.001). At univariate analysis, the number of organ failures was the main factor that predicted death or delisting, whereas MELD score did not differentiate between those who were ultimately transplanted *vs* those who were delisted. Mounzer *et al*[62] showed that, 38% of patients who had experienced an episode of SBP before waiting list admission, were subsequently removed from the list or died.

Regarding patients who fully recovered from an episode of BI, the study by Sun *et al*[63] showed that recipients with pre-transplant BI (n = 32) within 12 mo before LT had a higher MELD score (median 25 *vs* 22, P < 0.05) at transplant, higher time of post-LT intubation (3 d *vs* 2 d, P = 0.05), and longer post-transplant hospitalization (29 d *vs* 20 d, P = 0.05). However, post-transplant mortality was not different between groups (9.4% *vs* 2.9%) and was not associated with pre-LT infection. Lin *et al*[64] retrospectively analyzed the outcome of 34 living donor LT candidates who had experienced an episode of BI within 4 wk prior surgery, which was effectively treated (*e.g.*, disappearance of symptoms and signs suggestive of sepsis, normalization or improvement of laboratory and/or imaging findings after antibiotic therapy). The post-operative outcome was compared with 20 patients with pre-LT ACLF without infection. The only difference between groups was the longer total hospital stay (89.0 d *vs* 65.5 d, P = 0.024), whereas post-LT ICU stay, one-year survival, and post-LT infection rates were similar between groups. Few data are available on the possibility to offer a standardized MELD exception after recovery from infection[65-67]. A possible scenario is the onset of recurrent episodes of cholangitis in PSC candidates; in a study by Goldberg *et al*[68], 300 patients who received MELD exception points for an increased risk of waitlist mortality, had a lower proportion of death/drop out (20.0% *vs* 1.3% P < 0.001); however, this non-standardized exception has not been further confirmed.

Several studies recently investigated the outcome of patients who underwent LT under "controlled" infection. In an Italian study[69], 84 patients were considered eligible for LT after disappearance of symptoms and signs suggestive of severe sepsis/septic shock. The overall post-LT 90-d mortality, septic shock, and sepsis as cause of death were not significantly different between infected and not-infected LT recipients; however, patients with previous infection had in the post-operative course higher rates of infections (40% *vs* 36%, P = 0.003) and post-transplant MDR strains (26% *vs* 13%, P = 0.005). Artru *et al*[70] recently demonstrated that ACLF grade 3 patients were transplanted in France after they had recovered from an episode of BI according to a subjective criterion of "controlled sepsis" for at least 24 h within transplant; the authors demonstrated an excellent 1-year post-LT survival (83.8%), not different than that observed in patients with no ACLF or with lower stages of ALCF.

MDR bacteria colonization represents another important issue in the setting of WL, because of the risk of spreading of BI in the post-operative course and/or after the introduction of immunosuppression. Giannella *et al*[71] prospectively evaluate the role of carbapenems resistant K. *Pneumoniae* (CR-KP) colonization (*e.g.*, presence of MDR bacteria in the rectal swab in absence of symptoms and signs of active infection) in 237 patients awaiting LT, of whom 11 (4.6%) were positive at the time of LT. Hospital admission, higher MELD at LT, prior antibiotic exposure, post-operative complications, and ICU length of stay were the factors associated with the CR-KP active infection after LT. In addition, the same group, performing a multicenter prospective

study on CR-KP carriers[72], not only in the setting of LT, demonstrated that the number of additional colonization sites was an independent risk factor for invasive infection.

In conclusion, BI significantly modify the natural history of patients with cirrhosis listed for LT. Severe BI in a sick and frail patient can produce a multiorgan failure comprising further deterioration of liver function. Even if this can increase priority in the WL, this gain in priority should be used only after adequate control of infection. To date, standardized definition of "controlled infection" is lacking. As for other patients with severe ACLF in the WL[73,74], prioritization rules in the respect of distributive justice, definition of the ideal timing for LT and definition of delisting criteria have to be refined in the next future.

MEDICAL PROPHYLAXIS OF BACTERIAL INFECTION

Antibiotic prophylaxis in patients with decompensated cirrhosis is standard of care in patients with recent gastrointestinal bleeding[75], and in those with high risk of SBP(*e.g.*, Child-Pugh > 9, serum bilirubin > 3 mg/dL and impaired renal function), or in secondary prophylaxis for SBP[24].

Antibiotic prophylaxis after upper GI bleeding reduces the incidence of in-hospital infections, re-bleeding rate within 7 d (7% *vs* 34%), and 28-d mortality (13% *vs* 35%, $P = 0.04$).

However, some concerns about long-term prophylaxis has been recently raised, since it's been associated with high prevalence of MDR BI, before and after LT. Tandon *et al*[76] evaluating 110 episodes of BI (30% hospital acquired), reported 47% of antibiotic resistance and a significant association between previous exposure to systemic antibiotics and antibiotic-resistance. Infections due to MDR bacteria are associated with an increased risk of septic shock, acute kidney injury, and death, in the post-transplant setting[44]. Furthermore, antibiotic use has been identified as the strongest predictor of invasive post-transplant fungal infection, associated with a 60% mortality[77].

Even if several studies suggested the need to stratify patients who need antibiotic prophylaxis, both after variceal bleeding and after an episode of SBP, no robust data are available to date[12,23,78,79].

Patients with cirrhosis admitted to ICU could be at higher risk of BI. Recently, a metanalysis on prognosis of cirrhotics admitted to ICU showed that acute kidney injury and sepsis as indications to ICU admission were the only factors significantly associated with mortality[80]. Another retrospective study[81] on 42 patients who underwent LT from the ICU, showed that pre-LT intubation was a factor significantly associated with post-LT pneumonia ($P = 0.02$).

On the contrary, patients who recover liver function while in the WL (*e.g.*, after viral eradication/suppression), history of BI would not be a sufficient factor for administering long-term antibiotic prophylaxis. In addition, the spreading of MDR bacteria will reduce the potential role of antibiotic mono-prophylaxis with quinolones or cephalosporines.

Given the crucial role played by dysbiosis in BI in patients with cirrhosis, several studies assessed the role of intestinal decontamination. Grat *et al*[82] evaluated the fecal microflora in 40 LT candidates, showing that abundance of several species (*e.g.*, *Bifidobacterium* and *Enterococcus*) significantly correlated with the severity of liver disease. In systematic review and metanalysis, Safdar *et al*[83] compared parenteral (*e.g.*, cephalosporins/quinolones), topically applied or non-absorbable antibiotic strategies (polymyxin, gentamicin, and nystatin) for intestinal decontamination. The Authors found an association between selective decontamination and reduction of GNB infections ($P = 0.001$), however studies were underpowered and heterogeneous.

CONCLUSION

BI represent a turning point in the natural history of cirrhosis, being the first cause of development of ACLF, and significantly affecting the outcome of patients listed for LT. These patients are at the highest risk of infection, because of frequent hospitalizations and contacts with healthcare facilities, immune dysregulation, end-stage liver disease. SBP, pneumonia and bloodstream infection represent the commonest sites of BI. In such cases, early institution of empirical antibiotic therapy is mandatory, to reduce infection-related mortality. However, empirical antibiotic therapy should take into account the changing epidemiology of infections, related both to an increase of gram positive strains and to MDR bacteria.

In the setting of LT, patients should be considered suitable for transplant after resolution of infection. However, according to recent studies, selected patients with "controlled infection" should be considered for transplant, since this condition does not impair the post-transplant outcome[69,70]. Antibiotic prophylaxis is the standard of care in cirrhotic patients with gastrointestinal bleeding or with previous episodes of SBP. However, it should be considered also in other settings with a high prevalence of BI, as in patients listed for LT, admitted to ICU and requiring intubation, because of a higher risk of post-LT pneumonia.

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REVIEW

Digital liver biopsy: Bio-imaging of fatty liver for translational and clinical research

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Abstract

The rapidly growing field of functional, molecular and structural bio-imaging is providing an extraordinary new opportunity to overcome the limits of invasive liver biopsy and introduce a "digital biopsy" for *in vivo* study of liver pathophysiology. To foster the application of bio-imaging in clinical and translational research, there is a need to standardize the methods of both acquisition and the storage of the bio-images of the liver. It can be hoped that the combination of digital, liquid and histologic liver biopsies will provide an innovative synergistic tri-dimensional approach to identifying new aetiologies, diagnostic and prognostic biomarkers and therapeutic targets for the optimization of personalized therapy of liver diseases and liver cancer. A group of experts of different disciplines (Special Interest Group for Personalized Hepatology of the Italian Association for the Study of the Liver, Institute for Biostructures and Bio-imaging of the National Research Council and Bio-banking and Biomolecular Resources Research Infrastructure) discussed criteria, methods and guidelines for facilitating the requisite application of data collection. This manuscript provides a multi-Author review of the issue with special focus on fatty liver.

Key words: Biobank; Bio-imaging; Fatty liver; Genomics; Liver biopsy; Liver cancer; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Magnetic resonance; Radiomics; Ultrasound

Core tip: The manuscript provides an extended expert review on the issue of bio-imaging of the liver with special focus on fatty liver and the need for a new integrated approach to bio-banking.

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INTRODUCTION

Autopsy, from the Greek autopsía, namely seeing with our own eyes or direct examination after death, gave birth to modern pathology during the Italian Renaissance when autopsies were performed by such artists as Leonardo da Vinci to paint and sculpt the human body and by such medical doctors as Benivieni to understand the causes of death. Two and half centuries later, the father of anatomical pathology Morgagni executed hundreds of autopsies and released the monumental five-volume On the Seats and Causes of Disease[1]. The first liver aspiration biopsy on a living patient was performed in 1883 by Paul Ehrlich, but the breakthrough that allowed the spread of liver biopsy as a diagnostic tool in clinical practice was the simple and effective technique invented by Menghini in 1958[2]. The combination of liver histology from conventional biopsy and circulating biomarkers (liquid biopsy) helps in generating an understanding of the interplay between genes and epigenetic factors in physio-pathogenic processes, providing a bimodal approach for the study and understanding of liver physio-pathology. The liver biopsy specimen however, provides a random sampling of just 1/150000th of the liver, and as such is subject to considerable sampling errors in cases with an inhomogeneous distribution of the pathologic lesion[3]. Moreover, it is obtained by an invasive procedure, unsuitable for the repeated sampling necessary to follow the *in vivo* dynamics of many physio-pathologic mechanisms. In addition, in the process of fixation and staining, the melting of intracellular fat results in artifactual ghost droplets. In short, histology provides a dead, isolated frame from a living film that runs throughout the liver, and this has deeply limited the advancement of knowledge regarding the physiopathology of fatty liver.

Modern biomedical imaging techniques offer an attractive non-invasive option for the *in vivo* study of liver physiopathology and have the capacity to provide detailed anatomical and biochemical information on the whole organ, thereby overcoming the limit of sampling error. The combination of image-based digital liver biopsy with liver histology and liquid biopsy could yield a new multimodal approach to the study of the fatty liver in clinical pathology. To foster this approach in clinical and translational research there is a need to standardize the methods of both acquisition and storage of bio-images of the liver.

ACQUISITION OF HEPATIC BIO-IMAGES

Biomedical tomographic images are rich in information that is increasingly subject to quantitative radiological assessment. A major development in computer-aided

research and diagnosis in recent years is based on computing multiple image features from volumes of interest (VOIs) or regions of interest (ROIs) of an organ and/or pathological tissue, and linking them to clinical or physio-pathological features, namely radiomics[4-6]. Automatic and semiautomatic image algorithms are mandatory for computing large numbers of image features from standardized VOIs and or ROIs for 2D/3D[7-12]. After computation, the image features can be used in correlation studies with physio-pathological and clinical characteristics and to build predictive models[13,14]. Because many of the computed features are correlated, appropriate machine learning methods are useful for establishing sustainable pattern recognition analysis[15-17].

In parallel to these developments, a great deal of new knowledge about the physiopathology of fatty liver disease has been accumulated in recent years, revealing the complexity of the mechanisms involved in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) and the severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation[18-20]. The most recent guidelines and expert opinions for the management of NAFLD patients call for a new, systems medicine approach to the study of the interplays between the major physiology systems that control the vital relations of the liver with the environment, brain and nervous system, endocrine system, digestive system (gut and microbiota) and immune system[19]. New concepts for patient stratification are needed to identify different clinically significant profiles within the generic context of metabolic-syndrome[18]. This is especially important given that NAFLD affects almost one third of the general population, is an emerging cause of liver related mortality, and no reliable predictors of disease progression and response to therapy are yet available that can be applied on a large scale.

In keeping with this premise, we propose the digital liver biopsy as a new paradigm for full exploiting the information contained in multi-modality or multi-contrast conventional and molecular liver imaging, with the aim of establishing a comprehensive platform of liver radiomics, capable of capturing the heterogeneity of liver pathology associated with intra-hepatic fat accumulation. Central to our vision is that digital liver biopsies be archived together with stored specimens from both liquid and histologic biopsies in a national bio-imaging repository network including reference hepatology units and an accredited biobank hub[21]. This will foster the advancement of liver radiomics analysis, incorporating multiple texture and intensity-based features to identify those consistent with morphological transformations and highly correlated with clinic-pathologic characteristics. These imaging biomarkers will increase the potential of translational and clinical research and properly address several unmet needs for precision medicine[22-25]. The approach also encourages studies combining new imaging techniques with liver and blood metabolomics, as well as the analysis of the interplay between specific genes and the epigenetic factors conditioning their expression; opening a very interesting new way to target the dynamics of the pathogenic processes involved in NAFLD/NASH. While we hope that these new studies will identify different aetiologies, new diagnostic and prognostic biomarkers, and therapy targets; the underlying aim is to establish a basis for better patient stratification in respect to both prevention and outcome prediction that can support personalized treatment of NAFLD. The starting points for such work are the imaging technologies that are already well-established in clinical routine or well-advanced as imaging biomarkers. From current translational research and clinical practice there are several relevant PET/TC developments, as well as offshoots of ultrasound (US) and magnetic resonance (MR) imaging techniques[24-27].

LIVER ELASTOGRAPHY FOR ASSESSMENT OF FIBROSIS

Liver stiffness has been measured using magnetic resonance elastography (MRE), ultrasound-based transient elastography (TE, Fibroscan), and acoustic radiation force impulse imaging techniques in order to assess fibrosis for defining different degrees of the disease and predicting clinical outcomes[28-32]. Despite their many advances, these techniques have several limitations that must be taken into account[33-36]. TE for instance, is hampered by the presence of significant fat and/or fluid between the chest wall and the liver. These result in unreliable TE measurements in about a quarter of obese patients: A figure that likely can be reduced using a larger probe, at least in non-severely obese individuals[35,36]. Lee *et al*[37] compared the diagnostic performance of transient elastography (TE) with acoustic radiation force impulse imaging (ARFI) for staging fibrosis in nonalcoholic fatty liver disease (NAFLD). Liver stiffness correlated with fibrosis stage ($P < 0.05$) and the area under the ROC curve of TE (kPa) was slightly better than ARFI (m/s), namely 0.757 *vs* 0.657. MRE is more accurate than TE, especially for detection of initial stages of liver fibrosis, but it is influenced by iron overload and requires additional hardware[38]. Other limitations of MR elastography are technical, such as ensuring coupling of the driver to the abdomen, and ensuring that wave interference and attenuation do not compromise the stiffness values in some parts of the liver. Despite these obstacles and the limited clinical use seen at present due to expense and restricted availability, it is expected that MRE will come to represent the "gold standard" for tissue stiffness measurement[39,40].

The most important limitation of liver stiffness measures is that they are surrogates of liver fibrosis rather than a metric of fibrosis itself. Moreover, fibrosis is just one of the three major pathophysiological vectors, that determine liver stiffness; the others being congestion

and inflammation[33,34]. The impact of congestion can easily be ruled out in patients without right heart failure through examination under standard fasting conditions, but there remains a need for additional clinical and pathological information and/or biomarkers to precisely characterize the different and relative contributions of inflammatory and/or fibrotic processes to liver stiffness in the single patient[34].

MAGNETIC RESONANCE RELAXOMETRY FOR ASSESSMENT OF FIBROSIS

Contrast in MR images is the product of exponential relaxation processes, the time constants of which (T1, T2, and T2*) are determined by the physico-chemical environment of the tissues. While T2 values are typically dominated by tissue water content, the use of T2 measurements for characterizing liver inflammation is complicated by iron sequestration and storage in hepatocytes. In fact, the use of T2 shortening (seen with spin-echo sequences) is well established for estimating high levels of iron overload, while T2* shortening (evident with gradient echo sequences) being more iron-sensitive is used in characterizing low levels of iron content. Similarly, several studies[24,41,42] have reported that liver T1 varies with degree of fibrosis. A number of fast imaging approaches have been developed that permit whole-liver T1 and T2 mapping within a small number of breath-holds, raising the potential for their clinical use. As yet however, these techniques are not widely available and need to be validated for use as surrogate endpoints in clinical trials[43].

Building on these components, a multi-parametric MR strategy has recently been established[26] that includes T1 mapping for the *in vivo* characterization of fibrosis/inflammation, T2* mapping for liver iron quantification, and proton magnetic resonance spectroscopy (1H-MRS) for liver fat quantification (see below) without the intravenous injection of contrast agents. A good agreement with histology was shown in a cohort of patients with chronic liver disease of different etiologies[26]. Excitingly, other emerging MR biomarkers, including magnetization and saturation transfer, intra-voxel incoherent motion imaging, and perfusion mapping, most of which are well established in neurological imaging, offer potential to further characterize fibrosis, but remain to be optimized and adapted to liver imaging.

MAGNETIC RESONANCE SPECTROSCOPY FOR ASSESSMENT OF STEATOSIS

Sensitivity to the subtle differences in magnetic fields affecting atoms of a given species due to their different positions within a molecule or in different molecules has rendered magnetic resonance spectroscopy (MRS) one of the most powerful tools in biochemistry. In the face of the complex chemical environment of the liver, and in the modest static magnetic fields available for *in vivo* use, however, the scope for MRS is greatly limited. Nonetheless, in expert hands, it is considered a gold standard technique for *ex vivo* evaluation of tissue fat content and commonly used as an *in vivo* reference standard[45-47]. In terms of analysis, MRS is straightforward. One simply integrates the area under the water and the individual fat component peaks of the hydrogen spectrum, and then calculates the ratio of fat signal to the total hydrogen signal.

Although the acquistion of the hydrogen spectrum is relatively fast, there are a number of difficulties with this technique. Firstly, it measures only in a small region of the liver, and so lacks information on heterogeneity within the liver. Second, the positioning of the volume is subject to uncertainty due to patient respiration – in particular differences in breath-hold position between planning and acquistion can produce large errors in the fat fraction estimation[48]. Third, the presence of iron accumulation can affect the reliability of the measurement. One approach for overcoming the limit of a single sampling volume is to perform MRS in multiple voxels simultaneously so that an image can be formed (chemical shift or spectroscopic imaging)[47]. Relative to conventional MRS however, spectroscopic imaging is time consuming, and requires specialized pulse sequences that are not uniformly available across centres. More importantly, the resolution of the images remains rather poor, and contamination errors due to blurring reduce the accuracy of fat fraction measurements. In fact, at least one report has suggested that neither MR spectroscopy nor chemical shift imaging provide adequate accuracy for clinical decision-making[46].

PROTON-DENSITY FAT FRACTION MEASUREMENT

When observed in weak magnetic fields ($\leqslant$ 1T), the MR spectrum of fat is largely reduced in its primary peak associated with hydrogen bound to carbon along the backbone of the fat molecule (triglycerides). This observation has given rise to a widely used method of creating water and fat images in MRI that was initially proposed by Dixon[48]. Because of their different resonant frequencies, the water and the primary fat peak cycle in and out of phase after excitation. The difference between an image formed when the peaks are in-phase and one formed when they are out-of-phase is proportional to the quantity of fat present and one can readily calculate a fat fraction. A significant limitation of this "two-point" Dixon (or in-phase: out-phase) technique is ambiguity as to which (*i.e.*, water or fat) is the dominant component. Uncorrected, the assumption that water dominates leads to significant errors when the fat fraction exceeds 50%[45]. In the liver, a further complication of the two-point Dixon technique is associated with the presence of iron, which can be increased several hundredfold in patients with iron overload[44]. At elevated iron contents, the MR signal decays significantly between the in and

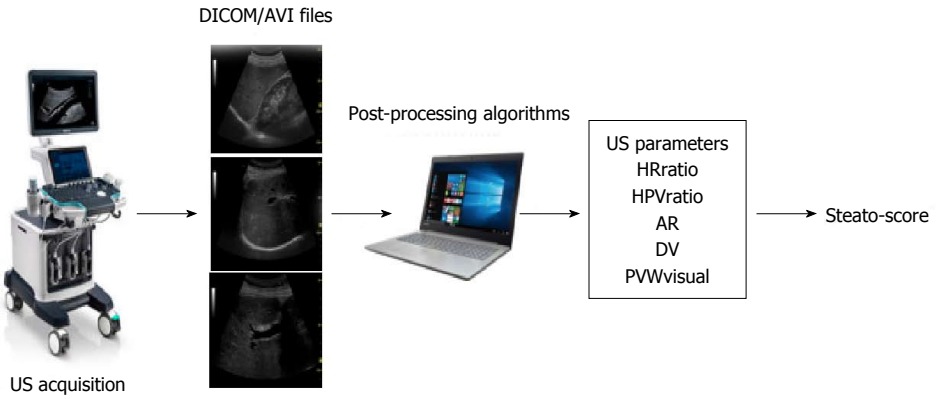

Figure 1 Quantitative multi-parametric assessment of intrahepatic fat by ultrasound. Workflow of the image acquisition, processing and data elaboration leading to a score provided by an algorithm (Steato-score)[65]. US: Ultrasound; AR: Attenuation rate; DV: Diaphragm visualization.

out of phase echoes, creating an artificial elevation of the fat fraction (or reduction if the out of phase echo is acquired first).

At higher magnetic field strengths, and in the presence of good magnetic field shimming, the hydrogen spectrum becomes more complex as the different physical-chemical environments occupied by hydrogen within fats contribute additional frequencies (chemical shifts) relative to water. As not all of the peaks are in-phase (nor out-of-phase) at any given time, the additional peaks produce variability in the fat fraction estimates when using the two-point Dixon technique[48]. To deal with the errors due to the contributions from the minor fat peaks in the hydrogen spectrum, Yu *et al*[49] proposed the use of multi-component model fitting to multi-echo MRI data to produce proton density fat fraction (PDFF) measurements. After a rapid evolution to further account (up to certain limits) for ambiguities in water or fat dominance, T2* effects associated with iron content, and the possibility to perform quantification with or without phase information[50-54], PDFF has emerged as the preferred MR imaging strategy for fat quantification[55].

Although PDFF measurements[56] have been reported to better correlate with biochemical analysis (Folch method[57]) than with the conventional histopathology grading of fat content, there is a relatively good concordance between MRI-PDFF and liver histology[58,59]. Relative to the conventional Dixon technique, PDFF measurements offer increased accuracy and repeatability, and can be used to assess the full range of fat values with less risk of inversion between water and fat values. The fact that the entire liver can be examined with PDFF scans provides a considerable advantage over MR spectroscopy for measurement stability as a high number of voxels contribute to estimates of fat fraction in the liver as a whole or in individual lobes thereof. Achieving this improved performance however, requires care in the choice of imaging parameters acquisition, and software specifically adapted to the fitting process.

There are now numerous PDFF software solutions that offer varying degrees of support, cost and availability for clinical and research use. Most free-ware solutions for example, do not offer support either in use of the software, or for the optimization of imaging parameters and data preparation. The maturity, cost and support for commercially available software is varied, and their reliability and accuracy are still being established in practice.

INTRAHEPATIC FAT MEASURED BY ULTRASOUND

US imaging has many advantages over MRI/MRS since it is simple, quick, less expensive and well tolerated. Moreover, the equipment is relatively widely available and, if needed, can be brought to the patient and conventional US is already widely used for diagnosis and management of liver pathologies. However, while US provides reliable qualitative diagnosis of liver steatosis when liver fat is above roughly 20%[60-62], its quantitative assessment is considered unreliable due to operator dependence. As described above, US can be used to measure shear wave propagation as an indicator of liver stiffness in fibrosis[58,59] and the Controlled Attenuation Parameter (CAP) score provided by Fibroscan (Echosens, Paris, France) was the first widely available quantitative US measure of intrahepatic fat in clinical practice[63,64]. It is based upon a single parameter, the US beam attenuation rate along a beam transmission line.

There is now a trend to adopting multi-parametric strategies to better assess steatosis quantitatively. One such approach is based on the acquisition of common scan projections (both subcostal longitudinal and oblique views) available with commercial US systems[65]. Two recordings (clips of 5 s) allow the quantification of 5 different parameters associated with intrahepatic fat accumulation (Figure 1). These include the hepatic-renal ratio (HR_{ratio}) and the hepatic-portal vein ratio (HPV_{ratio})[60,61] that describe the echogenicity of the liver parenchyma in comparison with renal cortex and inner portal vein, respectively. In addition, the attenuation rate (AR) takes into account the reduction of US beam penetration[61], while grades are assigned to diaphragm

visualization (DV, assessing the degree of diaphragm line visualization) and portal vein wall visualization (PVW_{visual}, assessing blurring of the portal vein walls). An algorithm (Steato-score) combining these five parameters showed good diagnostic performance discriminating presence (⩾ 5%) or absence (< 5%) of steatosis relative to ^{1}H-MRS with an area under the receiving operator characteristic (ROC) curve of 0.98 and roughly 90% specificity and sensitivity[65].

In a further refinement, the five parameters (HR_{ratio}, HPV_{ratio}, AR, DV, PVW_{visual}) will be combined with texture analysis of the liver parenchyma in the images to discriminate multiple steatosis classes using machine learning approaches. Taking ^{1}H-MRS values as ground-truth, the diagnostic performance of advanced algorithms will extend the quantification of higher intrahepatic fat levels by ultrasound[65-67].

PET TO STUDY LIVER METABOLISM

Positron emission tomography (PET) is able to image *in vivo* biochemical processes quantitatively; molecules labeled with positron-emitting radioisotopes are used in trace quantities (*i.e.*, without pharmacological effect) to visualize and measure rates of biochemical processes *in vivo*. Among the various PET-tracers, the fluorinated glucose analogue [^{18}F]-2-fluoro-2-deoxy-D-glucose (FDG), is the most widely used for metabolic, neurologic, and oncologic research as well as in clinical practice. Inflammatory and cancer cells are both metabolically active and show an increased uptake of FDG which is subsequently phosphorylated to FDG-6-phosphate and trapped intracellularly because most of FDG-avid cells, including inflammatory cell, do not express glucose-6-phosphatase. Hepatocytes on the other hand, do express this enzyme, and release FDG back into the blood.

Several studies have examined the potential of this difference in FDG kinetics in the study of fatty liver[68-70], comparing hepatic FDG uptake in NAFLD patients to controls using the semi-quantitative measurement of standardized uptake value (SUV). The results however, are mixed with some reporting a negative correlation between liver FDG uptake and NAFLD[69], others a positive[70] and others no association[69]. These conflicting results may be the consequence of the many factors, both physiological and technical that can affect SUV values[71,72]. Interestingly, FDG PET studies have nonetheless shown a link between vascular inflammation and NAFLD[73] as well as that liver SUV in patients with NAFLD may be a prognostic factor for cardio-cerebrovascular events[74,75].

From a metabolic point of view the options on PET tracers are manyfold providing a role to play in the study of NAFLD pathology. A first example is the study of hepatic metabolism in the pathogenesis of the metabolic syndrome and its clinic-pathologic correlates (*e.g.*, diabetes, hypertension and obesity). A negative correlation between higher hepatic glucose uptake HGU and liver fat content was reported in overweight subjects with type 2 diabetes[76]. These results support the notion that NAFLD is epiphenomenon of insulin resistance. The liver is an insulin sensitive organ[77] and thus, hepatic glucose uptake is reduced in subjects with insulin resistance.

Other aspects of liver metabolism such as fatty acid uptake, oxidation, and perfusion are also amenable to study with PET techniques. In a recent study in morbidly obese subjects, Immonen and coworkers, using a palmitate analog, [^{18}F]-fluoro-6-thiaheptadecanoic acid (^{18}F-FTHA), found morbidly obese subjects to have elevated hepatic fatty acid uptake (HFU) before bariatric surgery that decreased after surgery, though it did not normalize relative to lean subjects. Intriguingly, HFU and liver fat content in these patients were not related[78]. Iozzo *et al*[79] using ^{11}C-palmitate-PET scanning showed that obese subjects have higher rate of fatty acid oxidation than lean subjects. According to these authors, the assessment of fatty acid oxidation is of major importance in characterizing subjects with NAFLD, since fatty acid oxidation is a significant source of reactive oxygen species in obesity-related hepatic lipotoxicity. Finally, with appropriate modeling, PET like MRI can be used in the quantification of liver perfusion in humans[78,80].

In the oncologic field, FDG-PET scanning is the gold standard, due to the avidity of tumors for glucose. Moreover, as used in this context, serial SUV measurements is a reliable tool, with the individual patient serving as their control before and after treatment. In hepatocellular carcinoma, FDG-PET has shown its usefulness both for identifying more aggressive tumors predictive of lower survival rates, and for capturing early signs of response to treatment. In pre-clinical models, FDG-PET scanning showed capacity to quantify tumor response[81] with potential usefulness in tumor monitoring. In the clinical setting, FDG-PET has proven useful as survival predictor: patients with lower tumor to liver ratio (TLR) at time 0 showed three times longer survival than patients with high TLR[82] and the SUVmax parameter was an independent predictor of survival in HCC patients undergoing Sorafenib treatment[83]. Most interestingly, the discriminating capacity of FDG-PET associated with the clinic pathologic response to the anti-angiogenetic drug was shown to be detectable as soon as three weeks from the beginning of treatment[84], thus an ealry FDG-PET scan after starting anti-angiogenetic treatment seem to be a promising technique for monitoring early response[85].

Undoubtedly, PET imaging in the liver has provided new insight in quantifying different metabolic pathways in humans. Whereas measuring inflammation in the liver until now has been proven challenging, FDG-PET has a clear role in patients affected from hepatocellular carcinoma.

IMAGING MASS SPECTROMETRY

IMS combines the mass spectrometry with the Matrix-assisted laser desorption/ionization (MALDI) technique,

and allows the visualization of the spatial distribution of metabolites, drugs, peptides, proteins and lipids, directly on tissue sections without the need for extraction (biochemical analysis) or the use of specific markers (enzyme- or immune-histochemistry). The advantage of this technique is in mapping the subcellular localization and morphology on tissue sections and, at the same time, providing qualitative and quantitative evaluation of thousands of analytes. This bi-dimensional technique can also be adapted to fathom below the tissue surface to produce a three-dimensional map of the molecules (having molecular weights between 1000 to 70000 Da) present in the histological sections. The signals of ions obtained from the histological section/sample can be mapped in specific regions of the tissue and transformed into images on the basis of the density of the ions inside the tissue.

As an example of the use of IMS, accumulation of C14:1 acylcarnetine in a steatotic liver has suggested a deficiency of very long-chain acyl-CoA dehydrogenase permitting molecular genetics analysis to be addressed towards the detection of the underlying mutations[86].

There are in fact a growing number of techniques similar to IMS, including synchrotron infrared and ToF-SIMS (time of flight secondary ion mass spectrometry) micro-spectroscopies[87] capable of identifying molecular species of lipids concentrated in steatotic vacuoles or present in small lipid droplets that most probably correspond to the first step of lipid accretion. Likewise these techniques are of interest for tracking and understanding the effects (pharmacokinetics, toxicology, metabolomics) of drugs on cells in metabolic diseases and NAFLD. While not applicable *in vivo* they are likely to play an increasing role in the chemical imaging/analysis of pathology samples to provide high resolution targets, in both spatial and biochemical terms for the understanding of radiological findings of NAFLD.

RADIOMICS

The success of precision medicine requires a clear understanding of disease heterogeneity at the single patient level, yet despite the large radiological armamentarium available, most imaging-based clinical decision-making is still based solely on visual assessment. The combination of visual assessment and image-processing techniques that describe and quantify numerous image features, such as intensity-based and textural properties that are difficult to convey consistenty in verbal reports, can provide a comprehensive characterization of imaging data sets. This approach adds value to the individual quantitative measure or visual subjective report and the derived results can be combined with statistical modelling techniques to predict clinical end points. The field of research that examines the relationships between advanced quantitative imaging features from medical images and clincal data is called "Radiomics", while "Imaging Genomics" (or radiogenomics) refers to the study of imaging features in association with high-throughput genetic data[88] (Figure 2).

Radiomic features are derived from the information contained in the voxels of a segmented structure of an image, and are typically grouped into families having similar derivational or statistical roots. First-order statistical features are reflected in the histogram of voxel intensities, and include: the mean value, dispersion (standard deviation, mean absolute deviation), central moments (skewness and kurtosis describing asymmetry and sharpness, respectively) and randomness (entropy, uniformity). Texture or greyscale variation features, refer to higher-order statistical measures that describe the local spatial arrangement of intensities. This typically reduces to expressing how different parent matrices capture the spatial intensity distribution of an image, and is widely used as a low-level step in pattern recognition. In contrast to the pre-defined features associated with texture analysis, the rapidly growing approach to radiomics represented by deep learning draws on advanced optimization of self-generated patterns to create networks for recognition analogous to neurons. While originally developed for planar images, intensity-based and textural features measures and deep learning can generally be calculated for volumetric datasets.

Radiomics analysis, as a technique that objectively measures the structural and/or functional heterogeneity of the tissue by quantifying the spatial patterns of pixel intensities on cross-sectional imaging, can improve the clinical usefulness of multimodal biopsy data. Recent pilot studies employing radiomic approaches on multimodal and hybrid imaging (CT, optical, PET/MRI, PET/CT) have shown the usefullness of quantifying the overall tissue spatial complexity in identifying the regions which drive disease transformation, progression, and drug resistance in a variety of pathologies[17,90-93].

Both radiomics and imaging genomics are at very early stages in hepatology and many problems remain to be solved. To date, the vast majority of liver-related radiomic studies have focused on the analysis of malignant lesions for establishing prognostic or predictive models. For example, in 2007, Kuo *et al*[88] observed significant correlation between tumour margins in arterial phase images and doxorubicin-response gene expression. In 2015, Miura and coauthors[96] identified 53 up-regulated and 71 down-regulated subsets of genes in the highly aggressive HCC group compared with the low-grade HCC group as distinguished by gadolinium ethoxybenzyl-DTPA magnetic resonance imaging hyperintensity. They have also shown that clinico-pathological and global gene expression analyses revealed low-grade malignancies within high-grade HCCs compared with low-grade HCCs. Recently, Zhou *et al*[97] have extracted radiomics features from arterial and portal venous phase using CT images, identifying twenty-one radiomics features from a panel of 300 candidate features, to predict the early recurrence ($\leqslant$ 1 year) of hepatocellular carcinoma. A further example of the scope for synergy formed by the three-

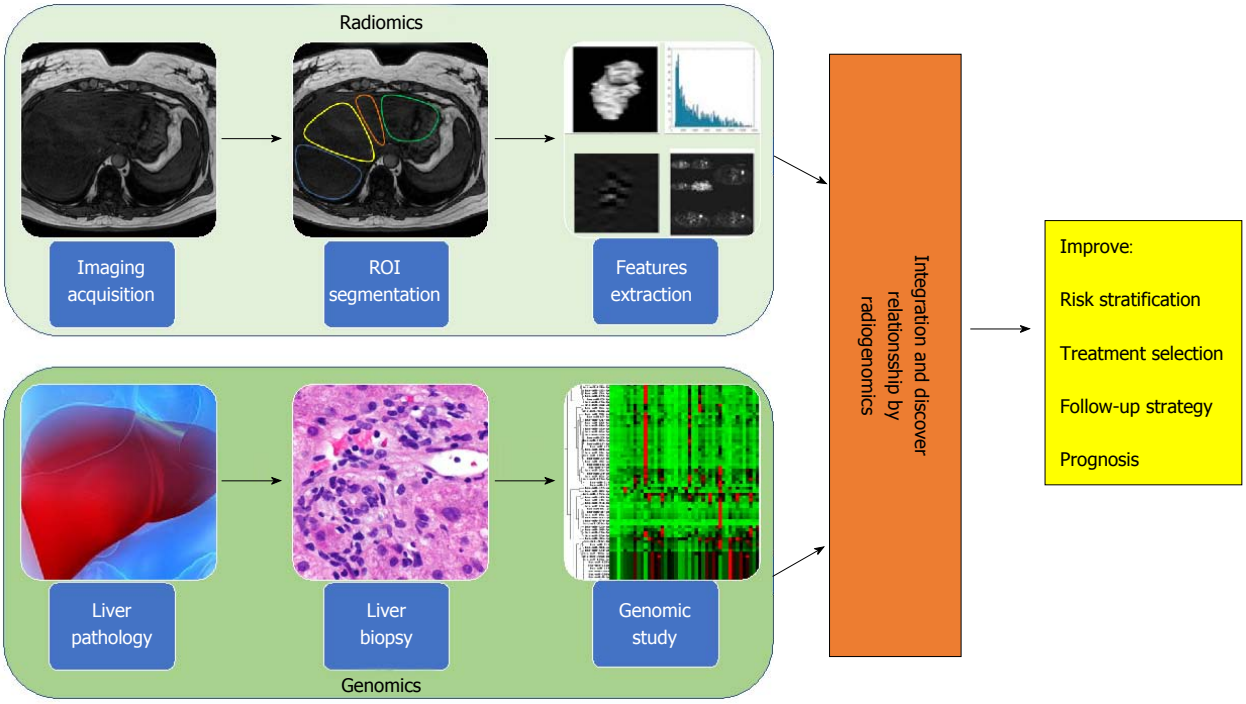

Figure 2 Radiogenomic approach to liver disease. Radiogenomics integrates radiomic data (upper panel), produced from the in silico extraction of features from bio-images, with genomic data (lower panel), coming from the study of bio-specimens with next generation sequencing technologies. Radiogenomics represents a powerful strategy to improve and personalize diagnostic accuracy, as well as measure response to therapy, leading to an overall improvement of patient management affected by liver disease.

dimensional vision of digital, liquid and phsyical biopsy, is the recognition of relationships between histologic and clinical phenotypes including microvascular invasion of hepatocellular carcinoma and prognosis of intrahepatic cholangiocarcinoma evidenced *via* MRI diffusion-weighted imaging and hepatobiliary phase after gadoxetic acid[94,95].

In one of the few reports applying the radiomic approach to the study of NAFLD, in 2015 Vanderbeck *et al*[90] developed an automated classifier that detected and quantified macrosteatosis with at least 95% precision. They applied an automatic quantifier of lobular inflammation and ballooning to digital images of hematoxylin and eosin stained slides of liver biopsy samples from 59 individuals with normal liver histology and varying severity of NAFLD. Their results demonstrated that automatic quantification of cardinal NAFLD histologic lesions is possible, and offer promise for further development of automatic quantification of NAFLD biopsies in clinical practice.

Genome-wide association studies of hepatic fat content as determined radiologically on the other hand, have already led to major breakthroughs in the field, with the identification of genetic variants influencing hepatic fat accumulation[98,99]. In addition, although fibrosis stage is considered the major prognostic determinant in patients with NAFLD/NASH[100], recent data suggest that the amount of hepatic fat accumulation plays a causal role in determining the development of NASH and its progression[101]. Thus, a reliable non-invasive measure of intrahepatic fat may represent an important outcome predictor in both clinical research and practice.

Radiogenomics findings in hepatic oncology illustrate the potential for further clinical insights and diagnostic pathways. For example, Banerjee *et al*[106] found an interesting correlation between a "Radiogenomic Venous Invasion" biomarker (a contrast-enhanced CT parameter) and a 91-gene HCC "venous invasion" gene expression signature. This biomarker may be able to predict histological microvascular venous invasion in HCC that may be useful for identifying patients less likely to a durable benefit from surgical treatment. Renzulli *et al*[107] also found a correlation between multiple imaging biomarkers, such as tumor dimension, non-smooth tumor margins, peri-tumoral enhancement, and gene expression called "two-trait predictor of venous invasion", as predictor of microvascular venous invasion.

Hepatology is a particularly interesting field of application for the new radiogenomic approach; patient management already requires the combination of information coming from *ex vivo* analyses of circulating biomarkers (serum proteins, transaminases, *etc.*), histology and direct *in vivo* multimodal imaging data (ultrasound, CT, MR, *etc.*) (Figure 3). Nowadays however, all these data remain locked within the various departments and isolated between institutions, creating an obstacle to the creation of the coherent program of collaborative research.

BIO-BANKING FOR LIVER DISEASE

Institutional biobanks are service units dedicated to the collection, processing, storage and distribution of

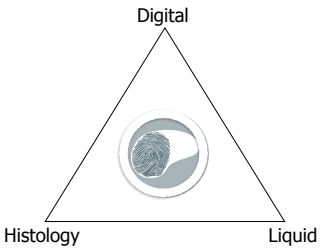

Figure 3 A new three-dimensional view of the liver biopsy. Digital biopsy, direct *in vivo* imaging of the whole liver, adds important pathophysiological and morphological context to liquid and invasive (percutaneous or surgical) liver biopsies that provide focal *ex vivo* analysis of circulating biomarkers and specimens of the liver respectively contributing to a three dimensional view for diagnosis and prognosis of liver disease.

biological materials and associated patient information. Biobanks are becoming more and more central to translational research in the fields of human health, biotechnologies and development in life science.

Generally, institutional biobanks can be classified in terms of the profile of material contained in the collection as: population-based, disease-specific or rare disease oriented. The biological material can be constituted of different types of samples such as solid tissues, plasma, serum, RBC, buffy coats, white cells, DNA, RNA, proteins, cell lines, saliva, urine and fecal samples. The widest European consortium of Biobanks is represented by the Bio-banking and Bio-Molecular Research Infrastructure (BBMRI-ERIC)[103]. Recently, an additional class of biobanks has emerged in the form of imaging biobanks. Specifically, the European Society of Radiology defines imaging biobanks as "organized databases of medical images and associated imaging biomarkers (radiology and beyond) shared among multiple researchers, and linked to other biorepositories"[104,105]. The primary role of imaging biobanks is the identification of novel imaging biomarkers to be used in research studies, as well as to support validation of novel biological or genetic biomarkers of disease. It is important to recognize the fundamental importance of integrating clinical, pathlogical and biological endpoint data (such as with genomic results coming from high-throughput sequence analysis of DNA or RNA) with *in vivo* imaging data derived from radiomic analysis of CT, MRI or PET images for the identification of biomarkers (both imaging and non-imaging) and their translation to clinical relevance. A major limitation of radiomics and radiogenomics research is that the large number of radiomic features and patholgical/biological variables that may or may not be involved necessitate the use of large datasets and condivision of data across disciplines. Thus, imaging biobanks should be embedded in wider biobank networks. This may be the product of an explicit program of data collection (such as the German national Cohort, and the United Kingdom Imaging Biobank projects) or through consistent practice across a number of individual clinical entities that interact as nodes in a network dedicated to a given pathology/ies. As an example of a product of the former, in a prospective study of 4.949 participants in the United Kingdom Imaging Biobank[105] in whom liver fat was measured using the PDFF MRI technique, Wilman *et al*[105] showed an association of increased liver fat with greater age, BMI, weight gain, high blood pressure and type 2 diabetes. An example of an individual clinical node for the latter form of network is the SDN Biobank, that is the institutional biobank of the IRCCS SDN. This structure is devoted to the collection of biological material (blood, urine, feces) as well as images (deriving from CT, PET, MRI) from patients affected by oncological, cardiological, neurological and metabolic diseases[21].

Due to the non-invasive nature of medical imaging and its ubiquitous use in clinical practice, the field of radiogenomics is rapidly evolving and initial results are encouraging. Biobanks will surely have a critical role for the development of the innovative radiogenomics protocols. Indeed, their collaboration across centres and disciplines is likely the only feasible approach to assembling the large numbers of images and samples, along with the clinical data required for testing associations in identifying and validating imaging and genomic biomarkers. To facilitate this collaboration, in the future it will be necessary to bring together images and biological samples from the same patients in innovative biobanks adapted to collect both kinds of material. The data coming from this kind of biobanks will have a critical role for the development of personalized protocols for diagnosis and patients care (Figure 4). In the context of fatty liver, well defined and correlated histologic and imaging targets from MRI and US (Table 1) already exist and they can form the basis of such a collection.

DIGITAL BIOPSY-THE ROAD AHEAD

Digital biopsies for NAFLD need to be fast and easy to obtain, and consistent among operators, technologies and methods, and most importantly provide clinically relevant results. To arrive at this level, there is a need for research, development and extensive collaboration.

A number of imaging techniques are already available for evaluation of fibrosis and steatosis. Wider adoption of these techniques and continued work to overcome their limitations can provide the basis for multi-centric collaboration on the optimization of imaging for fatty liver.

No individual centre has the breadth and depth of patients to go it alone in establishing a validated digital liver biopsy. Collaboration on imaging technique adoption should be performed in combination with establishing a cross-center imaging biobank linked to associated conventional biobanks.

An open need lays in the attraction of expertise and interest to carry out radiomics and radiogenomic studies and this follows the generation of data and the establishment of the bio-repository resources described above. Only at this point we will see the fruits of the labour, in terms of possible imaging biomarker signatures and associations with clinical and biological

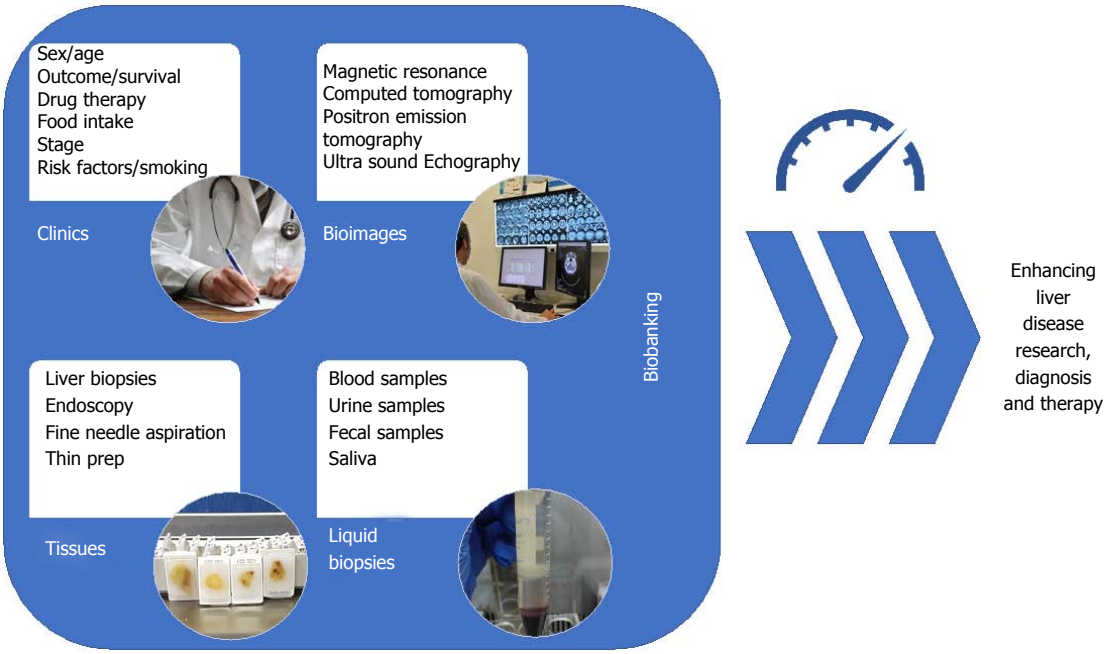

Figure 4 Modern vision of bio-banking. The collection of patient clinical data, tissue samples, liquid biopsies as well as bio-images, in organized datasets is defined as "bio-banking". With the advent of omics sciences (*i.e.*, proteomics and genomics) where a large number of biological specimens and associated data are needed for making a precision medicine approach to the patients collaborative studies across centers are essential to maximizing patient recruitment. Equally, accessible well-structures data stores permit re-use and re-examination of data reducing the cost of subsequent studies. In this context, the field of bio-banking has the possibility to enhance research on liver disease as well as improve diagnostics and therapeutics.

Table 1 Intrahepatic fat measurement

Ref.	Imaging modality	Classification	ROC-derived parameters		
			AUC	Sensitivity (%)	Specificity (%)
Mancini *et al*[60] 2009	US	^{1}H-MRS fat content > 5%	0.996	100	95
Xia *et al*[61] 2012	US	^{1}H-MRS fat content > 5.56%	NA	95.1	100
Edens *et al*[62] 2009	US	^{1}H-MRS fat content > 5.56%	NA	66.7	100
Di Lascio *et al*[65] 2018	US	^{1}H-MRS fat content > 5%	0.97	89	94
Sasso *et al*[108] 2012	CAP score (imaging derived)	Liver biopsy	S_0 *vs* $S_1S_2S_3$: 0.80	S_0 *vs* $S_1S_2S_3$: 76	S_0 *vs* $S_1S_2S_3$: 71
		S_0: < 10% of hepatocytes	S_0S_1 *vs* S_2S_3: 0.86	S_0S_1 *vs* S_2S_3: 87	S_0S_1 *vs* S_2S_3: 74
		S_1: 11%-33% of hepatocytes	$S_0S_1S_2$ *vs* S_3: 0.88	$S_0S_1S_2$ *vs* S_3: 78	$S_0S_1S_2$ *vs* S_3: 93
		S_2: 34%-66% of hepatocytes			
		S_3: 67%-100% of hepatocytes			

Both histologic and digital liver biopsy provided reliable measures of intrahepatic fat that are significantly correlated, but categorically different. Liver biopsy describes the histologic characteristics of the pathologic lesions and accounts for the percentage of hepatocytes with intracellular fat-derived vacuoles using categorical grading systems that are not directly representative of the hepatic triglyceride concentration[19-21]. On the other hand ^{1}H-MRS measures protons in acyl groups of liver tissue triglycerides and provides continuous quantitative values expressed as mg/g of hepatic tissue[109]. Moreover, ^{1}H-MRS uses a much larger volume of liver tissue than biopsy reducing sampling error and representing the most accurate measure of the overall liver triglyceride content. US: Ultrasound; ROC: Receiving operator characteristic; CAP: Controlled attenuation parameter.

endpoints. Prospective studies should then compare the results obtained using images acquired at different points in time by multiple technicians, and read by both radiologists and trained non-radiologists. The next step is studying and comparing quantitative image features to unravel the relationships between the digital biopsy features and histopathologic, metabolic and genetic characteristics and building models which link them to the disease outcomes. Validation studies with multiple users are required to show that intra- and inter-reader variations of the digital biopsy derived features are acceptable.

CONCLUSION

We foresee the combination of image-based digital liver biopsy with liver histology and liquid biopsy in a multimodal approach to the study of the fatty liver in clinical pathology as a key to personalizing patient care. To foster this approach in clinical and translational research there is a need to standardize the methods of acquisition, processing and storage of bio-images of the liver. A number of imaging techniques that offer a starting point for acquisition have been identified and their use in combination in prospective studies will

open a new venue of translational and clinical research. However, many of these tools are still limited to selected Centres and it is important to increase their accessibility by reducing costs.

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REVIEW

Alkaline sphingomyelinase (NPP7) in hepatobiliary diseases: A field that needs to be closely studied

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Abstract

Alkaline sphingomyelinase cleaves phosphocholine from sphingomyelin, platelet-activating factor, lysophosphatidylcholine, and less effectively phosphatidylcholine. The enzyme shares no structure similarities with acid or neutral sphingomyelinase but belongs to ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family and therefore is also called NPP7 nowadays. The enzyme is expressed in the intestinal mucosa in many species and additionally in human liver. The enzyme in the intestinal tract has been extensively studied but not that in human liver. Studies on intestinal alkaline sphingomyelinase show that it inhibits colonic tumorigenesis and inflammation, hydrolyses dietary sphingomyelin, and stimulates cholesterol absorption. The review aims to summarize the current knowledge on liver alkaline sphingomyelinase in human and strengthen the necessity for close study on this unique human enzyme in hepatobiliary diseases.

Key words: Sphingomyelin; Alkaline sphingomyelinase; Nucleotide pyrophosphatase/phosphodiesterase 7; Autotaxin; Platelet-activating factor; Cholangiocarcinoma; Liver diseases; Gallstone

Core tip: Alkaline sphingomyelinase is an enzyme expressing in the intestinal tract and additionally human liver. It hydrolyzes sphingomyelin, platelet activating factor and lysophospholipase. In the intestinal tract, it digests dietary sphingomyelin, stimulates cholesterol absorption, and inhibits development of colon cancer. Less is known about the implications of the enzyme in liver diseases. The review summarizes the current knowledge of its roles in hepatobiliary disease and raised special topics for future investigations.

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ALK-SMASE IN NPP FAMILY

An enzyme that catalyzes sphingomyelin (SM) hydrolysis to ceramide at optimal alkaline pH was first identified in the intestinal tract by Nilsson in 1969[1]. The enzyme was thereafter named as alkaline sphingomyelinase (alk-SMase)[2] in line with acid and neutral SMases. However, after purification, characterization, and gene cloning[3-6], it was found that alk-SMase actually had no structural similarities with either acid or neutral SMase, but shared about 30% amino acid sequence similarities with enzymes in ecto nucleotide pyrophosphatase/phosphodiesterase (NPP) family. As a novel member in NPP family, alk-SMase is nowadays also called NPP7.

Comparing with other six NPP members, alk-SMase is distinctive in several aspects. Not like most NPP members, alk-SMase has no nucleotidase activity but a phospholipase C activity. It cleaves phosphocholine from phospholipids including SM, platelet activating factor (PAF)[7], lysophosphatidylcholine (lyso-PC), and phosphatidylcholine (PC) less effectively[5]. In NPP family, NPP2 (autotaxin) can also hydrolyze lyso-PC, but with a phospholipase D activity[8]. Another NPP member NPP6 can cleave phosphocholine from lysophospholipids mainly lyso-PC and lyso-PAF but not from SM[9]. Alk-SMase is so far the only one that has decent activities against SM and PAF in this family. In addition, while the activities of other NPP members could be identified in many organs and tissues, expression of alk-SMase is only restricted to intestinal tract in most species[10]. Western blot of rat tissues only shows positive band in intestinal mucosa and content but not in other organs including brain, heart, lung, liver, spleen, kidney and pancreas[4]. Interestingly, additional high activity was found in the bile of human[11], but not in bile of other species including rat, mouse, pig, cow, sheep, dog, guinea pig, and baboon[10] (and unpublished data). Furthermore, most NPP members are functioning as a proliferative and inflammatory factors that are important for cell survival[12], alk-SMase displays inhibitory effects on cell proliferation and inflammation[13,14]. Cell culture studies show that alk-SMase can inhibit cell proliferation by about 50%[15] and its activity *in vivo* is positively correlated to the activity of caspase 3, the key enzyme that triggers apoptosis[16-18]. Rectal administration of alk-SMase in rats suppresses colitis induced by dextran sulfate sodium (DSS)[19]. Recently the studies with alk-SMase knockout mice clearly showed that both initiation and malignant transformation of colon cancer induced by azoxymethane and DSS was enhanced by about 5 times in the knockout mice comparing with the wild type mice[20]. In agreement with the animal studies, clinical studies also found reduced alk-SMase activity in patients with inflammatory bowel diseases (IBD) and colon cancer, and the reduction is progressive from 25% in IBD to 75% in colonic carcinoma[21-23].

The anticancer effects of alk-SMase are thought to be achieved with a three armed mechanism[13]. First, it hydrolyzes SM to ceramide, which is a well-known antiproliferative and apoptotic molecule[18,24]. Second, it cleaves phosphocholine moiety from PAF and inactivates PAF[7], which is widely expressed in many inflammatory tissues promoting inflammation and tumorigenesis[25]. And finally NPP7 converts lyso-PC to monoacylglycerol[5], thus reducing the production of lysophosphatidic acid (LPA), which otherwise can be formed by NPP2[8]. LPA has emerged as an important messenger with potent inflammatory and carcinogenic effects mediated *via* several signaling transduction pathways after binding to G protein coupled receptors[26,27]. In supporting this three arm hypothesis, decreased ceramide and increased PAF[20] and LPA (Zhang P *et al* Abstract presented in AACR symposium, Shanghai, China, 2016) have been found in NPP7 knockout mice.

Similar to other NPP members, alk-SMase is anchored on the surface of the cell membrane with a short hydrophobic domain. The remaining part of the enzyme including the catalytic domain is exposed extracellularly[13]. The enzyme can be released by bile salt[28], and also by pancreatic trypsin, as there is a tryptic site just above the hydrophobic domain embedded inside the membrane[29].

ALK-SMASE IN HUMAN BILE

Alk-SMase in human bile was discovered by Nyberg *et al*[11] in bile collected from patients in our hospital. The activity in human bile is not derived from bacteria since it is similarly present in the samples with and without bacterial infection. Although gallbladder bile has higher activity than the hepatic bile, no activity was found in the homogenates of gallbladder mucosa, confirming that it is liver not gallbladder that expresses the enzyme. Because PCR experiment identifies alk-SMase mRNA in human HepG2 cells[30], the enzyme is believed to be expressed by hepatocytes, transported to the surface of the microvilli that extend into the bile canaliculi and released by bile salt into the lumen.

Alk-SMase in human bile shares similar characteristics as the one in the intestinal mucosa[31,32]. The enzyme becomes active at the pH around 7 and the maximal activity occurs at pH 9.0. Its activity requires the presence of bile salts[4,6]. The bile salt dependency is type specific, which differs from other lipases such as bile salt stimulated lipase[33,34]. Although different bile salts more or less increase alk-SMase activity with the maximal effects at their critical micelle concentrations, taurocholate (TC) and taurochenodeoxycholate (TCDC) are much more effective than other bile salts. On the other hand, the nonionic detergent Triton X100 and zwitterionic non-denaturing detergent CHAPS with similar structure as TCDC and TC have no stimulatory effect but inhibit alk-SMase activity in the presence of other bile salts[4,31,35]. The finding indicates that the bile salt induced activation is not a simple detergent effect on the physical state of the substrate SM in mixed micelles. Additional interaction between the enzyme

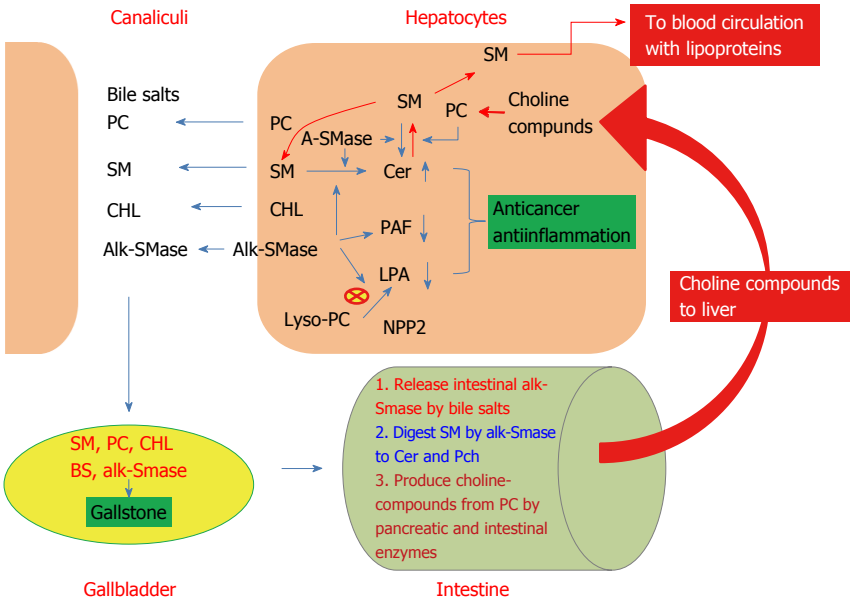

Figure 1 Metabolism of sphingomyelin in the liver and potential implications of alk-SMase in liver diseases. Liver alk-SMase is localized on the hepatocyte canaliculi membrane. It hydrolyzes SM, PAF, and lyso-PC, resulting in increased ceramide (Cer) and decreased PAF and LPA, thus having anticancer and anti-inflammatory effects. Together with PC, cholesterol, and bile salts, SM is released in canaliculi and transported to gallbladder, where the interactions of these compounds affect gallstone formation. When the bile is delivered into the intestinal tract, bile salt will release additional alk-SMase from intestinal mucosa, and digest intestinal SM to ceramide and phosphocholine (Pch). Meanwhile, PC will be hydrolyzed by enzymes from pancreas and intestinal mucosa to choline compounds such as free choline, lyso-PC, and Pch. These choline compounds will be transported to liver where to be used for synthesis of PC. Pch moiety in PC can be transferred to ceramide to form SM by SM synthases. Part of the SM formed will be released into blood together with lipoproteins, part to bile, and part to be degraded by alk-SMase and acid SMase (ASMase) in the liver.

protein and the bile salt is likely involved. Supporting this hypothesis, recent studies on crystal structure of human alk-SMase by Gorelik *et al*[36] showed that the enzyme forms a hydrophobic loop and a positively charged surface which can interact with bile salts. This needs to be proved in further investigations.

Similar also to intestinal alk-SMase, human bile alk-SMase is inhibited by PC, the most abundant phospholipid in the bile[31,37]. This might be related to a competition between PC and SM for the substrate binding site of the enzyme. As shown by both computer homology modelling studies[38] and crystal structural studies[36], alk-SMase forms a specific pocket and a long narrow groove that fits the phosphocholine head group, and the tails of these substrates, respectively. The binding affinity is stronger for SM than for PC[39,40].

Presence of alk-SMase in human bile enhances SM digestion in humans. Intestinal SM is derived from diet, shedding mucosal cells and bile. The enzyme responsible for digesting SM in the gut is alk-SMase and in alk-SMase knockout mice, about 90% of ingested SM cannot be digested but accumulated in the colon[41]. In many species except human, alk-SMase activity is absent in duodenum, increasing in the jejunum and declining in the colon[2]. SM digestion in the gut normally starts in the middle of the jejunum where alk-SMase is high and PC, the major inhibitor of alk-SMase, has been decreased due to the absorption[42]. The process of SM digestion is slow and incomplete in most species, resulting in about 40% ceramide and SM being identified in feces[43,44]. However, due to the presence of additional alk-SMase in the bile of human, human duodenum has considerable alk-SMase activity. Ohlsson *et al*[45] found that digestion of SM in human is more efficient than other species and about 81% of ingested SM can be digested. The more effective digestion of SM could be important for human health, as dietary SM stimulates the development of the gut of the new born and inhibits colonic tumorigenesis[46-48].

ROLE OF LIVER IN SPHINGOLIPID METABOLISM

It is well known that liver is an important organ for lipid metabolism such as fatty acid beta-oxidation, ketone body generation, cholesterol metabolism, lipoprotein synthesis, and phospholipid metabolism. For the phospholipid metabolism in liver, most previous studies focused on PC not SM, but the interest in SM was increasing in the latest decades. Liver is an organ with relatively high levels of SM. Comparing with subcutaneous and intra-abdominal adipose tissues, SM in human liver is 7-8 fold higher than in these adipose tissues[49].

The high levels of PC and SM in the liver are attributable to the fact that liver efficiently takes up choline containing compounds such as choline, lyso-PC and phosphocholine derived from digestion of phospholipids in the intestinal tract and uses them for synthesis of PC and SM[42,50] (Figure 1). As shown in animal studies, after feeding choline labeled SM, up to 30% of the

labeled choline is accumulated in the liver and more than 95% of them is utilized for PC synthesis[42]. This is important for SM synthesis, as at the last step of SM synthesis, phosphocholine is transferred from PC to ceramide, catalyzed by SM synthase, which is highly expressed in the liver[51,52]. For the hydrolysis of SM, liver has high acid SMase activity than most other organs in many species[41]. Acid SMase is an enzyme with two isoforms. One is the lysosome enzyme that breaks down internalized SM in lysosome and the other is a secretory form that can be secreted to the plasma membrane and hydrolyzes membrane bound SM[53]. That is why in Niemann Pick diseases with acid SMase deficiency, liver is one of the most affected organs with SM accumulation[54]. There are many other factors that influence SM levels in the liver, such as high fat diet[55], endotoxin infection[56], hepatitis B virus infection[57] and liver cancer[58].

SM synthesized in the liver can be released into plasma and bile (Figure 1). Comparing with other species, human plasma has at least two fold higher levels of SM than other species[59]. The plasma SM from liver is mainly transported with lipoproteins mainly VLDL and less with LDL and HDL[43]. SM in plasma is also derived from intestinal mucosa and other tissue cells, being about 1-1.5 g in total[43]. Most SM secreted from intestine is in chylomicron. SM in chylomicron is mainly not from the dietary products but from the membrane of enterocytes, because most sphingosine, the final digestion product of SM by alk-SMase and neutral ceramidase in the gut is not utilized to resynthesize SM after absorption in mucosal cells, but to convert to fatty acid and then to chyle triglyceride[44,60].

Secretion of phospholipids from hepatocyte canaliculi membrane is by a mechanism related to the interactions of bile salt and ABCB4[61]. Under physiological conditions, more than 95% of the phospholipids in bile is PC, and SM is accounted for about 3%[62]. The levels of SM in human bile is relatively low comparing with other species which don't have alk-SMase in the bile such as sheep[63]. But under pathological conditions, the level of SM in bile is subject to change. Barnwell *et al*[64] showed that *in vivo* perfusion of bile salts in rat significantly reduced PC content and meanwhile induced about 10 time increase of SM in the bile without obvious damage to the liver.

The implications of SM in plasma and bile are getting increasing interest. It has been known that high concentration of plasma SM is a risk factor for atherosclerosis[65]. Recent studies also found that plasma SM could be a biomarker for various liver diseases such as hepatitis, primary sclerosing cholangitis (PSC), and steatosis[57,66,67], indicating SM metabolism in liver significantly contributes to plasma SM levels. SM in the bile may have important implications, as it has stronger van der Waal interactions with cholesterol than PC[40] and its physical properties of SM is affected by bile salt which may affect gallstone formation in gallbladder[68].

ALK-SMASE IN HEPATOBILIARY DISEASES

Comparing the extensive studies on intestinal alk-SMase, which showed its important roles in SM digestion, colon cancer prevention, and cholesterol absorption[13,14,20], the progress of the research on human bile alk-SMase obviously lags behind. The main obstacle is lack of an animal model that expresses alk-SMase in the liver, and lack of a cell line that highly expresses alk-SMase, as the enzyme has already been downregulated in tumorigenesis. In a pilot study, we measured alk-SMase activity in 30 human liver biopsies and the results indicated a reduction of the enzyme activity in steatosis and PSC[30]. Recently we determined alk-SMase activity in 59 bile samples taken under ERCP and found significant reduction of alk-SMase activity in bile of patients with PSC and tumorigenic diseases, with the most remarkable reduction in cholangiocarcinoma[69].

Besides the reduction of activity, an abnormal transcript of alk-SMase was identified in both liver cancer HepG2 cells and colon cancer HT29 cells, which is caused by a shift of RNA splice site at transcriptional level, resulting in exon 4 deletion[30,70]. The enzyme translated from this transcript is totally inactive, as 73 amino acids coded by exon 4 were absent, of which a histidine is critical for formation of the substrate binding site[70]. According to the size of their mRNA, the wild type and the mutant isoform have been called 1.4 kb and 1.2 kb form, respectively. These two forms were found in the bile of many of the 59 patients with different hepatic diseases[69], but the activity of alk-SMase is positively correlated with the ratio of 1.4/1.2 kb form. Decrease in the 1.4 kb product and increase in the 1.2 kb product are likely associated with the development of cholangiocarcinoma. In the bile of one PSC and one cholangiocarcinoma patient, no alk-SMase activity and no 1.4 kb product but only high levels of 1.2 kb form were identified[69].

On the other hand, hepatobiliary diseases may also affect the levels of alk-SMase and SM digestion in the intestine because bile diversion strongly reduced alk-SMase activity in the small intestinal content by 85% and in the feces by 68% in rat[71]. The changes are believed to be related to bile salts, which release the alk-SMase from the intestinal mucosa to gut lumen[28].

HUMAN HEPATIC ALK-SMASE IN PERSPECTIVES

Considering the results from previous studies on alk-SMase, it is predictable that human liver alk-SMase may also have important implications for hepatobiliary diseases. The following questions are worth close investigation: (1) Can the remarkable reduction of alk-SMase activity and the increase in the aberrant isoform

in the bile be warning signals for carcinogenesis in the liver, particularly cholangiocarcinoma? It is well known that cholangiocarcinoma is a disease lacking an early biomarker, and most patients are not curable at the time when the disease is diagnosed[72]. Making the situation worsen is the fact that the incidence of cholangiocarcinoma is increasing[73]. To find an early biomarker for cholangiocarcinoma is therefore a challenge for clinical doctors and medical researchers. Alk-SMase activity in bile is significantly decreased in cholangiocarcinoma to an extent greater than in other hepatic diseases associated with increased expression of the 1.2 kb isoform[69]. The reduction of alk-SMase activity seems already occurring in both bile and liver biopsies in PSC patients[31]. PSC is a major risk factor for cholangiocarcinoma and about 15% of PSC patients may finally develop this type of cancer[74]. Future studies in a relatively large scale are necessary to evaluate whether the changed activity and 1.2 kb isoform expression in PSC patients can be biomarker for cholangiocarcinoma. To follow these changes might be helpful for identifying the early carcinogenesis. In addition, it is worthwhile to point out that about 70% of PSC patients may have IBD, particularly ulcerative colitis[75,76]. Reduction of alk-SMase activity in chronic ulcerative colitis has been reported[23]; (2) Are there a cross communication between NPP7 (alk-SMase) and NPP2 (autotaxin) in hepatobiliary diseases? NPP2 hydrolyzes lyso-PC with a phospholipase D activity and generating LPA, a potent inflammatory and proliferative factor[77]. Increased levels of NPP2 are a feature of many important hepatobiliary diseases such as PBC, PSC[78], steatosis[79], liver fibrosis[80], hepatitis C[81] and liver cancer[82]. Alk-SMase shares the same substrate lyso-PC with NPP2 but it cleaves phosphocholine instead of choline and thus generates monoacylglycerol not LPA[5]. Alk-SMase therefore may counteract NPP2 and thus reduce the formation of LPA. Recently we did find that in alk-SMase knockout mice treated with DSS, the levels of LPA in the colonic mucosa is higher in the knockout mice than in the wild type mice (Zhang P *et al*, Abstract presented in AACR symposium, Shanghai, China, 2016). Interestingly, a recent cohort study showed that primary biliary cirrhosis (PBC) patients who did not respond to ursodeoxycholic acid (UDCA) treatment display higher NPP2 levels than the responders[78]. Changes of alk-SMase may be implicated in the results, as alk-SMase activity can be increased by UDCA in liver cells[17]. No response to UDCA in these PBC patients may indicate a failure to upregulate alk-SMase by UDCA in these patients, leading to increased levels of NPP2; (3) Can alk-SMase protect liver against noxious effects of PAF? PAF is a type of bioactive lipid and can be synthesized rapidly in various inflammatory tissues. After binding to its G protein coupled receptors, PAF triggers several signal transduction pathways leading to activation of various phospholipases including C, D and A2 and to calcium mobilization, MAP kinase activation, and neutrophil mobilization[83,84]. In the liver PAF induces vasoconstriction[85] and may play a key role in several hepatic diseases such as CCl4 induced cirrhosis, ethanol and acetaminophen induced liver injury, viral induced hepatitis, and hepatocellular carcinoma[86-89]. All these diseases are associated with increased formation of PAF levels and PAF receptor expression. To inhibit the effects of PAF, previous studies were focused on PAF acetyl hydrolase and PAF receptor antagonists[90]. PAF is a substrate for alk-SMase which inactivates PAF by degrading it to phosphocholine and alkyl acetyl glycerol[7]. The activity is bile salt dependent with optimal pH at 7.5, which well fits the niche of the hepatobiliary system[7]. In alk-SMase knockout mice, PAF has been found to be significantly increased in the intestinal lumen[20]. It is therefore worthwhile examining the impact of alk-SMase on PAF action in these hepatic diseases and whether upregulation of alk-SMase may counteract the effects of PAF and benefit the patients; (4) What is the role of human bile alk-SMase in regulating SM levels in bile? Alk-SMase affects SM levels in the cell membrane. Overexpression of alk-SMase in COS7 cells[5] and incubation of the cells with purified alk-SMase result in reduced SM in the cell membrane[13]. Alk-SMase in human bile most likely can do the same things and thus affecting SM levels both in the hepatocyte canaliculi and bile. Phospholipids particularly PC and SM affect crystallization of cholesterol which is a key event involved in gallstone formation[68]. SM levels in the bile can be increased in the presence of high concentrations of bile salt[64] , and be decreased in the presence of high levels of alk-SMase released from canaliculi. Considering the influence of the membrane SM on cholesterol translocation and synthesis[40], and the more appreciable interaction of SM than PC with cholesterol[39,40], the impact of bile alk-SMase on gallstone formation through regulating SM levels both in the canalicular membrane and bile might be also worthwhile for close investigation.

CONCLUSION

Additional expression of alk-SMase in liver is unique for humans. As shown in the figure, by hydrolyzing its substrate SM, PAF, and Lyso-PC, alk-SMase generates anticancer and apoptotic molecule ceramide, reduces levels of PAF and LPA, which have been shown to be involved in a series of liver diseases including viral infection, steatosis, fibrosis, sclerosis, and tumorigenesis. Alk-SMase thus may play important roles in protecting the organ from these diseases. In addition, the enzyme is released into bile together with SM, PC, bile salt, and cholesterol and may interfere SM and PC levels and the physical-chemical interactions of these molecules in bile, thus affecting gallstone formation. Liver is an active organ for SM metabolism and for regulating plasma SM levels. Changed alk-SMase activity in bile and SM levels in plasma have been found in several hepatobiliary diseases, and such changes may have diagnostic and prognostic values. The contributions of alk-SMase, a unique human liver enzyme, for these changes need close investigation.

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MINIREVIEWS

Spontaneous bacterial and fungal peritonitis in patients with liver cirrhosis: A literature review

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Abstract

Spontaneous bacterial (SBP) and spontaneous fungal peritonitis (SFP) can be a life-threatening infection in patients with liver cirrhosis (LC) and ascites. One of the possible mechanisms of developing SBP is bacterial translocation. Although the number of polymorphonuclear cells in the culture of ascitic fluid is diagnostic for SBP, secondary bacterial peritonitis is necessary to exclude. The severity of underlying liver dysfunction is predictive of developing SBP; moreover, renal impairment and infections caused by multidrug-resistant (MDR) organism are associated with a fatal prognosis of SBP. SBP is treated by antimicrobials, but initial empirical treatment may not succeed because of the presence of MDR organisms, particularly in nosocomial infections. Antibiotic prophylaxis is recommended for patients with LC at a high risk of developing SBP, gastrointestinal bleeding, or a previous episode of SBP, but the increase in the risk of developing an infection caused by MDR organisms is a serious concern globally. Less is known about SFP in patients with LC, but the severity of underlying liver dysfunction may increase the hospital mortality. SFP mortality has been reported to be higher than that of SBP partially because the difficulty of early differentiation between SFP and SBP induces delayed antifungal therapy for SFP.

Key words: Liver cirrhosis; Spontaneous bacterial peritonitis; Spontaneous fungal peritonitis; Bacterial infections

Core tip: Spontaneous bacterial (SBP) and spontaneous fungal peritonitis (SFP) are infectious complications in patients with liver cirrhosis (LC). Renal impairment, severity of underlying liver dysfunction, and infections caused by multidrug-resistant (MDR) organisms are associated with a fatal prognosis in SBP. Antibiotic prophylaxis is recommended for patients with LC and with a high risk of developing SBP, gastrointestinal bleeding, or a previous episode of SBP, but the increase in the risk of infections caused by MDR organisms is of concern. Increased mortality of SFP compared with that of SBP may partially result from delayed diagnosis and

starting of antifungal therapy.

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INTRODUCTION

Patients with liver cirrhosis (LC) are at a high risk of developing bacterial infections because of hypoactivity of phagocytic cells in the hepatic reticuloendothelial system, decreased production of complement, and bacterial influx into the general circulation through portacaval shunts[1]. The most common are spontaneous bacterial peritonitis (SBP), urinary tract infections, respiratory infections (pneumonia), soft tissue infections, and bacteremia[2].

SBP can be a life-threatening LC complication, and the severity of underlying liver dysfunction and renal impairment such as hepatorenal syndrome (HRS), are associated with poor prognosis[3]. An increasing prevalence of multidrug-resistant (MDR) bacterial infections has been associated with failure of empirical antibiotic therapy, and the prognosis of SBP caused by MDR bacteria is poor[4]. Less is known about spontaneous fungal peritonitis (SFP) in patients with LC[5,6], but the available case reports indicate that the mortality of SFP is worse than that of SBP.

This article reviews the published data on the incidence, diagnosis, causative organisms, treatment, prognosis, and prognostic factors of SBP and SFP in patients with LC.

BACTERIAL INFECTIONS IN PATIENTS WITH LIVER CIRRHOSIS

Possible mechanisms underlying bacterial infections including SBP

In cirrhosis with portal hypertension, the microcirculation in the intestinal mucosa is disturbed, resulting in a reduction of mucosal blood flow, intestinal bacterial overgrowth, and impaired mucosal integrity[7,8]. Intestinal bacterial overgrowth, impairment in permeability of the intestinal mucosal barrier, and deficiencies in local host immune defenses are estimated to the major mechanisms to promote bacterial translocation (BT or pathological BT) in LC[7,9]. BT may be involved in the onset or aggravation of bacterial infections such as SBP and or HRS in patients with LC[7,8,10]. Pijls *et al*[11] reported increased large and small intestine permeability in patients with LC, and BT has been reported to be associated with bacterial infections caused by aerobic Gram-negative bacteria in patients with LC and was found to be more frequent in experimental LC models with than in those without ascites[7].

Studies of BT and bacterial DNA (bact DNA) translocation are limited in humans[7], but acute-phase proteins such as C-reactive protein (CRP) or procalcitonin have been used as BT biomarker[7], and detection of bact DNA by polymerase chain reaction (PCR) in biologic fluid has been proposed as a useful surrogate biomarker of BT in patients with advanced LC[7]. The presence of bact DNA in ascites increases the risk of developing SBP and HRS[12] and is an indicator of poor prognosis[7,13]. Assay of bact DNA by PCR has low diagnostic accuracy for SBP because of possible contamination and has poor sensitivity[14]. *In situ* hybridization of bact DNA in leukocytes recovered from the ascitic fluid in patients with LC has high sensitivity and specificity even in patients with culture-negative SBP[14,15]. Proinflammatory cytokines or oxidative stress may be involved in the pathogenesis of BT, and anti-tumor necrosis factor (TNF) has been reported to be an effective treatment in an animal model of LC[8]. In addition to the severity of liver dysfunction, the genetic diversity and virulence of causative bacteria also may influence the development of BT[16]. Genetic variation of superoxide dismutase 1 may involve the development of SBP[17]. Bacterial metabolites that reach the liver through portal system activate toll-like receptors (TLRs)[18], and the genetic polymorphism of TLR and nucleotide-binding oligomerization domain 2 genes may also be involved in the pathogenesis of BT, thus increasing the risk of infection in patients with LC by altering TLR binding to lipopolysaccharides or endotoxins[13,16]. Finally, intestinal colonization and translocation of drug-resistant bacteria may induce MDR SBP infections[19,20].

Bacterial infections in patients with liver cirrhosis

It is estimated that 30%-60% of inpatients with LC will develop a bacterial infection[1,21], and that the occurrence of these infections is four to five times higher in patients with LC than that in the general population[21]. Bacterial infections can trigger rapid deterioration of liver function and are a common precipitating factor of acute-on-chronic liver failure in cirrhosis patients[10,16].

Mortality and the incidence of LC-related complications are both significantly higher in patients with than without bacterial infections[22].

Karvellas *et al*[23] reported that septic shock secondary to SBP has a high mortality rate of approximately 80% in patients with LC, and bloodstream infections occur in 4%-21% of patients with LC, which is 10-fold higher than the rate in noncirrhotic patients[24]. The mortality of bloodstream infections in patients with LC ranges from 23% to 58%[25].

The incidence of bacterial infections in patients with LC is significantly correlated with the severity of the underlying liver dysfunction[10,22,26,27]. However, a recent study by Dionigi *et al*[27] found that patients with LC who become infected had an increased risk of death

independent of the severity of the underlying liver disease, even if they survived the acute infection. In patients with LC, infections caused by MDR bacteria are associated with a higher risk of mortality compared with those infections caused by susceptible bacteria[28,29]. An increased risk of MDR infections is a serious concern, is closely related to failure of antimicrobial therapy[29,30], and may induce deterioration of liver function[29]. MDR bacteria are resistant to at least three widely used antibiotic families[22]. These include the resistant bacteria that produce extended-spectrum β-lactamase (ESBL), such as *Pseudomonas aeruginosa,* and methicillin-resistant *Staphylococcus aureus* (MRSA)[31,32]. Recently, extensively drug-resistant (XDR) bacteria such as carbapenemase-producing *Klebsiella pneumoniae* and vancomycin-resistant *Enterococci* have been isolated from patients with LC[31]. The development of MDR infections is associated with suboptimal antibiotic use including antibiotic prophylaxis, nosocomial infection, recent infection with an MDR organism, current or recent hospitalization, exposure while receiving health care, and upper gastrointestinal bleeding in LC[33,34]. In a large prospective study, MDR bacteria were isolated in 4% of community-acquired infections, 14% of health care-associated infections, and 35% of nosocomial infections[33]. In two series of patients with LC evaluated in 2005-2007 and 2011-2012, Fernández *et al*[33] reported that MDR bacteria were more common in nosocomial infections and were associated with higher in-hospital mortality than in infections caused by antibiotic-susceptible bacteria. Pouriki *et al*[20] reported drug-resistant bacteria in intestinal cultures from 44% of uninfected patients with decompensated LC. Recent reports have found that asymptomatic intestinal colonization with MDR or XDR bacteria was an increased mortality risk in patients with LC in part because of BT of circulation of bacterial components[20].

SPONTANEOUS BACTERIAL PERITONITIS

Diagnosis

The development of ascites in patients with LC is a marker of poor prognosis and has been associated with high liver transplantation-free mortality ranging from 15% to 20% in 1 year and 40% to 60% in 5 year from the first onset[35].

SBP is as bacterial infection of the ascitic fluid with no apparent intraabdominal source of infection or malignancy[23]. A polymorphonuclear cell (PMN) count of $\geqslant$ 250 cells/mm^3 in the ascitic fluid, regardless of the isolation of bacteria from the fluid[36], is diagnostic for SBP. PMN cell count is routinely performed by using the manual laboratory counting which is time-consuming and costly[37]. Some studies have reported that the manual and the automated counting methods have a good agreement in the determination of PMN in the ascitic fluid, and automated methods have the potential to replace the manual counting method[37,38]. Culture-negative SBP, also known as culture-negative neutrocytic ascites (CNNA), should be treated in the same way as culture-positive SBP[39]. Estimates of the mortality of culture-positive SBP and CNNA are conflicting. Some report a lower mortality for CNNA compared with culture-positive SBP[40]; others report comparable rates[41]. Bacterascites has also been diagnosed in patients with positive microbiological culture of ascites and a PMN count < 250 cells/mm^3[36,42]. The incidence of bacterascites in inpatients with LC has been estimated at 3%-4%[42]. Most reports on bacterascites were published in the 1980s and early 1990s; very few are recent[43]. The diagnosis of SBP is necessary to distinguish from the cases with secondary bacterial peritonitis, partially because surgical treatments should be considered in secondary bacterial peritonitis but never in SBP[44,45]. Secondary bacterial peritonitis consists of ascitic fluid infection due to intraabdominal infections, for example, perforation of gastrointestinal tract or abscess[44,45]. It is much less frequent but has still high mortality rate compared with SBP in patients with LC[44,45].

Causative organisms

Ascitic fluid is culture positive in 35%-65% of patients with SBP[35,46-49]. The positive ascitic fluid bacterial cultures have been reported to increase by placement of the fluid directly into blood culture flasks at the bedside immediately after collection for a diagnosis of SBP[46,50]. BT from the intestinal tract is believed to be involved, as enterobacteria account for a relatively large percentage of the causative bacteria[8]. However, some reviews report a recent shift in the bacterial spectrum to include a high prevalence of Gram-positive bacteria (16.6%-68.3%) globally[32,51,52]. The change in etiology may have resulted from increases in the use of quinolones for bacterial prophylaxis and instrumentation in patients with LC[32]. The most frequently cultured organism in the ascitic fluid of patients with LC and SBP is *Escherichia coli*[18,30,52-56], followed by Gram-negative *Klebsiella* spp.. *Streptococcus* spp., *Staphylococcus* spp., and *Enterococci* are frequently isolated Gram-positive bacteria[30,53-56]. Kalvandi *et al*[57] reported *E. coli*, and Preto-Zamperlini *et al*[58] reported *Streptococcus pneumoniae* as the most frequent isolate in the ascitic fluid of children with SBP.

The incidence of recurrent SBP has decreased in parallel with the use of norfloxacin[59,60], but the increased prevalence of MDR bacteria in patients with SBP may be related to the use of long-term antibiotic prophylaxis or invasive procedures such as catheterization and ablation of hepatocellular carcinoma[61]. MDR bacteria are found frequently in nosocomial SBP (20%-35%)[61,62], but also occur in community-acquired SBP (4%-16%)[18]. Nosocomial SBP is also more likely to be antibiotic resistant. Balaraju *et al*[63] reported that up to 48% of the *E. coli* in patients with nosocomial SBP were resistant to third-generation cephalosporins. Li *et al*[62] found higher frequencies of ESBL-producing *E. coli* and *Klebsiella* spp. in cases of nosocomial compared with

non-nosocomial SBP.

Diagnostic markers

The gold standard for a diagnosis of SBP is the PMN count in the ascetic fluid[36], but paracentesis is not always possible. Laboratory markers are useful for early diagnosis of SBP and early prediction of the response to initial treatment because a lack of response is a predictor of SBP mortality[64-66]. TNF-α and interleukin-6 are significantly higher in the ascitic fluid of patients with SBP than in those with sterile ascites[67,68], and increases of those proinflammatory cytokines have been associated with renal impairment complicated by SBP and with mortality[67,69]. The lactoferrin concentration is also higher in patients with SBP than in those with sterile ascites[70-72], and the lactoferrin level in ascitic fluid has shown high sensitivity and specificity for the diagnosis of SBP[39]. The optimal timing of lactoferrin assays is not yet clear, and diagnostic assay kits are not commercially available[39].

Procalcitonin, a prohormone of calcitonin synthesized in the C cells of the thyroid gland[73,74], is an acute-phase reactant protein that has been studied in patients with SBP. Seven studies assayed serum procalcitonin[69,75-80]; three assayed procalcitonin in ascitic fluid[75,76,80]. Serum procalcitonin was significantly higher in SBP than in sterile ascites in six of the seven[69,75,76,78-80], which supports use of serum procalcitonin as an SBP marker. In a review by Yang *et al*[81] of the available data from 339 patients with LC accompanied by SBP, it was concluded that serum procalcitonin was a relatively sensitive and specific marker for the diagnosis of SBP. It has been reported that serum procalcitonin was significantly higher in cirrhotic patients with culture-positive SBP than in those with CNNA[77,82]. Two of the three evaluations of procalcitonin in ascitic fluid found no significant differences in procalcitonin levels in patients with SBP and those with sterile ascites[76,80]. The usefulness of ascitic fluid procalcitonin to distinguish between SBP and sterile ascites has not been demonstrated.

Calprotectin is a calcium- and zinc-binding protein with antimicrobial and antiproliferative functions. It is almost exclusively expressed in neutrophils, and its level in body fluids is proportional to the influx of neutrophils[69,83]. Burri *et al*[83] reported that ascitic fluid calprotectin level was correlated with the PMN count and that it reliably predicted a count of $\geqslant$ 250 cells/mm^3, which is the standard for a diagnosis of SBP. Subsequent studies found that ascites calprotectin is significantly higher in cirrhotic patients with SBP than in those without SBP[47,69]. Lutz *et al*[47] have shown that the ratio of calprotectin to total protein in ascitic fluid was a better diagnostic marker of SBP than calprotectin alone and that a high ratio was independently associated with 30-d mortality.

Leukocyte esterase activity, which can be assayed with commercially available reagent strips, may have diagnostic value[39]. Castellote *et al*[84] reported that the use of reagent strips is a rapid and inexpensive tool for the diagnosis of ascitic fluid infection and it had a high negative predictive value (99%), indicating that a negative result may be useful as screening to exclude SBP. Oey *et al*[85] reviewed 23 studies of leukocyte esterase in patients with SBP published between 2002 and 2015 and concluded that it had poor sensitivity and positive predictive value for the diagnosis of SBP. They found that the sensitivity of the reagent strips for diagnosing SBP was variable, and a negative test result strongly suggested the absence of SBP[85]. In another review of 26 studies published from 2002 to 2010, Koulaouzidis[86] confirmed the poor sensitivity and poor positive predictive value of leukocyte esterase activity as well as the high 93%-100% negative predictive value. A negative test result may thus indicate a high probability of the absence of SBP[84-86].

There is evidence for the diagnostic value of other markers including monocyte chemotactic protein-1 in serum[87] and ascitic fluid[88,89], lipopolysaccharide-binding protein in serum[90] and ascitic fluid[91], macrophage inflammatory protein type-1 beta in ascitic fluid[76], interferon-γ-induced protein-10 in serum[92] and ascitic fluid[92], triggering receptor expressed on myeloid cells-1 in ascitic fluid[93], high-sensitivity CRP in serum[94] and ascitic fluid[55], and neutrophil gelatinase-associated lipocalin in ascitic fluid[95]. Further study is needed to validate the diagnostic usefulness of these candidate markers.

Incidence

The incidence of SBP in LC with ascitic fluid has been estimated as 7%-30% in hospital inpatients[52,56] compared with 1.5%-3.5%[56,96,97] in outpatients with LC. The annual recurrence rate is approximately 70%[98], and the 1-year survival after recovery from the first episode of SBP is 30%-40%[99].

Prediction of incidence

Factors associated with the incidence of SBP in patients with LC and ascites include age, history of SBP[100], gastrointestinal bleeding[61,100], and endoscopic intervention for varix control[101]. Severity of liver dysfunction[42,54,56,61,102] including the Child-Pugh score or model for end-stage liver disease (MELD) score has been reported as a predictive factor, but few studies have not found an association of MELD score and the incidence of SBP[103] in patients with LC and refractory ascites. The MELD score does not include some clinical variables that are evaluated in the Child-Pugh score[104,105]. A PMN count[42,102] and a low protein concentration (< 1.5 g/dL) in the ascitic fluid, which may be related to decreased opsonic activity in the ascitic fluid[26,54,56], have also been reported as predictive factors, but the evidence for ascitic fluid protein is not conflicting[106]. The severity of liver dysfunction and serum and ascitic albumin levels have also been reported as risk factors for the recurrence of SBP[39].

Long-term use of proton pump inhibitors (PPIs) may increase the risk of SBP[107-110] because of facilitation of intestinal BT, but the association is controversial[26,111,112]. Multivariate analysis has identified the use of PPIs by patients with LC as an independent risk factor for the development of SBP[108,109], and Kwon *et al*[107] reported that PPI use was associated with the development of SBP and increased mortality, but other studies have not found a significant association of PPIs and development of SBP in patients with LC[112,113]. A recent meta-analysis by Yu *et al*[114] of 10 case-control and six cohort studies involving 8145 patients and published between 2008 and 2014 concluded that it is not possible to establish causality between PPI use and increased risk of SBP. A similar analysis by Khan *et al*[115] of six case-control and eight cohort studies concluded that there was a statistically significant association between PPI use and increased incidence of SBP, but that the difference in incidence was small. Kim *et al*[116] reported that PPI use was not associated with risk or SBP recurrence. PPI use may be associated with slightly increased risk for SBP[117], but the significance of the association has not been confirmed.

Mortality

SBP mortality has decreased in the four decades since it was first described as a result of early diagnosis and prompt treatment[56] and treatment by liver transplantation[103]. Hospital mortality is estimated as 10%-50% for the first episode and 31%-93% for the second or subsequent episodes[53,118]. Recent studies have estimated 1-mo mortality at > 20%[52,102], inpatient mortality at > 30%[61], and 1- and 2-year mortality following an SBP episode as 50%-70% and 70%-75%, respectively[23,39]. A recent nationwide cohort study in Taiwan reported that SBP mortality was 24.2% at 1 mo and 66.5% at 3 year[119].

Prediction of mortality

Predictors of mortality in SBP include severe underlying liver disease with a high Child-Pugh[48,52,64,96,100,102,104,105,120-122] or MELD score[47,48,52,104,121-123], renal impairment[16,52,54,64,65,96,100,124,125] such as HRS, and onset of severe sepsis[96]. Some studies did not find differences in the mortality of nosocomial and community-acquired SBP[63]; others have reported increased mortality in nosocomial compared with community-acquired SBP, which was correlated with the involvement of drug-resistant bacteria[4,21,63,65]. Other predictors of SBP mortality include old age[16,102], gastrointestinal bleeding[4,52], complications of shock[65], rapid deterioration of liver function[64], positive ascitic fluid culture[16,48], elevated blood leukocyte level[47,104,123], low serum sodium[63,102], complications of hepatic encephalopathy[52,63,122], presence of hepatocellular carcinoma[54], and complications of other infections, such as pneumonia[122]. Tandon *et al*[123] reported that peripheral blood leukocyte count $\geqslant 11 \times 10^9$ cells/L and MELD score $\geqslant 22$ were independent predictors of 30-d mortality in patients with LC and SBP.

Renal impairment develops in 30%-40% of patients with LC and SBP[16] and is a significant prognostic factor in patients with LC and SBP. The available evidence strongly associates renal impairment with SBP mortality[16,52,54,64,65,96,100,124,125], but Lim *et al*[97] exceptionally reported that renal impairment, including HRS, was not predictive of mortality possibly because of early and effective treatment of SBP. Tandon *et al*[121] reported 67% mortality in patients with SBP and renal failure, but only 11% in patients with SBP and normal renal function. Some clinical studies indicated the use of intravenous albumin in addition to antibiotics for treatment of SBP[70,126-130]. Possible mechanisms of albumin use for improvement of SBP include its oncotic properties, immunomodulatory and antioxidant effects, and its endothelium stabilization capacity[131]. Dosage and duration of intravenous albumin in previous studies were as follows: (1) 1.5 g/kg body weight at the time of diagnosis and 1 g/kg body weight on day 3[126-128,130]; and (2) 20% 50 mL every day for 3 d[70]. Moreover, some clinical studies indicated that the use of intravenous albumin and antibiotics can reduce the incidence of renal impairment and mortality in patients with LC and SBP[126,129,130]. The efficacy of albumin treatment in patients with LC and other types of bacterial infection remains unknown. Thévenot *et al*[132] reported that albumin did not improve renal function or survival at 3 mo in patients with LC and non-SBP bacterial infections.

A meta-analysis of studies published between 2002 and 2011 identified a four-fold increased risk of mortality of patients with LC and SBP caused by MDR bacteria[133], and a retrospective multicenter study in Korea found that SBP caused by ESBL-producing bacteria was an independent prognostic factor of high in-hospital mortality[54,108]. Chon *et al*[4] reported that both in-hospital mortality and follow-up mortality after recovery from SBP were significantly higher in nosocomial SBP than in community-acquired SBP. Alexopoulou *et al*[31] did not find significant differences in the 30-d mortality of infections caused by MDR bacteria and nonresistant bacteria in 60 cases of culture-positive SBP and 70 cases of bacteremia without SBP.

However, 30-d mortality from infections caused by XDR bacteria was significantly higher than that of infections caused by MDR or nonresistant bacteria[31]. In another study, an overall 20% 30-d survival of nosocomial SBP was related to inadequate empirical antibiotic treatment[32].

Antibiotic prophylaxis

Antibiotic prophylaxis is effective, but long-term antibiotic administration leads to the emergence of MDR, which is a serious concern worldwide[33,134-136]. The current practice guidelines of the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver recommendations[26] limit its recommended use to patients who are at the highest risk of developing a bacterial infection. However, some studies have reported significant decreases

in the incidence of SBP in patients with antibiotic prophylaxis[18,137]. Patients with LC and low ascites protein concentrations of < 1.5 g/dL are at risk of developing a first episode of SBP[26,138]. Primary antibiotic prophylaxis is recommended in patients with LC and no history of SBP and low ascites protein plus other predisposing factors, such as severe liver failure (Child-Pugh score > 9), impaired renal function (serum creatinine > 1.2 g/dL or blood urea nitrogen > 25 mg/dL), or hyponatremia (≤ 130 mEq/L), are present[23,26,102]. Primary prophylaxis is also recommended in patients with LC and acute gastrointestinal bleeding[138] or to reduce the incidence of SBP after gastrointestinal bleeding[134]. Antibiotic prophylaxis has also been associated with reduced rebleeding within 7 d and to lower 28-d mortality[18,134]. The standard therapy in patients with LC and acute gastrointestinal bleeding is oral norfloxacin[18], but Fernández *et al*[139] reported that intravenous ceftriaxone is more effective than oral norfloxacin. A randomized, controlled trial demonstrated that primary prophylaxis with trimethoprim/sulfamethoxazole also decreased the risk of SBP[140]. Recurrence rates as high as 70% have been reported in patients with LC who have recovered from a previous episode of SBP without secondary antibiotic prophylaxis[61]. Secondary prophylaxis is indicated for patients with a previous episode of SBP[26,53]. Norfloxacin was shown to decrease overall recurrence from 68% to 20%[26] and recurrence with a Gram-negative microorganism from 60% to 3% in the first year[61]. However, studies published between 2010 and 2016 reported that the frequency of recurrence with bacteria resistant to quinolones was 30%-33%[52]. The efficacy and safety of rifaximin for primary prophylaxis for SBP and for prophylaxis of recurrent SBP have been studied[141-145]. A study of primary prophylaxis by Assem *et al*[141] demonstrated that alternating norfloxacin and rifaximin as combination therapy was remarkably more effective compared with norfloxacin monotherapy in part because of an increased incidence of quinolone-resistant and Gram-positive SBP. Mostafa *et al*[142] showed that rifaximin prophylaxis was more effective than norfloxacin in patients with LC and at least one previous episode of SBP. A randomized, controlled trial of secondary prophylaxis by Elfert *et al*[143] demonstrated that SBP recurrence and mortality were significantly lower with rifaximin than with norfloxacin.

A systematic review by Goel *et al*[144] concluded that rifaximin was as effective for both primary and secondary prophylaxis as other systemically absorbed antibiotic. Rifaximin might be considered, particularly in cases involving quinolone-resistant bacteria[18]. The optimal duration of prophylaxis is currently unclear[26], but secondary prophylaxis should be continued until the ascites is resolved or liver transplantation can be performed[3].

Empirical antibiotic treatment for spontaneous bacterial peritonitis

Empirical antibiotic treatment must be initiated immediately after the diagnosis of SBP[53]. If the ascites neutrophil count decreases to < 25% of the pretreatment value after 2 d of antibiotic treatment, then there is an increased probability of failure to respond to any treatment[66]. Third-generation cephalosporins have been the most frequently used antibiotics in the treatment of SBP since 1985[4] and were highly effective until about 10 year ago[64]. They are still effective for community-acquired infections in patients with LC, with resolution rates of around 80%[33], but the development of resistance to third-generation cephalosporins is of great concern. Resistance can result in failure to respond to initial empiric therapy with a third-generation cephalosporin in 33%-75% of cases, and failure to respond is associated with reduced survival[48,49]. Recent studies indicate that third-generation cephalosporins are not appropriate for the treatment of nosocomial infections in patients with LC[34] because of effectiveness as low as 40% related to an increase in the prevalence of MDR bacteria in nosocomial infections[61,64]. SBP treatment recommendations distinguish between nosocomial and community-acquired infections[61] following evidence from studies like that by Lutz *et al*[146] who reported that approximately one third of health care-related and nosocomial SBP infections were resistant to third-generation cephalosporins. They also found that patients with health care-related SBP infections resistant to first-line treatment had worse survival than were those with infections susceptible to first-line treatment[146]. Moreover, Ariza *et al*[147] reported that resistance to third-generation cephalosporins occurred in 7.1% community-acquired SBP, 21.1% health care-related SBP, and 40.9% nosocomial SBP among 246 episodes in 200 patients with LC and SBP (2001-2009). Some studies recommend that third-generation cephalosporins not be used for empirical treatment of health care-related SBP[136,146]; Lutz *et al*[146] recommend piperacillin-tazobactam rather than third-generation cephalosporins. The most recent recommendations restrict third-generation cephalosporins to selected patients, primarily those with community-acquired SBP[21,32].

Daptomycin is effective against Gram-positive *Enterococci* resistant to vancomycin and against MRSA. A recent randomized, controlled study reported that empirical treatment of nosocomial SBP with meropenem plus daptomycin was more effective in resolving SBP and had better 3-mo survival than did third-generation ceftazidime[31,64]. Piano *et al*[64] and European expert opinion[49] both recommend a combination regimen of meropenem and daptomycin for the management of nosocomial SBP. A randomized trial by Jindal *et al*[122] reported that cefepime, a fourth-generation cephalosporin with good activity against most nosocomial Gram-negative bacteria and Gram-positive cocci, was as effective as imipenem for resolution of SBP. Following evaluation of antibiotic susceptibility in 575 SBP cases, Shi *et al*[30] recommended cefoperazone/sulbactam or piperacillin/tazobactam for the empirical treatment of SBP.

SPONTANEOUS FUNGAL PERITONITIS

Patients with LC are at an increased risk of fungal infection[138] because the antibiotics used for prevention of SBP can select for excessive growth of fungi in the intestinal flora with subsequent fungal translocation into peritoneal cavity and development of SFP[6]. Fungi are much larger than bacteria, which makes fungal translocation across the gut mucosa more difficult than BT, and may require higher intestinal permeability, which is more common in patients with advanced LC[148,149]. Fungal translocation may be facilitated by upper gastrointestinal bleeding, which is also common in patients with advanced LC[150]. Immunosuppression and malnutrition in LC patients also promote this fungal translocation[6]. Direct percutaneous inoculation of fungi is the proposed route of fungal infection in patients with refractory ascites and a history of paracentesis[150].

There are few data on the characteristics of SFP in LC patients[5,6], but case studies are available. Fungal colonization in ascitic fluid is not a rare complication in end-stage liver disease[5], and studies of the clinical characteristics of SFP are becoming more frequent. SFP is defined as a fungal infection of ascitic fluid with no apparent intraabdominal source of infection or malignancy[148]. A PMN count of $\geqslant$ 250 cells/mm^3 in the ascitic fluid with a positive fungal culture regardless of co-colonization of bacteria is diagnostic of SFP[151]. A positive fungal culture with a PMN count of < 250 cells/mm^3 is diagnosed as fungiascites or fungal ascites[151]. Fungal ascites has a higher mortality rate than does bacterascites[151]. Of spontaneous peritonitis cases, 0%-7.2% are culture positive for fungus[6,30,32,62,82,148,152-154], and the most frequent isolate is *Candida albicans*[5,6,148,149,153-156]. Other causative fungi include *Candida glabrata*[30], *Candida krusei*[153], *Cryptococcus* spp.[148,157], *Aspergillus* spp.[150,154], and *Penicillium* spp.[154]. Polymicrobial infections, i.e., bacterial co-colonization, occurs in 32%-74% of SFP cases[148,149,153,154,156], but early diagnosis by conventional microbial culture is difficult because of the time required for growth[155], and the efficacy of PCR or assay of the fungal biomarker 1,3-beta-D-glucan in ascitic fluid has not been established[149,155].

High Child-Pugh or MELD scores increase the risk of SFP in patients with LC[5,6,148], and a retrospective case-control study by Gravito-Soares *et al*[153] found no significant difference in the Child-Pugh or MELD scores of patients with SFP and SBP. The risk of SFP is increased in patients with LC who undergo invasive procedures[153] and increases with the length of the hospital stay[153,156]. Some studies have reported that a significantly greater proportion of SFP infections than SBP infections were nosocomial[148,156], but the data are conflicting[6]. In previous studies of spontaneous peritonitis with ascitic culture-positive diagnosed between 2003 and 2016, nosocomial SFP was confirmed in 7.7% (53/689) of nosocomial spontaneous peritonitis, and non-nosocomial SFP was confirmed in 1.7% (17/1018) of non-nosocomial spontaneous peritonitis[156].

Risk factors associated with hospital mortality in SFP include severe underlying liver disease[148], Child-Pugh score[155], MELD score[149], antibacterial prophylaxis[6], incidence of HRS[6], low ascites protein concentration[6], Acute Physiology And Chronic Health Evaluation Ⅱ score[149], and sepsis shock[153]. SFP mortality is estimated to be 56%-90%[5,148,149,151,153], and 1-mo mortality may[148,153] or may not be significantly higher than SBP mortality[149,157]. Hwang *et al*[148] reported a high SFP mortality that was related to unresponsiveness to initial empirical treatment for suspected SBP. The condition of nearly all the patients with SFP worsened after initial empirical treatment, and they died during the early stage of peritonitis regardless of undergoing antifungal treatment[148]. Some patients with SFP improved after receiving the initial empirical treatment without any antifungal agents[148]. Those patients may have had SBP and colonization by innocent fungi[148], as it was not possible to distinguish fungal colonization from true SFP in the clinical setting[148]. SFP is usually diagnosed after the identification of fungi in cultures of ascitic fluid. The mortality is high because of delayed diagnosis, lack of clinical signs, lack of suspicion of SFP, and delay in treatment with antifungal therapy[5,6,148,155,156,158]. Fungal resistance to empirical specific antifungal therapy together with delayed diagnosis and treatment is related to poor prognosis of SFP[153].

Recent treatment guidelines on management of infections in LC do not include antifungals for prophylaxis or optimum treatment but do include recommendations for fungal infections[138]. Echinocandins are recommended as first-line treatment for patients with LC and nosocomial SFP or critically ill patients with LC and community-acquired SFP[148]. Fluconazole is recommended for less severe infections[6,138,152]. De-escalation from echinocandins to fluconazole is advised in critically ill patients with LC and SFP when their condition is stable and sensitivity tests are available[6,152]. However, directed antifungal therapy may not improve the outcomes of some patients with SFP[149,156] because of lack of response to the administration of empirical treatment and delay in starting antifungal therapy[156].

Although early differentiation between SFP and SBP may be difficult partially because identification of fungi in cultures of ascitic fluid is time-consuming, clinician is able to suspect SFP or SBP due to MDR if spontaneous peritonitis is not improved after 48 h empirical antibiotic treatment[151]. Therefore, new cultures in ascitic fluid and blood may be performed in these patients[151]. Moreover, additional administration of antifungal agents or administration of antifungal agents and alternation of antibiotics may be considered in these patients[151,159].

CONCLUSION

The evidence of previous studies confirms that SBP and SFP in patients with LC is often life-threatening. The severity of liver dysfunction, the presence of renal impairment, and the emergence of MDR bacteria have

a clinically significant influence on the prognosis of SBP. The efficacy of the recommended antibiotic therapy for SBP may be decreased in nosocomial infections because of increases in the prevalence of MDR bacteria. In SFP, mortality is associated not only with the severity of the underlying liver disease but also with delay in diagnosis and initiation of antifungal therapy. Further research is needed to better our understanding of the nature of SFP and improve the response to treatment with the available antifungal agents.

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MINIREVIEWS

Impact of direct acting antivirals on occurrence and recurrence of hepatocellular carcinoma: Biologically plausible or an epiphenomenon?

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Abstract

Hepatocellular carcinoma (HCC) is a major cause of morbidity and mortality worldwide. Chronic hepatitis C virus infection (HCV) is the most common cause of HCC in many European countries, Japan and Pakistan. Introduction of the new direct acting antivirals (DAAs) has revolutionized the management of HCV worldwide, with high rates of sustained virologic response in patients who could not have tolerated the previous interferon based treatments. However, recently there have been reports raising caution about the long term effects of DAAs, particularly a possible increased risk of HCC. Therefore this review explores the current molecular studies as well as clinical data that investigate the impact of DAAs on occurrence and recurrence of HCC.

Key words: Hepatocellular carcinoma; Direct acting antivirals; Hepatitis C

Core tip: Our aim is to consolidate the existing literature as well as to identify whether there is a particular subset of the population in which this phenomenon was witnessed. The ground-breaking discovery of the new group of direct acting antiviral agents (DAAs) had led to a paradigm shift in the management of chronic hepatitis C (CHC). Wide variations have been observed in the studies assessing the long-term role of DAA based therapy on occurrence and recurrence of HCC. There is a need to differentiate weather the reported higher occurrence and recurrence rates are due to DAA or host and disease related factors and to identify subset of individuals particularly at risk. Also, future investigations should be directed towards

assessing the long-term effects of DAAs on group of patients that have not been studied thus far. Some important Centers in Europe and United States have been delaying antiviral treatment for 6 mo or more after the recent treatment for HCC. Hence, until more robust data is available, clinical practices should continue as per current guidelines in those patient groups who can benefit from DAA therapy with close surveillance of patients with advance fibrosis.

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INTRODUCTION

The ground breaking discovery of the new group of direct acting antiviral agents (DAAs) had led to a paradigm shift in the management of chronic hepatitis C (CHC) which is the most common cause of hepatocellular carcinoma (HCC) in Japan, Pakistan, United States and many European countries[1,2]. With annual incidence of HCC ranging from 1% to 7% in patients with HCV related cirrhosis, (HCC) is a leading cause of morbidity and the second most common cause of cancer related deaths worldwide[3,4]. Besides contribution of several host and viral factors in the pathogenesis of disease progression, achieving sustained virologic response (SVR) has been found as the single most important factor in reducing HCV associated HCC incidence[5].

The novel DAAs not only provided a potent, oral alternative to injectable interferons, but also had a shorter duration of treatment, better efficacy with over 90% achievement of SVR and a more favorable side effect profile[6]. However, since 2016, concerns were raised regarding the effect of DAAs on progression to HCC[7]. In addition, their long-term benefits including impact on HCC have been questioned in the context of specific populations and subgroups which were not included in the landmark trials investigating DAA based therapy[3].

Therefore, this review aims to explore existing molecular studies as well as clinical observations in order to determine whether there is an association between the use of DAAs and the occurrence or recurrence of HCC among patients with HCV related liver disease. We also aim to evaluate whether there is a subset of the population in which this phenomenon has been observed.

DAA AND CARCINOGENESIS: BIOLOGICALLY PLAUSIBLE OR NOT?

The carcinogenesis of HCC is a chronic process with several steps that may serve as potential targets for drug therapy. Unlike the hepatitis B virus, HCV is an RNA virus which is unable to integrate into the host genome and thus it is unlikely to have direct carcinogenic activity[8]. While the mechanism of carcinogenesis due to HCV is not completely understood, observations from transgenic mice models suggest that liver cancer occurs because of rapid hepatocyte turnover, dysregulation of apoptosis and generation of reactive oxygen species, arising in the setting of a chronic inflammatory state induced by HCV[9,10]. This indirect mechanism of cirrhosis driven carcinogenesis is supported by clinical data which shows a greater risk of HCC with chronic HCV infection and worsening liver fibrosis[8].

One of the mechanisms proposed regarding the risk of DAAs towards development of HCC is that DAAs downregulate interferon genes, disrupting the innate immunosurveillance of the body[8]. In the chronic phase of HCV, an estimated 10^{12} virions are produced per day by infected hepatocytes[11]. These trigger an immune response mediated primarily by natural killer (NK) cells which release cytokines such as interferon (IFN)-γ. IFNs upregulate interferon stimulated genes which have an anti-proliferative response by prolonging all phases of the cell cycle and decreasing viral replication. When HCV infection becomes persistent, NK cells become dysfunctional due to continuous antigenic stimulation by the high load of virions, resulting in impaired production of IFNs[12]. In mice models, it was observed that decreased levels of IFN-γ independently control tumorigenesis[13]. Thus the downregulation of these genes in IFN free therapy could contribute to development of HCC.

Analysis of the peripheral blood mononuclear cells (PBMCs) of CHC patients treated with DAAs has shown that in comparison to healthy controls, these patients have attenuated activity of both NK cells and monocytes, reflected by a decreased level of inflammatory cytokines in patients who had achieved rapid virological response (RVR), *i.e.*, undetectable HCV RNA at the end of 4 wk of DAA treatment[14]. Natural Killer cell group 2D (NKG2D) is an activating receptor of immune responses which has been studied in the context of HCV associated HCC. It has been found that in HCV patients treated with IFN free DAA therapy, an on treatment decrease in the expression of NKG2D correlated to the early occurrence and recurrence of clinically evident HCC within the 6-mo surveillance period following treatment[15].

With DAA based therapy, HCV RNA becomes undetectable in days to weeks, a much more rapid response than that observed with IFN based regimens. It is hypothesized that rapid eradication of HCV and subsequent abrupt resolution of the chronic inflammatory state disrupts the natural immune response of the body, possibly favoring the proliferation of neoplastic cells[16]. Since clinical studies have shown no difference among different DAA regimens and development of HCC, it is hypothesized that if this effect does exist then it would have to be a class effect of the DAAs[17,18].

Table 1 Possible factors contributing to hepatocellular carcinoma after hepatitis C virus eradication by direct acting antivirals

Down regulation of *IFN* genes
Presence of fibrosis
Sudden disruption of chronic inflammatory state
Impaired immune response by NK cells
T cell dysfunction
Decreased microRNA-122

IFN: Interferon; NK: Natural killer.

An experimental study analyzing the soluble inflammatory milieu from plasma cells of cirrhotic HCV patients found that HCV specific CD8+ T cells failed to recover from the baseline after DAA therapy. These cells play a role in HCC surveillance; therefore, their reduced activity in IFN free therapy could potentially affect development of HCC[19]. Furthermore, serum levels of microRNA (miRNA) 122 were found to be reduced in DAA treated HCV patients after achieving SVR[20]. Previous evidence shows that miRNA-122, which is the most abundant miRNA in the liver, functions as a tumor suppressor against HCC[21]. Thus it is hypothesized that decreased miRNA-122 in DAA treated patients could contribute to an increased risk of HCC recurrence[22]. Table 1 summarizes the possible factors that could lead to HCC in DAA treated HCV patients.

DAA AND CARCINOGENESIS: REVIEW OF EXISTING EVIDENCE/DATA

Studies that observed increased incidence of HCC

While DAAs represented a major breakthrough in the treatment of CHC, one of the first reports questioning their long-term role in development of HCC came from Reig *et al*[8] in 2016. The authors retrospectively assessed a cohort of 58 patients who had a history of HCC secondary to HCV and had received different regimens of DAA based therapy. After a median follow up of 5.7 mo (range 0.4-14.6 mo), 16 out of their 58 patients (27.6%) showed radiographic evidence of HCC. This study alerted the scientific community regarding the potential risks associated with use of DAAs and the authors called for a large-scale assessment to confirm their findings[8]. However, their method of statistical analysis was questioned by Camma and colleagues who felt that reporting the crude rate of recurrence was a weakness of the study because of the variation in time elapsed from treatment of HCC and starting DAAs (median 11.2 mo, range 1.2-87.7 mo)[23]. Cammà *et al*[23] used the data presented by Reig *et al*[24] to calculate the actuarial probability of HCC recurrence by plotting a Kaplan Meier curve. For their analysis, Cammà *et al*[23] used the time of HCC treatment as the initiation point, not the time of initiating DAA treatment as used by the original authors. With this method they found a much lower recurrence rate than that reported in the original study (7% and 13% at 6 and 12 mo respectively).

Reig *et al*[24] later went on to present a follow up of their original cohort. In 2017, they reported that not only did they observe a higher recurrence rate of HCC among the DAA treated patients in their study; they also found a more aggressive pattern of recurrence in terms of tumor staging and subsequent treatment options. Renzulli *et al*[25] have also reported a rapid development of HCC following DAA treatment with a more aggressive pattern of microvascular invasion. The median duration between completion of DAA treatment and diagnosis of HCC in their patients was 82 d (range 0-318).

A retrospective study from Italy by Conti *et al*[18] reported HCC occurrence and recurrence rates of 3.16% (95%CI: 1.45-5.90) and 28.81% (95%CI: 17.76-42.07) respectively in a cohort of 344 CHC patients treated with different DAA regimens over a follow up of 24 wk. Approximately 69% of this patient population had HCV genotype 1 and 91% had achieved SVR. However, the lack of a control group makes the interpretation of these findings difficult. The authors attempted to account for this limitation by comparing their findings to those of a historic cohort of untreated cirrhotic patients at their center. They found an HCC occurrence rate of 3.2%, which was similar to their current study. Conti *et al*[18] have interpreted their results with caution. The authors claim that while DAA treatment of HCV does not seem to reduce the occurrence or recurrence of HCC, anitiviral treatment should be started as early as possible to prevent the development of cirrhosis and recommend active surveillance of all cirrhotic patients, during and after DAA therapy.

In Portugal, Cardoso *et al*[26] found HCC incidence to be 7.4% within a one year follow up of cirrhotic patients that had achieved SVR after being treated with sofosbuvir and ledipasvir. These *de novo* HCC patients had been asymptomatic, and were detected on radiological screening. This emphasizes the recommendation of Conti *et al*[18] that there should be close monitoring of CHC patients for development of HCC, despite achieving SVR[25]. In a larger study from Belgium, it was found that there was no difference in the early occurrence of HCC among patients treated with DAAs with or without Peg-IFN. However, this study reported HCC recurrence of 15% in patients treated with DAAs alone as compared to 0% in those who received a combination of Peg-IFN and DAAs. This study had a predominantly HCV Genotype 1 population. In these patients the authors also noted that those who developed HCC had a higher baseline risk of HCC which is a potential confounder[27].

The most recent study on this research question is by Ida *et al*[28], published in October 2017. The study population comprised 100 patients from Japan with HCV genotype 1, treated with Daclastavir and Asunaprevir, who were followed for 15 mo. In this group, there were 5 new cases and 12 recurrences of HCC. The authors

have hypothesized that the high rate of HCC seen in this study could be related to a history of HCC, as these patients already had advanced fibrosis which is known to be implicated in the process of hepatocarcinogenesis. Table 2 summarizes the studies that report an increased incidence of HCC in DAA treated HCV patients.

Studies that did not note any significant effect

In 2016 and 2017 there have been several other similar reports, fueling the debate regarding the role of DAAs in HCC. Two large retrospective cohort studies have been conducted in the United States to investigate this matter; one by Ioannou *et al*[33] with a sample size of 62354 and the other by Kanwal *et al*[34] with 22500 study participants. Both studies concluded that DAAs are not associated with a significant risk of HCC as compared to IFN based treatment. Ioannou *et al*[33] found that DAA induced SVR reduced the risk of HCC by 71%. This effect was similar in groups who had received DAAs alone, DAAs in combination with IFN or IFN alone regimens, thus suggesting that achieving SVR could be the crucial factor for risk reduction of HCC, regardless of the therapeutic agents used[33]. Kanwal *et al*[34] found that while there was a relative risk reduction in HCC, the absolute risk of HCC still persisted in those patients who had DAA induced SVR. In both studies the risk of HCC was greater in cirrhotic patients. Additionally, Kanwal *et al*[34] found that diabetes mellitus, alcohol use and a higher Fib-4 Index for assessment of fibrosis were risk factors for occurrence of HCC in patients who had achieved SVR. However, both study populations were restricted to United States veterans and were mostly patients with HCV genotype 1. The specific population included in these studies might limit the generalization of their findings.

The ANRS Collaborative Study Group assessed HCC recurrence rates among 3 French multicenter prospective cohorts who had received DAA based therapy after HCC curative treatment[35]. Their study included a diverse patient population with cirrhotics, non-cirrhotics and liver transplant recipients. They did not find any evidence that DAAs increases the risk of HCC recurrence. The strength of this study was that analysis of data from 3 distinct patient cohorts yielded fairly consistent results. Secondly, they included patients who had received curative therapy for HCC as opposed to non-curative therapies such as chemoembolization, leading to speculation that some of the earlier studies that had reported a higher recurrence rate might have included patients in whom the initial tumor staging was incorrect or was incompletely treated[35]. Table 3 summarizes the studies that do not show an increased risk of HCC in DAA treated patients.

Geographical variation of incidence of HCC in DAA treated patients

Many of the studies investigating the link between DAAs and HCC have been from Japan or European countries. Racial differences are known to be implicated in the progression to HCC among HCV infected patients[52]. In a large cohort of United States veterans with HCV, it was found that Hispanics were at greater risk of developing cirrhosis and HCC. Black race and Hispanic ethnicity have also been identified as independent predictors of treatment failure[53]. Indeed the first report raising caution about the possible association of DAAs with recurrence of HCC in an aggressive form did come from the Spanish cohort followed by Reig *et al*[8].

A systematic review and meta-analysis by Waziry *et al*[54] found that there was no difference in HCC occurrence and recurrence in CHC patients who received IFN based or DAA based regimens. However, the authors acknowledge that in their analysis they were unable to account for geographical variations. Due to the heterogeneity in the studies included in this meta-analysis, the results should be interpreted with caution. In this meta-analysis most of the IFN based studies were from Japan whereas DAA based studies were from Europe. Hence, due to baseline difference in between two populations it's hard to draw an accurate conclusion regarding occurrence or recurrence of HCC among DAA and IFN based therapy. Furthermore, they had to exclude several studies which had incomplete data regarding BCLC staging of HCC.

In our literature review, the populations that we have found to be under-represented in terms of the current research question include Indians, Arabs and Africans. Additionally, while the 2 studies with the largest sample size are from the United States, these were limited to veterans only[33,34].

Genotype based variation of HCC in DAA treated patients

HCV genotype is an important consideration when considering progression to HCC. HCV genotype 3 is generally more aggressive and is associated with a higher risk of progression to cirrhosis and HCC[55]. To the best of our knowledge, the studies published so far suggesting a greater risk of HCC with DAA treatment have not identified a particular genotype of HCV that is significantly associated with this disease progression. However, as most of these reports are from Japan or European countries such as France and Italy, therefore the major disease burden studied has been of HCV genotype 1[35,37]. Table 4 lists the factors predisposing to development of HCC in DAA treated HCV patients.

WAY FORWARD

There is wide variation in the studies assessing the long-term role of DAA based therapy on occurrence and recurrence of HCC, both in terms of the baseline characteristics of study population and the different DAA regimens used. At present, most of the studies have been reported from regions where HCV genotype 1 is the most prevalent one[34,35]. Data is very limited from Asian populations where HCV Genotype 3 is the most common genotype. This is an important consideration as the different genotypes of HCV have a unique

Table 2 Studies showing an increased incidence of hepatocellular carcinoma in direct acting antivirals treated patients

Ref.	Treatment groups	Country/sample size	Male gender *n* (%)	Age (yr)	Genotype *n* (%)	Duration of follow up	Cirrhosis *n* (%)	History of HCC *n* (%)	HCC occurrence	HCC recurrence
Reig *et al*[8]	All DAA treated	Spain *n* = 58	40 (69)	Median 66.3 (45-83)	G1a = 8 (13.8%), G1b = 45 (77.6%), G3 = 2 (3.4%), G4 = 3 (5.2%)	median 5.7 mo	55 (94.8)	58 (100)	Not applicable	16 (27.6%)
Conti *et al*[18]	All DAA treated	Italy *n* = 344	207 (60.2)	Median = 63 (29-85)	G1= 237 (69), G2= 40 (11.6), G3= 38 (11), G4= 29 (8.4)	24 wk	All cirrhotic	59 (17)	9 of 285 patients (3.16%, 95%CI: 1.45–5.90)	17 of 59 patients (28.81%, 95%CI: 17.76–42.07)
Cardoso *et al*[26]	All DAA treated	Portugal *n* = 54	> 70%	No HCC: 59 yr (41-81). Patients with HCC = 58 yr (55-72)	No HCC: G1 = 78%, G3 = 18%. Patients who developed HCC: G1 = 75%, G3 = 25%	Median = 12.0 mo (9.4-12.5 mo)	All cirrhotic	0 (0)	4 (7.4%)	Not applicable
Ida *et al*[28]	All DAA treated	Japan *n* = 100	46 (46)	Median 72.5 (26-87)	G1 = 100 (100)	15 mo		26 (26)	5 (5)	12 (12)
Kozbial et al[29]	All DAA treated	Austria	-	56-74 yr	G1a = 3 (15.8), G1b = 13 (68.4), G3a = 2 (10.5), G4 = 1 (5.2)			3 (15.8)	6.60%	3 patients
Issachar *et al*[30]	All DAA treated	Israel *n* = 273	-	-	-	15 mo	-	-	6 (2.1)	3 (1.05)
Kwong *et al*[31]	patients divided into 3 eras: IFN era (2003-2010), protease inhibitor era (2011-2013), and DAA era (2014-2015)	United States *n* = 48158	29858 (62)	Median 55 (49-60) yr		Median 493 d	All cirrhotic		Incidence Rate of HCC was 49% higher in the DAA era (IR 6.6/100 person-years [py], 95%CI: 5.6-7.9) *vs* the IFN era (4.5/100 py, 95%CI: 4.2-4.7; IRR 1.49, 95%CI: 1.24-1.79, $P < 0.001$).	Not applicable
Strazulla *et al*[32]	daclatasvir and simeprevir for 24 weeks to reach SVR	Italy *n* = 1 case report	1 male	53	G1	24 wk	Decompe-nsated cirrhosis	Treated	Not applicable	1 recurrence
Bielen *et al*[27]	PEG-IFN+ DAA = 77 (13.5), DAAs only = 490 (86.4)	Belgium *n* = 567	PEG IFN + DAA group: 55 (71.4) DAA only group: 307 (62.7)	PEG IFN + DAA group age : 52 ± 9, DAA only group: 59 ± 12	PEG IFN + DAA group: all genotype 1. DAA only: G1 = 69%, G4 = 14.7%	6 mo	Metavir fibrosis score F3/F4 included only	PEG IFN + DAA = 1/77 (1.3%),DAA alone: 41/490 (8.4%)	Early occurrence rate of HCC = 1.7% and 1.1% in patients treated with DAA with and without PEG-IFN, respectively	Early recurrence rate was 0% in patients treated with PEG-IFN+DAA and 15.0% in patients treated with DAA without PEG-IFN

DAAs: Direct acting antivirals; IFN: Interferon; HCC: Hepatocellular carcinoma.

Table 3 Studies that do not report an increased risk of hepatocellular carcinoma with direct acting antivirals

Ref.	Treatment N /sample size	Country	Male gender *n* (%)	Age (yr)	Genotype *n* (%)	Duration of follow up	Cirrhosis *n* (%)	History of HCC *n* (%)	HCC occurrence	HCC recurrence
Ioannou *et al*[33]	IFN only = 35871 (58%), DAA + IFN = 4535 (7.2%), DAA only = 21948 (35%) *n* = 62354	United States	96.60%	Mean 55.8 ± 7.6	G1 = 77.4 %, G2 = 13.5%, G3 = 8.3%, G4 = 0	Mean follow-up DAA only group = 1.53 years, DAA+IFN group= 3.6 yr, IFN only group = 9.1 yr	Cirrhosis: 16.8%, decompe-nsated cirrhosis: 4.7%	None	Total 3271 incident cases. IFN group = 0.81/100 person years, DAA + IFN = 1.06 /100 py, DAA only = 1.32/100 py	Not applicable
Kanwal *et al*[34]	All DAA treated *n* = 22500	United States	21761 (96.7%)	Mean 61.6 ± 6.1	G1 = 19531 (86.8%), G2 = 1422 (6.3%), G3 = 940 (4.2%), G 4-6 = 217 (1%)	22963 person years of follow-up	8766 (39.0%)	None	271 (1.2)	Not applicable
ANRS CO22 HEP ATH-ER *et al*[35]	DAA group = 189, no DAA = 78 *n* =267	France	DAA group = 147 (78%)	DAA group = 62 ± 9 yr, no DAAs = 66 ± 10 yr	65 % genotype 1	Median: 20.2 mo after DAA initiation and 26.1 mo for untreated patients	Cirrhosis: DAA group = 152 (80%), no DAAs = 55 (72 %)	All treated	Not Applicable	DAA group = 24 (12.7%), no DAAs = 16 (20.5%)
ANRS CO12 CIRVIR *et al*[35]	DAA group = 13, no DAA = 66 *n* = 79	France	DAA group = 11 (85%), no DAA = 39 (59%)	DAA group = 61 ± 10 yr, no DAA = 65 ± 9 yr	Genotype 1: DAA group = 11 (85%), no DAA group = 53/63 (84 %)		All cirrhotic	All treated	Not Applicable	DAA group = 1 (7.7%), no DAAs = 31 (47%)
ANRS CO23 CUPILT *et al*[35]	All DAA treated *n* = 314	France	257 (82%)	61 ± 8 yr	212 (67.5%) genotype 1		49 (15.6%)	Treated	Not Applicable	7 (2.2%)
Cabibbo *et al*[36]	All DAA treated *n* = 143	Italy	80 (60.1)	Mean 70.4 ± 8.9	G1a: 9 (6.3), 1b: 114 (79.7), G2: 9 (6.3), G3: 7 (4.9), G4: 4 (2.8)	6, 12 and 18 mo	All cirrhotic	All treated	Not applicable	6-, 12- and 18-mo HCC recurrence rates were 12%, 26.6% and 29.1%, respectively
Nagata *et al*[37]	IFN-based: 1145. IFN-free DAA group: 752 *n* = 1897	Tokyo	IFN group: 621 (54), IFN free: 340 (45)	Median: IFN group: 59 (19-79); IFN free: 69 (24-87)	IFN group: G1a = 8 (7), G1b = 833 (73), G2a = 182 (16), G2b = 105 (9), G3 = 1 (0)	Median for IFN group: 6.8 (0.2-22.0); IFN free: 1.8 (0.1–7.7)		5% of IFN group, 11% of IFN free group	IFN group: 18 (2.5%). IFN-free group: 7 (1.1%)	IFN group: 18 (53%). IFN-free group: 22 (29%)
Ikeda *et al*[38]	All DAA treated *n* = 177	Japan	M:F = 52: 37 in each group	DAA group: 71 (39-85)		Median 20.7 mo		All treated	Not applicable	HCC recurrence rates at 1st and 2nd year were 18.1 and 25.0% in pts with DAA therapy and 21.8 and 46.5% in those without DAAs, (P = 0.003)
Zanetto *et al*[39]	DAA treated= 23, control = 23 *n* = 46	Italy		DAA group = 59 (49-69), controls= 58 (46 -70)	DAA group: G1a = 5 (22), Gb = 9 (39), G2 = 1 (4), G3 = 5 (22), G4 = 3 (13)	Median= DAA group = 10 mo, Control group = 7 mo	All cirrhotic	All treated	Not applicable	12.5% of DAA-treated patients and 8.3% of control group had HCC recurrence (P = 0.60)

Zavaglia *et al*[40]	All DAA treated *n* = 31	Italy	20 (64.5)	Mean 65 ± 8	G1a = 4 (13), G1b = 23(74), G2 = 2 (6.5), G4 = 2 (6.5)	Median 8 mo	All cirrhotic	All treated	Not applicable	1 (3.2)
Ogata *et al*[41]	All DAA treated *n* = 1170	Japan	493 (42)	Median = 67 (21-88)	All genotype 1	Time from the end of DAA therapy until last visit: 1.3 yr		None	22 cases (1.8%)	Not applicable
Minami *et al*[42]	DAA group = 27, IFN group = 38, Controls = 861 *n* = 926	Japan	DAA group: 18 (67), IFN group: 27 (71), Controls: 489 (57)	Median age: DAA group = 71 (48-82) IFN group = 66 (49-79), Control = 71 (44–91)	Genotype 1: DAA = 21 (78), IFN = 29 (76), Controls = 633 (74). Genotype 2: DAA= 6 (22), IFN= 9 (24), Control= 147 (17)	1 and 2 yr		All treated	Not applicable	Cumulative recurrence rates at 1 and 2 yr were 21.1% and 29.8%, respectively, in the DAA group, 26.3% and 52.9%, respectively, in the IFN group, and 30.5% and 61.0%, respectively, in the control group
Deterding *et al*[43]	*n* = 974	Germany			G1 = 743 (76.2)		All had advanced cirrhosis		12 (1.2)	
Degasperi *et al*[44]	*n* = 565	Italy	60%	Median age = 65 (30-87) yr	G1a = 15%, G1b = 49%, G2 = 13%, G3 = 11%, G4 = 12%, G5 = 1%	Median 42 wk for occu-rrence, 39 wk for recurrence	All cirrhotic	48 (8%)	20 (4%) estimated annual incidence of 1.6%	9 (19%), annual incidence of 7 .7%
Bourliere *et al*[45]	DAA + RBV ± PEG IFN = 21%. IFN free DAA therapy = 79% *n* = 1393			47 (19-79) yr		2.5 (0.6-4.3) yr			0 (0)	0 (0)
Nagaoki *et al*[46]	PEG-IFN/ RBV = 244, DCV/ASV = 154 *n* = 398	Japan			All genotype 1	Median for PEG-IFN/RBV = 96 (10–196) and DCV/ASV group = 23 (4–78) mo			PEG-IFN/RBV = 13 (5.3%), DCV/ASV group = 7 (4.5%)	
Cheung *et al*[47]	All DAA	United Kingdom			198 (48.8) 171 (42.1)	15 mo	All decompensated cirrhosis	29 (71.4%)	17 (5%)	2
Mettke *et al*[48]	158 DAA treated, 184 controls	Germany				Median = 440 (91–908) and 592 (90-1000) d	All cirrhotic		6 and 14 patients during follow-up, resulting in an HCC incidence of 2.9 (AVT) and 4.48 (Con) per 100 py, respectively	
Ji *et al*[49]		China	51%	Mean age 51	82.2% genotype 1b	Median 14 (3-35) mo	48% cirrhotic	None		Not applicable
Innes *et al*[50]		Scotland				1.8 yr		None	44 (5.1%)	Not applicable
Torres *et al*[51]	All DAA treated	United States	7 (87.5%)	Median 64 (57-87) years	G1 = 6 (75%), mixed genotype = 2 (25%)	12 mo	7 (87.5%)	All treated	Not applicable	0 (0)

DAAs: Direct acting antivirals; IFN: Interferon; HCC: Hepatocellular carcinoma.

Table 4 Factors predisposing to hepatocellular carcinoma in hepatitis C virus patients treated with direct acting antivirals after sustained virologic response

Past history of hepatocellular carcinoma[18,28,36]
Male gender[28]
Cirrhosis[34]
Hypoalbuminemia[41]
Thrombocytopenia[41]
Raised AFP levels[41]

response to DAA based therapy and are associated with a unique burden of HCC[56]. Beside male patients, group of individuals with prior history of HCC, cirrhosis and elevated AFP at baseline were found with greater risk of HCC if treated with DAAs. Currently, the phenomenon appears to be a class effect rather than an individual drug effect. Hence, focusing on these HCV patients and measuring the impact of DAAs on progression or development of HCC will help to estimate the more accurate risk. In certain studies higher rates of HCC recurrence was found. There is a need to differentiate whether the reported higher recurrence rate and more aggressive pattern of recurrence are due to DAA or host or disease related factors including presence of fibrosis, gaps in initial tumor staging and receiving non-curative therapies such as chemoembolization. Also, future investigations should be directed towards assessing the long-term effects of DAAs on these populations that have not been studied thus far[3]. In the meantime, a consensus recommendation seen in most of the studies at present is that even after achieving SVR, there should be close surveillance of patients with CHC especially with advance fibrosis and those who received a recent treatment for HCC in order to detect HCC at an early stage[18,19,26,32]. Some important Centers in Europe and United States have been delaying antiviral treatment for 6 mo or more after recent treatment for HCC in these patients. Moreover, until more robust data is available to investigate the role of DAAs in HCV related HCC cases, clinical practice should continue as per current guidelines in those patient groups who can benefit from DAA therapy[51].

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ORIGINAL ARTICLE

Basic Study

Homologous recombination mediates stable *Fah* gene integration and phenotypic correction in tyrosinaemia mouse-model

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Author contributions: Junge N was involved in conception and design of the research, performed the majority of the experiments, analysed the data and wrote the manuscript; Yuan QG performed mouse surgery and immunostaining and revised the work critically for important intellectual content; Huong Vu T had substantial contributions to the experiments, animal care and analysis and interpretation of data for the work; Krooss S, Bednarski C, Balakrishnan A and Cathomen T helped with the experiments and design of the research and revised the work critically for important intellectual content; Manns MP and Baumann U revised the work critically for important intellectual content; Sharma AD and Ott M were initiator and supervisor of the work, the developed initial concept and design of the research and conducted important preliminary studies.

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Abstract

AIM

To stably correct tyrosinaemia in proliferating livers of fumarylacetoacetate-hydrolase knockout (*Fah*$^{-/-}$) mice by homologous-recombination-mediated targeted addition of the *Fah* gene.

METHODS

C57BL/6 *Fah*$^{\Delta exon5}$ mice served as an animal model for human tyrosinaemia type 1 in our study. The vector was created by amplifying human Fah cDNA including the TTR promoter from a lentivirus plasmid as described. The *Fah* expression cassette was flanked by homologous arms (620 bp and 749 bp long) of the *Rosa26* gene locus. Mice were injected with 2.1 × 10^8 VP of this vector (*rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR*) *via* the tail vein. Mice in the control group were injected with 2.1 × 10^8 VP of a similar vector but missing the homologous arms (*rAAV8-TTR.Fah*). Primary hepatocytes from *Fah*$^{-/-}$ recipient mice, treated with our vectors, were isolated and 1 × 10^6 hepatocytes were transplanted into secondary *Fah*$^{-/-}$ recipient mice by injection into the spleen. Upon either vector application or hepatocyte transplantation NTBC treatment was stopped in recipient mice.

RESULTS

Here, we report successful HR-mediated genome editing by integration of a *Fah* gene expression cassette into the "safe harbour locus" *Rosa26* by recombinant *AAV8*. Both groups of mice showed long-term survival, weight gain and FAH positive clusters as determined by immunohistochemistry analysis of liver sections in the absence of NTBC treatment. In the group of C57BL/6 *Fah*$^{\Delta exon5}$ mice, which have been transplanted with hepatocytes from a mouse injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR* 156 d before, 6 out of 6 mice showed long-term survival, weight gain and FAH positive clusters without need for NTBC treatment. In contrast only 1 out 5 mice, who received hepatocytes from *rAAV8-TTR.Fah* treated mice, survived and showed few and smaller FAH positive clusters. These results demonstrate that homologous recombination-mediated *Fah* gene transfer corrects the phenotype in a mouse model of human tyrosinaemia type 1 (*Fah*$^{-/-}$ mice) and is long lasting in a proliferating state of the liver as shown by withdrawal of NTBC treatment and serial transplantation of isolated hepatocytes from primary *Fah*$^{-/-}$ recipient mice into secondary *Fah*$^{-/-}$ recipient mice. This long term therapeutic efficacy is clearly superior to our control mice treated with episomal *rAAV8* gene therapy approach.

CONCLUSION

HR-mediated *rAAV8* gene therapy provides targeted transgene integration and phenotypic correction in *Fah*$^{-/-}$ mice with superior long-term efficacy compared to episomal *rAAV8* therapy in proliferating livers.

Key words: Gene therapy; *AAV8*; Liver based metabolic disease; Targeted integration; *ROSA26*; Paediatric liver disease

Core tip: Recombinant adeno-associated virus (rAAV) has been explored for gene delivery in various murine models of hereditary liver disease, but in young children transgene expression from *AAV*-epigenomes diminishes over time. We thus explored, whether homologous recombination-mediated targeted gene addition of the fumarylacetoacetate hydrolase (*Fah*) gene would stably correct tyrosinaemia in rapidly proliferating livers of *Fah*$^{-/-}$ mice. Here, we report successful homologous recombination-mediated genome editing of a *Fah* gene expression cassette at the *Rosa26* locus by *rAAV8*. We demonstrate that this approach corrects the phenotype and is long lasting in a proliferating state of the liver, as shown by serial transplantation.

Junge N, Yuan Q, Huong Vu T, Krooss S, Bednarski C, Balakrishnan A, Cathomen T, Manns MP, Baumann U, Sharma AD, Ott M. Homologous recombination mediates stable *Fah* gene integration and phenotypic correction in tyrosinaemia mouse-model. *World J Hepatol* 2018; 10(2): 277-286 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/277.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.277

INTRODUCTION

Therapy for many liver-based metabolic diseases (LBMD) is limited to supportive measures and may entail significant side effects, such as organ failure, metabolic crisis, malignancy and impairment of quality of life. Until now, the only established curative treatment is liver organ transplantation (LTX). Although LTX for LBMDs has excellent long-term outcomes, the procedure is associated with significant morbidity and mortality and dependent on limited donor organ availability. Gene therapy could provide a minimally invasive therapeutic alternative to whole organ transplantation.

Recombinant adeno-associated viruses (rAAV) have evolved as promising vehicles for gene therapy to date and shown to produce long-term therapeutic effects in many mouse models of inherited liver diseases as well as in patients with haemophilia B[1-3]. AAV of serotype 8 has been shown to target mainly hepatocytes in the liver and is considered to be safe for clinical application[3-6]. Recombinant AAVs express the transgenes from epige-

nomic circular DNA with only rare genomic integration events[7]. Insertional mutagenesis resulting from random vector integrations has been observed in only one study[8] and these results remain to be confirmed by other studies[9]. Notably, AAV gene therapy in 77 dogs did not cause tumour formation during an observation period of up to 10 years[10]. Nathwani *et al*[11] presented a study in non-human primates with no signs of insertional mutagenesis 5 years after AAV application. Further, serotype 8 shows lower seroprevalence of preformed antibodies in humans than other AAV serotypes[3,12] thus minimizing risk of significant immune response.

Epigenomic expression of the therapeutic transgene from rAAV is thought to gradually decline in tissues with high cell turnover. Therapeutic efficacy of AAV-mediated gene transfer would thus decrease in growing livers of newborns or in diseases with intrinsic stimuli causing hepatocyte turnover. In some studies, gene correction by homologous recombination of rAAV transduced therapeutic genes was shown to result in long-term cellular persistence. Although the feasibility of *in vivo* gene correction in mice has been demonstrated in several models, superior therapeutic efficacy of gene therapy by gene addition mediated by homologous recombination remains to be demonstrated. Therefore, we examined whether the application of a *Fah* expression cassette flanked by homologous arms for the ROSA 26 Locus improves the efficacy and persistence of *Fah* gene delivery by integration at the *Rosa26* gene locus through homologous recombination in a mouse model of human tyrosinaemia type 1. We used C57BL/6 $Fah^{\Delta exon5}$ mice, which served as an animal model for human tyrosinaemia type 1[13]. Liver physiology and function in these animals can be maintained by providing water that is supplemented with the drug NTBC [2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione]. Control mice die 20-45 d after deprivation of NTBC due to liver failure. In the absence of NTBC, gene corrected hepatocytes proliferate and repopulate the liver.

MATERIALS AND METHODS

Animal model

All mouse experiments were granted permission and were performed according to the guidelines of the Hannover Medical School, Germany and the local government. Mice were kept on standard laboratory chow and free access to drinking water. They were housed in a restricted access room with controlled temperature and a light/dark cycle. We used C57BL/6 $Fah^{\Delta exon5}$ mice, which served as an animal model for human tyrosinaemia type 1[13]. Tyrosinaemia type 1 is caused by genetic alterations of the gene coding for FAH. The mutated *Fah* gene produces an unstable protein, which results in deficiency of fumarylacetoacetate hydrolase activity. The mice were provided with water supplemented with 1 mg/100 mL of NTBC [2-(2-nitro-4-(fluoromethyl) benzoyl) cyclohexane-1,3-dione] before performing experiments. Surgery was done under general anaesthesia with 2% isoflurane and 2 litres/min oxygen flow.

Cloning of AAV plasmids

For cloning of the *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR* plasmid, 620 and 749 bp Rosa26 gene locus homologous arms flanking the *Fah* expression cassette were subcloned into a pBlue-Script II plasmid. The entire transgene was further subcloned into the AAV backbone plasmid for virus generation. For the *Fah* expression cassette, we amplified hFah cDNA, including the TTR promoter, from a lentivirus plasmid described earlier from our group[14] by PCR (Phusion® High-Fidelity PCR Kit, Thermo scientific).

For cloning the *rAAV8-TTR.Fah expression cassette,* we created a similar plasmid with the same transgene cassette but not flanked by the homologous arms.

Preparation of adeno-associated virus serotype 8 vector

The adeno-associated virus serotype 8 (AAV8) vectors (Figure 1A), *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and *rAAV8-TTR.Fah*, were prepared as described previously[15]. The titre was determined by qRT-PCR using primers spanning the region of the TTR promoter, as published before[16].

AAV8 vector administration into $Fah^{-/-}$ mice

Mice were injected with 2.1×10^8 VP *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR via* the tail vein. Mice in the control group were injected with 2.1×10^8 VP *rAAV8-TTR.Fah*. Viruses were diluted in sorbitol to a total volume of 220 μL for injection. Non-treated control mice were injected with 0.9% sodium chloride. We used one control mouse group (n = 3) for the first generation experiment. Subsequently, the mice were monitored and weighed daily until they reached stable conditions or gained body weight. After 45 to 47 d, a 1/3 hepatectomy was conducted to analyse the presence of FAH protein-positive cell clusters. Tissues were fixed in 4% paraformaldehyde or snap frozen for subsequent analyses.

Serial transplantation of hepatocytes from virus-injected mice

Primary hepatocytes from primary $Fah^{-/-}$ recipient mice were isolated with the two-step collagenase (Roche) perfusion method, as described previously[4]. Hepatocytes (1×10^6) were transplanted into secondary $Fah^{-/-}$ recipient mice by injection into the spleen. Control mice were injected with sodium chloride into the spleen. We used one control mouse group (n = 3) for the second generation experiment.

Immunohistochemistry

Tissues were embedded in paraffin (ROTH) and cut in 2-μm-thick slices. Immunohistochemistry was carried out as described previously[17]. Briefly, after deparaffinization and blocking for endogenous H_2O_2, the slides were incubated in 1 x target retrieval solution

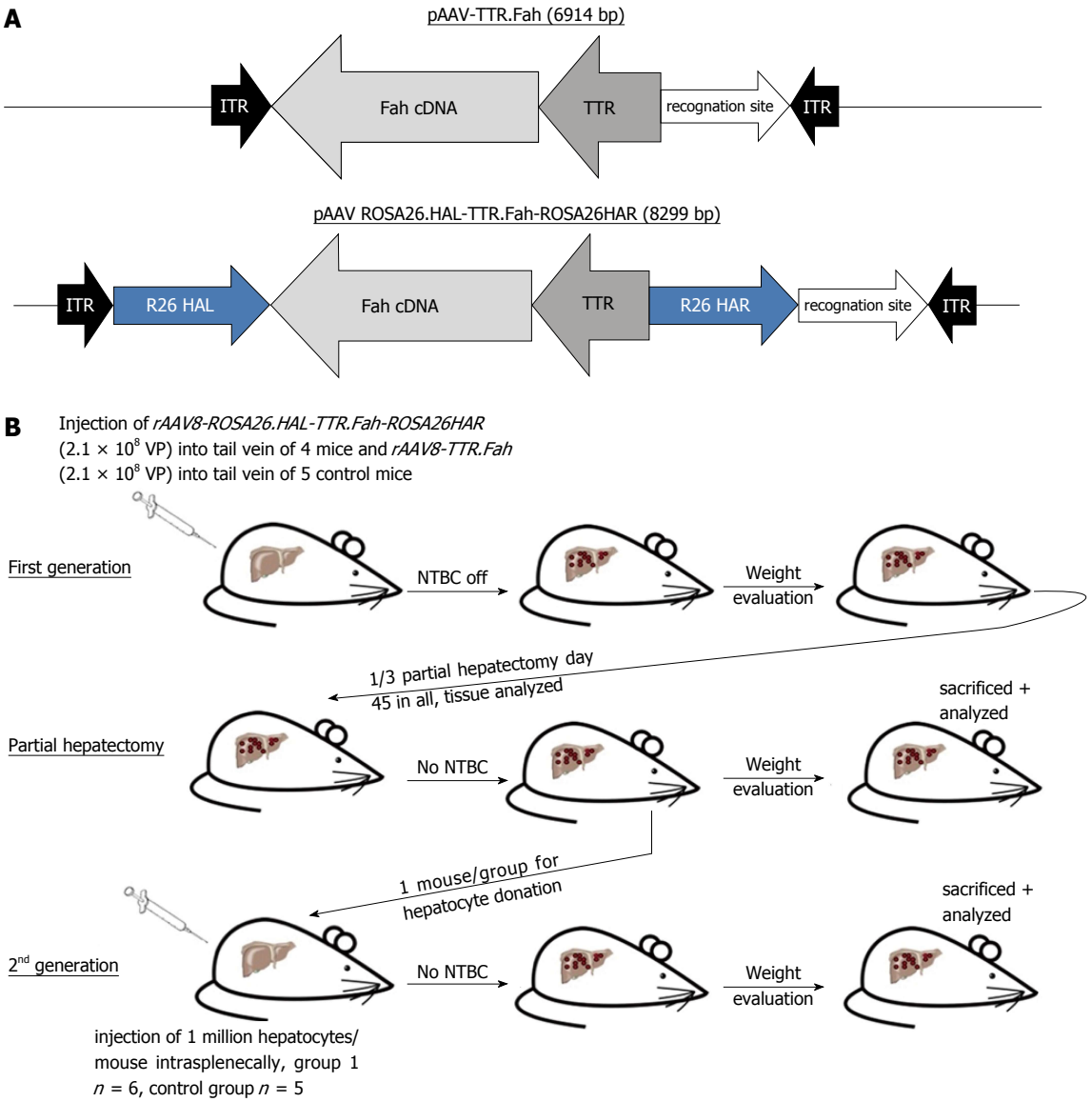

Figure 1 Flowchart of treating mice. A: Vector map for *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and for *rAAV8-TTR.Fah*. *Fah* cDNA is driven by the TTR promotor and for *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* located between the homologous arms of the Rosa26 locus. The vector was cloned into an AAV backbone; B: Scheme for the *in vivo* experiments. First-generation mice (*C57BL/6 FAH*$^{\Delta exon5}$ strain) were injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (group 1, $n = 4$) or *rAAV8-TTR.Fah* (control group, $n = 5$). The NTBC treatment was stopped, and after 45 d, a partial hepatectomy was performed. In each group, one mouse was used as the donor for hepatocyte transplantation into *C57BL/6 Fah*$^{\Delta exon5}$ mice. These recipients were the second generation of mice in our study. NTBC treatment was discontinued after hepatocyte transplantation. TTR: Transthyretin promoter (liver specific); R26 HAL: Homologous arm left for target locus in *Rosa26*; HAR: Homologous arm right for target locus in *Rosa26*; ITR: Inverted terminal repeat.

(Dako) at 98 ℃ for 20 min. For FAH (primary antibody, Abcam, ab81087) staining, tissues were blocked with the Avidin/Biotin blocking kit (Vector laboratories). Goat serum (Abcam) or rabbit serum (Abcam) was then used for blocking. Biotinylated goat anti-rabbit and rabbit anti-goat secondary antibodies (Vectastain, Vector laboratories) were used. Colour development was conducted using AEC substrate chromogen (Dako). Counterstaining was performed using haematoxylin (Merck Millipore, Germany).

Integration PCR

Genomic liver DNA was extracted from snap-frozen liver tissue with the DNeasy Blood and Tissue Kit (Qiagen) according to the protocol of the vendor. Two primers were designed, A and B. A was located in the *Rosa26* locus of recipient mouse 5′ to the donor gene. B was located in the *Fah* sequence of the donor DNA. Primer sequences were A: 5′-GGAGAGAGGCATTCAT GGGAGTGGAAAGTTAAGC-3′ and B: 5′-GCAGCATGG TCCAGTACATGTGCTTAAAGTTAGACC-3′. The expected length of the PCR amplicon was 1107 bp. PCR amplification was conducted with the Phusion® PCR Kit (New England BioLabs), and 200 ng of liver genomic DNA was used. The amplification was carried out under the following conditions: one cycle for 190 s at 98 ℃, followed by 50 cycles for 10 s at 98 ℃ and 90 s at 72 ℃, finished by one cycle for 10 min at 72 ℃. The

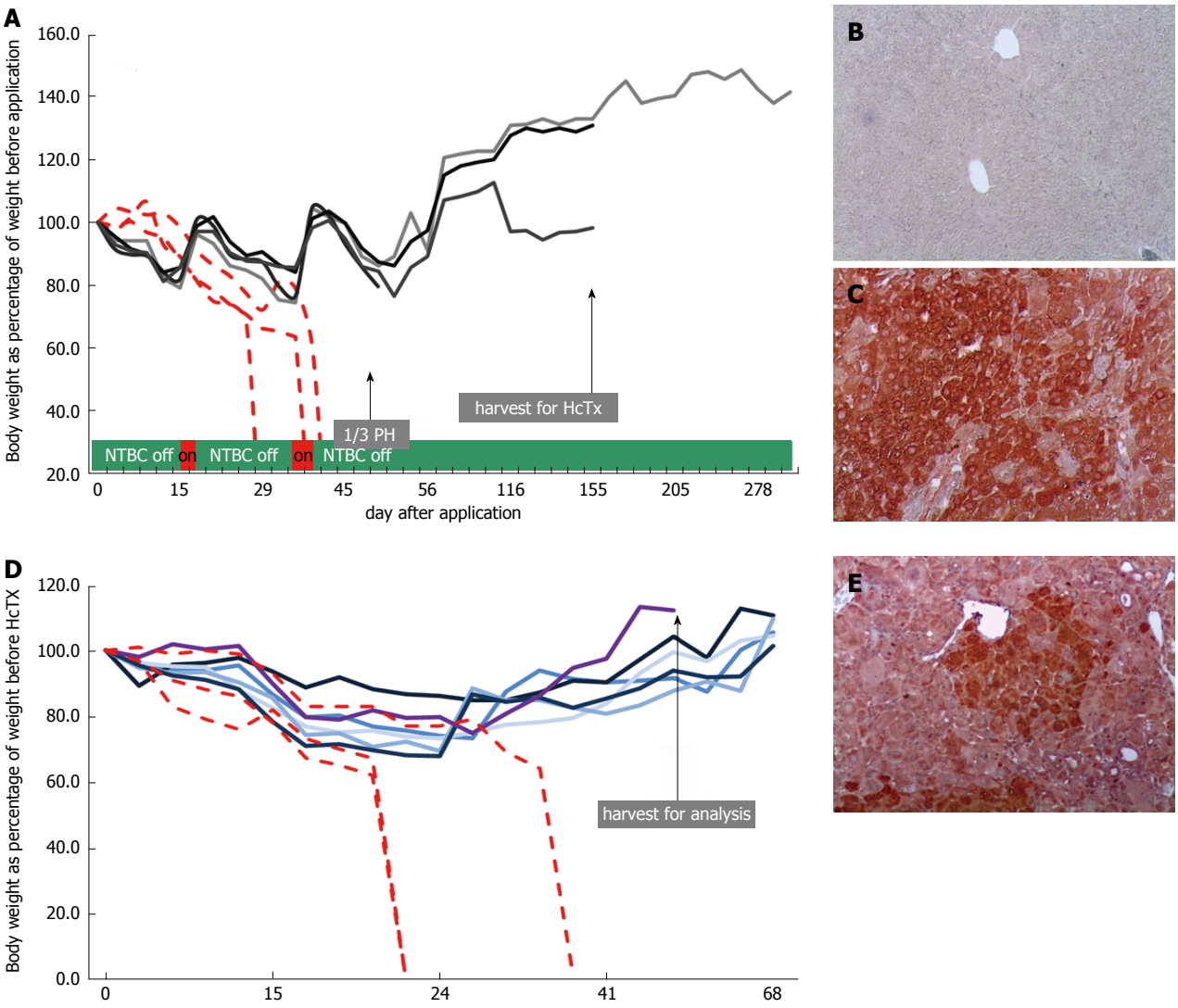

Figure 2 Mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR*. A: Weight graph and survival for first-generation mice (n = 4) injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and 3 untreated controls (injected with sodium chloride). Continuous line = *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* mice, broken line = controls (same control mice as displayed at Figure 3A). Body weight is displayed as percentage of body weight at the time of virus injection or sodium chloride injection (controls). The timeline (x-axis) is displayed in days beginning with the day of virus/sodium chloride injection as day zero; B: FAH staining of liver tissue from controls (mice with sodium chloride injection) after death (100 × magnification); C: FAH staining of liver tissue from partial hepatectomy in mouse injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (100 × magnification); D: Weight graph and survival for second-generation mice (continuous line), which were transplanted with one million hepatocytes from mice primarily injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and controls (same control mice as displayed at Figure 3D) without hepatocyte transplantation (broken line). Body weight is displayed as percentage of body weight at time of hepatocyte transplantation. The timeline (x-axis) is displayed in days, beginning with the day of hepatocyte transplantation as day zero; E: FAH staining of liver tissue from a partial hepatectomy from a second-generation mouse, which received one million hepatocytes from mice primarily injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (100 × magnification). 1/3PH: One third partial hepatectomy; HcTx: Hepatocyte transplantation.

PCR product was analysed utilizing gel electrophoresis on a 1% agarose gel (Biozym) for 50 min at 90 V.

qRT-PCR for FAH expression

RNA was isolated from snap frozen liver tissue of sacrificed mice. RNA was isolated with RNeasy® mini Kit (Qiagen) and QIAshredder® according to manufacturer instructions. After DNase treatment cDNA writing was performed (iScript™ reverse transcriptase supermix, BIO-RAD). SYBR green qRT-PCR (Qiagen QuantiTect Sybr green®) was performed at Stratagene Mx3000P (Aligent) with following primer (forward primer AGAATGCGCTGTTGCCAAA, reverse primer GGAAGCTCGGCCATGGTAT) spanning exon 5-6 and beta actin as housekeeping gene.

RESULTS

Long-term functional correction of the Fah gene defect by homologous recombination at the ROSA26 Locus in mice

We confirmed the correct design of our plasmids (Figure 1A) by sequencing and by evaluating FAH-Expression in Hepa1.6 cells by RT qPCR. For our experiments we used *Fah*$^{-/-}$ mice that contain a disruptive insertion in exon 5 of the *Fah* gene[13].

We prepared a high titre AAV8 vector suspension using the aforementioned AAV vector plasmids.

Next, we injected 4 mice with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR via* the tail vein (Figure 1B). To stimulate the proliferation of FAH-expressing hepatocytes, protective NTBC-treatment was discontinued immediately after injection. Whereas control mice (injected with saline) died before 45 d, all mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* survived beyond 45 d after injection (Figure 2A). On the 45th-47th days, 1/3 of the liver was removed and analysed for the presence of FAH cell clusters by immunohistochemistry. All animals injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* showed robust repopulation of the liver as indicated by survival, weight gain (Figure 2A) and multiple large FAH protein positive cell clusters in immunohistochemistry analyses (Figure 2C). Importantly, these mice survived without NTBC until the end of the study (day 288; Figure 2A).

Due to high selection pressure for gene corrected hepatocytes in the *Fah*$^{-/-}$ model, phenotypic correction of the enzyme deficiency as result of diluted, but still sufficient, FAH protein expression from epigenomic AAV DNA could not be excluded in the first generation. To test whether homologous sequences facilitated targeted integration and increased therapeutic efficacy, we isolated primary hepatocytes from one recipient mouse after recovery from partial hepatectomy and transplanted 1 x 10^6 cells each into the spleens of the secondary *Fah*$^{-/-}$ recipient mice (Figure 1B). All recipient animals (6/6) that were transplanted with hepatocytes from repopulated *Fah*$^{-/-}$ mouse showed liver repopulation and survived long-term in the absence of NTBC (Figure 2D and E).

Missing long-term in vivo correction of Fah in the absence of homologous sequences after hepatocyte transplantation.

To establish unequivocally that homologous recombination is indeed capable of long-term stable correction of *Fah* deficiency and superior to non-homologous, episomal gene therapy, we generated a control group with five mice, who were injected with *rAAV8-TTR.Fah*. All five primary recipient mice survived with weight gain (Figure 3A) and showed clusters of FAH-positive cells at partial hepatectomy on day 45 (Figure 3C). To show inferiority of this episomal approach we further increased the proliferation conditions by transplanting hepatocytes (1 × 10^6 cells for each recipient) from one first generation recipient mouse into 5 secondary *Fah*$^{-/-}$ recipient mice in this group also. Only one of the five secondary recipient mice (hepatocyte recipients) survived NTBC withdrawal and showed few and small FAH-positive cell clusters (Figure 3D and E). Hence, these results suggest that in the absence of homologous arms, the observed FAH-positive clusters in the primary recipient *Fah*$^{-/-}$ mice mostly resulted from epigenomic AAVs or an unexplained mechanism of integration/anchorage on cellular DNA, which was lost upon transplantation into secondary *Fah*$^{-/-}$ recipient mice.

Successful targeted integration of Fah cDNA at the Rosa26 locus

So far, our results revealed that mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* had robust liver repopulation and improved survival after secondary transplantation. However, it is important to prove that homologous arms facilitated targeted integration/gene addition of *Fah* cDNA into the *Rosa26* locus. We therefore examined targeted integration by genomic PCR amplifying portions of the *Rosa26* gene locus and the *Fah* transgene cassette. Indeed, we found an expected band of 1071 bp in mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (Figure 4) but not in mice injected with *rAAV8-TTR.Fah*. Our data thus indicate that homologous arms facilitated targeted integration at a frequency sufficient for increased therapeutic outcome and phenotypic correction in *Fah*$^{-/-}$ mice. This is further confirmed by Sybr green qRT-PCR results. These showed a clearly higher expression of FAH in mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* compared to mice treated with *rAAV8-TTR.Fah* alone (Figure 5).

In summary, we can conclude that in the first generation we could not detect a difference for survival, weight gain and FAH positive cell cluster between mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* or *rAAV8-TTR.Fah* but in secondary generation (recipients of 1 x 10^6 hepatocytes from first generation) we could detect a clear improved survival for the group with homologous arms in the vector. In this group 6 out of 6 mice survived and in the other group 1 out 5 mice survived. Furthermore the detection FAH positive cell clusters showed the same distribution.

DISCUSSION

In *in vitro* and *in vivo* studies[18,19], the AAV vector is used as the vector of choice for gene correction approaches by homologous recombination; one important reason is its single-stranded nature. Reports on gene correction or gene addition by homologous recombination for liver-based metabolic diseases are rare and have shown correction frequencies[20] too low for phenotypic correction, except for the study of Paulk *et al*[19]. However, they used a mouse model with a point mutation for *Fah* gene; therefore, their approach was a gene correction. Here, we provide proof of concept for *in vivo* targeted gene addition mediated by homologous recombination in a liver-based metabolic disease. Our findings demonstrate that in a state of extensive hepatocyte proliferation, targeted integration by homologous recombination was superior to gene therapy based on episomal AAV gene therapy.

Primary recipient mice that were injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* or *rAAV8-TTR.Fah* survived and showed phenotypic rescue after NTBC withdrawal. Notably, livers of mice from

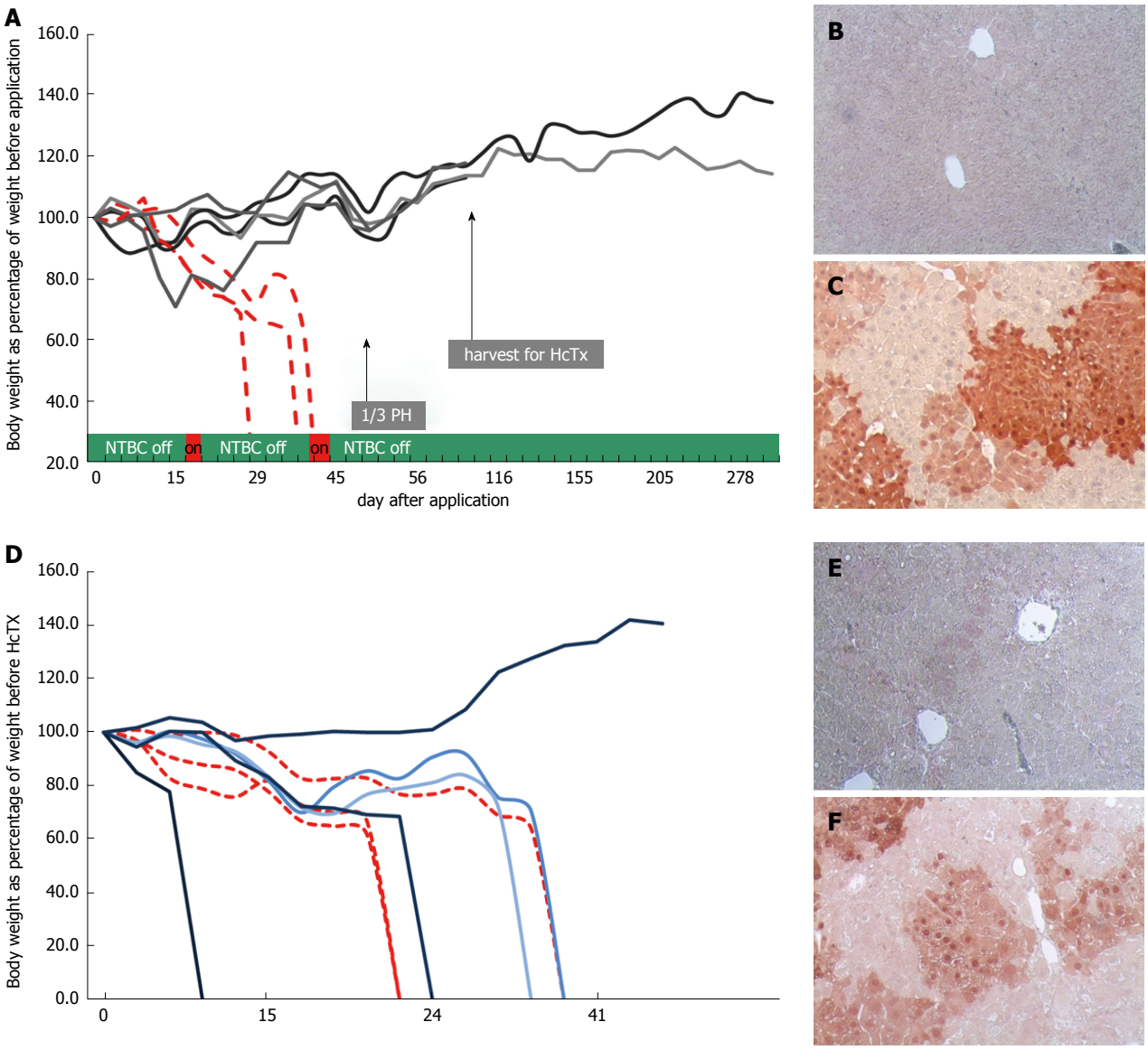

Figure 3 Mice treated with *rAAV8-TTR.Fah*. A: Weight graph and survival for first-generation mice injected with *rAAV8-TTR.Fah* (*n* = 5) and 3 untreated controls (injected with sodium chloride). Continuous line = *rAAV8-TTR.Fah* mice, broken line = controls (same control mice as displayed at Figure 2A). Body weight is displayed as percentage of body weight at the time of virus injection or sodium chloride injection (controls). The timeline (x-axis) is displayed in days beginning with the day of virus/sodium chloride injection as day zero; B: FAH staining of liver tissue from controls (mouse with sodium chloride injection) after death (100 × magnification); C: FAH staining of liver tissue from a partial hepatectomy from a mouse injected with *rAAV8-TTR.Fah* (100 × magnification); D: Weight graph and survival for second-generation mice (continuous line), which were transplanted with one million hepatocytes from mice primarily injected with *rAAV8-TTR.Fah* and controls (same control mice as displayed at Figure 2D) without hepatocyte transplantation (broken line). Body weight is displayed as percentage of body weight at time of hepatocyte transplantation. The timeline (x-axis) is displayed in days, beginning with the day of hepatocyte transplantation as day zero; E: FAH staining of liver tissue from a partial hepatectomy in a second-generation mouse, which received one million hepatocytes from mice primarily injected with *rAAV8-TTR.Fah* (100 × magnification); F: FAH staining of liver tissue from a partial hepatectomy from the single second-generation mouse that showed cluster and weight gain.

both groups showed clear FAH-positive cell clusters in immunohistochemistry. We determined the presence of FAH positive areas in the both groups of primary recipients. We did not find significant differences in FAH positivity indicating similar number of FAH positive hepatocytes in both groups of mice. So far, cell clusters have always been explained by clonal expansion of corrected hepatocytes, which would implicate the necessity of vector integration. In the tyrosinemia mouse model *Fah* corrected hepatocytes have a strong selective advantage so they grow clonally, form nodules and can repopulate the entire liver at least[21,22]. Therefore, it is reasonable that a small number of hepatocytes with random integrations or another unexplained mechanism such as of integration/anchorage on cellular DNA proliferate preferentially and repopulate the diseased liver, leading to FAH-positive cell clusters. A human liver contains approximately 300 billion hepatocytes, which means, in case of 10% transduction efficiency with an integration rate of 0.1%, a single individual will have approximately 30 million hepatocytes with at least one integration event[23]. Therefore, one can assume that the phenotypic correction in these mice can be explained by the selective proliferation advantage of a small

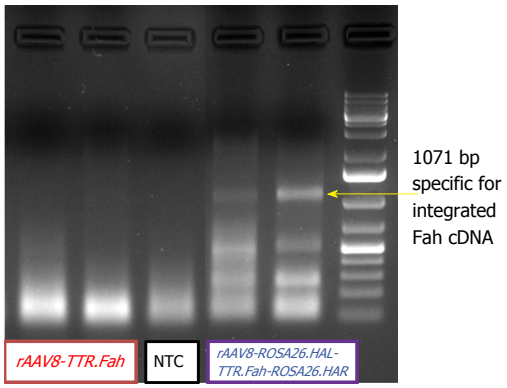

Figure 4 Integration PCR gel electrophoresis. A representative gel picture from the analyses of genomic liver DNA, that was extracted from snap-frozen liver tissue harvested between 60-70 d after hepatocyte transplantation. Primers were located in the *Rosa26* locus and in the FAH sequence of the donor DNA. Product could only be amplified if targeted integration occurred. The expected length of the PCR amplicon was 1107 bp. The PCR product was analysed utilizing agarose gel electrophoresis.

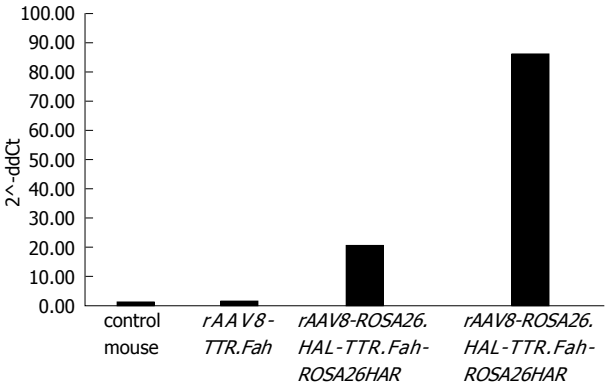

Figure 5 Sybr green qRT-PCR. Shown are the 2^-ddct values of two mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and one mouse treated with *rAAV8-TTR.Fah* calculated on an untreated control mouse.

number of hepatocytes with successfully integrated *Fah* cassettes. A spontaneous reversion of the genetic defect, as the underlying cause for phenotypic correction and FAH-positive cell clusters, as described in humans[24], is not possible in the *Fah*^{exon5} mouse model[25].

Therefore we increased the proliferation conditions by hepatocyte transplantation from one first generation recipient per group into secondary *Fah*$^{-/-}$ recipient mice (1 x 10^6 hepatocytes for each secondary recipient mouse). In this experiment, the advantage of homologous recombination became clearly visible, since phenotypic correction could be achieved in all mice (6/6). In the *rAAV8-TTR.Fah* group, only 1/5 mice survived. In accordance with these results, 6/6 mice co-injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* showed clear FAH-positive cell clusters in livers, whereas only 1/5 mice injected with *rAAV8-R26.Fah* had FAH-positive clusters. Furthermore Sybr green qRT-PCR showed higher FAH expression in liver tissue of *ROSA26.HAL-TTR.Fah-ROSA26HAR-mice* than in *rAAV8-R26.Fah-mice.*

Partial hepatectomy and serial transplantation together are supposed to have triggered at least 30 rounds of cell doubling for the hepatocytes[26], nevertheless we could not find any tumour formation in any of our mice. This is in line with other studies showing a good safety profile for *rAAV8* gene therapy[5]. Our proof of concept approach demonstrated that the targeted integration/addition of a therapeutic gene allows for safer (compared to random integration) and more efficient (compared to epigenomic) gene therapy, especially for gene therapy of liver-based metabolic diseases in paediatric patients, since the *Rosa26* locus exists in mice[27,28] as well as in humans[29]. In contrast to the assumption that homologous recombination alone is not sufficient for a long-lasting phenotypic correction of a liver-based metabolic disease, we could show the opposite with this study, at least for diseases with selection advantage for corrected hepatocytes, like tyrosinaemia type 1. Further potential target diseases with selection advantage could be Wilson disease or bile-acid transporter defects. Continuing studies should evaluate the efficiency of this approach in liver-based metabolic diseases without selection advantage such as Crigler Najjar Syndrome.

In summary, we demonstrate that targeted *in vivo* integration of a *Fah* expression cassette mediated by homologous arms is a highly efficient approach to stably correct a metabolic liver disease in an FAH mouse model with extensive hepatocyte proliferation. Since many metabolic disorders must already be treated in children with fast-dividing hepatocytes, targeted transgene integration is an important step to safe and long-lasting gene therapy in the developing liver.

ARTICLE HIGHLIGHTS

Research background

We describe an important proof of concept in the field of AAV gene therapy for liver based metabolic diseases (LBMD). First gene therapy studies in humans are done (Hemophilia B) or very ready to start (Crigler-Najjar Syndrome); even an EMA approved drug for AAV gene therapy (Glybera) exists already. But all these approaches have a major weakness, the missing permanence of the gene therapy effect, especially in young children. But they are the main target group for gene therapy in LBMD, since early therapy could avoid irreversible damage to the organs of the patient. In these patients the advantage of recombinant AAV gene therapy, the almost missing integration into the host genome turns into a disadvantage since donor cDNA will be lost during cell turn over.

Research motivation

Targeted integration into safe harbors like the ROSA26 locus could overcome the problem of diminishing donor-cDNA in rAAV gene therapy. There are studies, showing proof of concept for targeted integration with nucleases like zinc fingers or CRISP/CAS9, but these approaches contain also new potential sources of side effects. However in our study only natural appearing cellular repair mechanism has been used to generate a targeted integration.

Research objectives

Up to know it was assumed that the efficiency of gene addition by targeted integration into a safe harbor mediated by homologous recombination would be to low for phenotypic correction of liver based metabolic diseases (LBMD) in growing livers. But we could show in a disease model for LBMD with selection advantage of corrected hepatocytes that this is not the case. This could be

transferred to other diseases like the group of familial intrahepatic cholestasis or Wilson disease or even to diseases with less selection advantage.

Research methods

C57BL/6 *Fah*$^{\Delta exon5}$ mice served as an animal model for human tyrosinaemia type 1 in our study. We treated these mice with a rAAV Vector containing human *Fah* cDNA, a liver specific promotor (TTR) and homologous arms for ROSA26 locus. We compared this group to mice treated with a vector without homologous arms. Hepatocyte proliferation was induced by partial hepatectomy and serial hepatocyte transplantation. Survival of mice without NTBC and existence of FAH positive cell cluster at immunohistochemistry staining on liver tissue of the mice were the main endpoints.

Research results

We could show for the first time proof of concept for phenotypic correction of a LBMD in a mouse model under conditions of extensive hepatocyte proliferation with rAAV mediated gene addition by targeted integration at a safe harbor without the use of nucleases or gene repair. Further studies have to show if this concept is transferable to LBMD with less section advantage of corrected hepatocytes.

Research conclusions

Our study shows that phenotypic correction of a LBMD by rAAV gene therapy under conditions of extensive hepatocyte proliferation is possible with homologous recombination (HR) alone and does not necessarily have the need for nucleases. In conclusion we showed that HR-mediated rAAV8 gene therapy provides targeted transgene integration and phenotypic correction in *Fah*$^{-/-}$ mice with superior long-term efficacy compared to episomal rAAV8 therapy in proliferating livers. In opposite to approaches with the aim of point mutation repair on genes of LBMD our system with gene addition into a safe harbour can be easily transferred to other LBMDs and is not mutation specific.

Research perspectives

Our results are an important step into the solution of a main clinical problem for gene therapy of LBMD, since mostly this therapy is mandatory in growing children, where episomal gene therapy is not lasting. In opposite to studies with nucleases our study focus on a natural mechanism for targeted integration which avoids potential side effects of nucleases. A very important question for following studies would be if these results could also be observed in LBMD with less selection advantage for corrected hepatocytes (*e.g.,* Crigler-Najjar Syndrom).

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ORIGINAL ARTICLE

Basic Study

Multipotent stromal cells stimulate liver regeneration by influencing the macrophage polarization in rat

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Abstract

AIM

To investigate the influence of the umbilical cord-derived multipotent stromal cells (MSCs) on recovery of the liver after the subtotal resection, that is, removal of 80% of the organ mass, a renowned model of the small-for-size liver remnant syndrome.

METHODS

The MSCs were obtained from the intervascular tissue of umbilical cords, dissected from rat fetuses, by the explant culture technique. The vital labeling of MSCs with PKH26 was carried out on the 3rd passage. The subtotal resection was performed on male Sprague-Dawley rats. The experimental group animals received a transplant 10^6 MSCs infused into the spleen. Hepatocyte proliferation was assessed by counting of either mitotic figures or Ki67-positive cells in microscopic images. MSC differentiation was assessed with antibodies to hepatocyte-specific marker cytokeratin 18 (CK18), cholangiocyte-specific protein CK19, smooth muscle cell-specific protein α-SMA, the endothelial cell marker CD31, or the active fibroblast marker FAPα. Total macrophages of the liver were selectively stained in cryosections incubated with anti-CD68 antibodies (1:100, Abcam), while the M2a and M2c macrophage populations were selectively stained with anti-CD206 antibodies. Expression of interleukin and growth factor genes was evaluated with PCR-RT.

RESULTS

Intrasplenic allogeneic transplantation of the umbilical cord-derived multipotent stromal cells stimulates reparative processes within the residual liver tissue after subtotal resection (removal of 80% of the organ mass), as indicated by increased rates of hepatocyte proliferation and accelerated organ mass recovery. These effects may result from paracrine influence of the transplanted cells on the resident macrophage population of the liver. The transplantation favors polarization of macrophages to M2 phenotype (the M2-polarized macrophages specifically express CD206; they are known to suppress inflammation and support tissue repair). No differentiation of the transplanted cells into any of the liver cell types have been observed in the study.

CONCLUSION

We found no direct evidence for the paracrine effect of MSCs on liver regeneration after the subtotal liver resection in rats. However, the paracrine mechanism of the therapeutic activity of transplanted MSC is indirectly indicated by a decrease in the total number of CD68 + macrophages and an increase in the proportion of M2 pro-repair macrophages in the regenerating liver as compared to animals in which the transplantation was only mimicked.

Key words: Liver; Regeneration; Multipotent stromal cells; Macrophages

Core tip: Umbilical cord-derived multipotent stromal cells stimulate reparative processes within the liver after subtotal resection (removal of 80% of the organ mass). Multipotent stromal cells stimulate hepatocyte proliferation in rats after subtotal resection and favor polarization of macrophages to M2 phenotype. The transplanted multipotent stromal cells do not differentiate into any of the liver cell types under these conditions.

Elchaninov A, Fatkhudinov T, Usman N, Arutyunyan I, Makarov A, Lokhonina A, Eremina I, Surovtsev V, Goldshtein D, Bolshakova G, Glinkina V, Sukhikh G. Multipotent stromal cells stimulate liver regeneration by influencing the macrophage polarization in rat. *World J Hepatol* 2018; 10(2): 287-296 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/287.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.287

INTRODUCTION

Multipotent stromal cells (MSCs) are found to be a helpful supplement in various repair processes in mammals, particularly in solid organs[1]. These cells are considered as a promising tool of regenerative medicine[2]. Their ability to stimulate reparative regeneration of damaged liver have been confirmed[3], but the mechanisms remain uncertain.

The experimentally proven enhancement of liver regeneration by MSCs must be essentially paracrine, because their transplantation to residual livers causes an increase in concentrations of HGF and several other growth factors within the regenerating tissues[4]. Therapeutic activity of MSCs is apparently related to their anti-inflammatory properties, which also represent a sort of paracrine regulation, as manifested by a local increase in IL-4, IL-13, and TSG-6 production paralleled by relative shortage of TNFα and IL-6[5]. Modulation of inflammatory reactions may be implemented *via* influence of these, or similar, paracrine factors on immune cells, especially on macrophages[6,7]. Several studies, however, confirm the ability of MSCs to differentiate into hepatocytes, which may support quite a different explanation for the positive influence of MSCs on liver repair[3,8].

In a number of clinical cases, a residual portion of the liver left after an extended resection, *e.g.*, due to a tumor or metastasis, or even a transplanted portion of donor liver tissue supposed to replace the bulk of liver tissue in the host, is too small to effectively support the body homeostasis[9]. The resulting post-hepatectomy liver failure is a major manifestation of the small-for-size liver remnant syndrome, when the critically reduced liver mass is not only insufficient to maintain normal liver function, but also incapable of compensatory growth. The

problem could be solved by some specific controllable stimulation of this growth combined with intensive liver care and management for acute hepatic failure.

This study deals with influence of the umbilical cord-derived MSCs on the reparative regeneration of the liver after the subtotal resection, that is, removal of the 80% organ mass, a renowned model of the small-for-size liver remnant syndrome.

MATERIALS AND METHODS

Outbred male Sprague-Dawley rats, body weight 250-270 g, were obtained from the Institute for Bioorganic Chemistry branch animal facilities (Pushchino, Moscow region, Russia). All experimental work involving animals was carried out according to the standards of laboratory practice (National Guidelines No. 267 by Ministry of Healthcare of the Russia, June 1, 2003), and all efforts were made to minimize suffering. The study was approved by the Ethical Review Board at the Scientific Research Institute of Human Morphology (Protocol No. 16, November 19, 2015).

The MSCs were obtained from the intervascular tissue of umbilical cords, dissected from rat fetuses, by the explant culture technique. Their identity as MSCs was verified by their capacity of clonogenic growth on the untreated plastic, expression of specific set of surface antigens, and their ability to differentiate into mesodermal derivatives (Arutyunyan *et al*[2], 2016). The vital labeling of MSCs with PKH26 (Sigma-Aldrich Co LLC, United States) was carried out on the 3rd passage. The labeled cells were washed twice with saline (PanEco, Russia) and transferred to culture dishes for the labeling quality assessment or into syringes for transplantation to experimental animals.

The animals (n = 83) were operated as described earlier[15]. Hepatic tissue from sham-operated animals (n = 43) served as additional control in the assessment of hepatocyte proliferation, immunostaining and genes expression.

Animal survival was calculated as (the total number of operated animals minus the number of spontaneous deaths divided by the total number of operated animals. The animals were drawn from the experiment in CO_2-chamber at 3 h, 6 h, 24 h, 48 h, 3 d, 7 d, or 10 d after the surgery (5-6 animals for each term) between 9 am and 11 am. The regenerating livers were promptly dissected, weighed (along with the whole animal, to determine the liver-to-total mass ratio, with intact animals of similar age (n = 10) being used for comparison), and preserved for analysis. A part of the material was fixed in 10% buffered formalin for a routine histological procedure (dehydration, paraffin sectioning, HE staining, mounting, and microscopy). Another part of the material was frozen in liquid nitrogen for *in vivo* cell tracing and/or immunochemistry (see below).

Hepatocyte proliferation assessment

Hepatocyte proliferation was assessed by counting of either mitotic figures or Ki67-positive cells in microscopic images.

Mitotic index of hepatocytes was calculated for each animal individually as a number of mitoses per 6×10^3 hepatocytes, expressed in promille (‰).

Ki67-positive cells were counted on cryosections immunostained with corresponding primary antibodies followed by FITC-conjugated secondary antibodies, both supplied by Abcam, United Kingdom, and used in 1:100 and 1:200 dilutions, respectively; cell nuclei were counterstained with DAPI (Sigma-Aldrich Co LLC). The Ki67 proliferation index was calculated for each animal individually as a number of Ki67-positive hepatocytes per 3×10^3 hepatocytes.

MSC differentiation assessment

The cryosections were stained with antibodies to hepatocyte-specific marker cytokeratin 18 (CK18), cholangiocyte-specific protein CK19, smooth muscle cell-specific protein α-SMA, the endothelial cell marker CD31, or the active fibroblast marker FAPα. All of the primary antibodies were obtained from Abcam and used in 1:100 dilutions as recommended by the manufacturer and followed by FITC-conjugated secondary antibodies (1:200, Abcam); cell nuclei were counterstained with DAPI (Sigma-Aldrich Co LLC). Expression of the cell type-specific proteins was meticulously sought out in MSCs identified within regenerating liver tissue by PKH26 label. The observations were done using Leica DM 4000 B fluorescent microscope with LAS AF v.3.1.0 build 8587 software (Leica Microsystems CMS GmbH, Germany).

Selective immunostaining of macrophages

Total macrophages of the liver were selectively stained in cryosections incubated with anti-CD68 antibodies (1:100, Abcam), while the M2a and M2c macrophage populations were selectively stained with anti-CD206 antibodies (Santa-Cruz, United States) used in 1:100 dilution as recommended by the manufacturer. The signal was visualized by using FITC-conjugated secondary antibodies (1:200, Abcam) combined with DAPI-counterstaining of the nuclei. The CD68+ and CD206+ cells were counted in microscopic images and related to the total cell counts to obtain corresponding indexes of macrophage content.

Real-time PCR assay

Total RNA was isolated with RNeasy Plus Mini Kit (QIAGEN). Estimated concentration of RNA in the eluate was 0.1 g/L; the quality was controlled by electrophoresis. To remove traces of genomic DNA, the samples were treated with RNase-free DNase I (Thermo Scientific, Waltham, MA, United States; 1 U per μg of RNA); the efficacy of DNA elimination was confirmed by PCR with nontranscribed genomic region-specific primers. Reverse transcription reactions were set up using MMLV RT Kit (Evrogen CJSC, Moscow, Russia) and held at 39 ℃ for 1 h.

Table 1 Oligonucleotide sequences

Target designation	5'-end primer	3'-end primer
Il1β	CTGTCTGACCCATGTGAGCT	ACTCCACTTTGGTCTTGACTT
Il6	TACATATGTTCTCAGGGA GAT	GGTAGAAACGGAACTCCAG
Il10	GCCCAGAAATCAAGGAGCAT	TGAGTGTCACGTAGGCTT CTA
Tnfα	CCACCACGCTCTTCTGTCTA	GCTACGGGCTTGTCACTCG
Hgf	GGCCATGGTGCTACACTCTT	TTGTGGGGGTACTGCGAATC
Tgfβ1	CCGCAACAACGCAATCTATG	AGCCCTGTATTCCGTCTCCTT
Actβ	GAGATTACTGCCCTGGCTCC	GCTCAGTAACAGTCCGCCTA
B2m	CTCGCTCGGTGACCGTGAT	GGACAGATCTGACATCTCGA
Gapdh	GCGAGATCCCGCTAACATCA	CCCTTCCACGATGCCAAAGT

Table 2 Liver-to-body weight ratio dynamics (Mean ± SD, %)

Time after operation, d	Multipotent stromal cells transplantation group	Comparison group
Intact controls	4.5 ± 0.9	
3	1.8 ± 0.3	1.4 ± 0.1[a]
7	2.4 ± 0.7	2.7 ± 0.3[a]
10	3.1 ± 0.2	2.6 ± 0.2[a]

[a]$P > 0.05$.

PCR mixtures based on qPCRmix-HS SYBR system (Evrogen CJSC) containing oligonucleotide primers (custom made by SYNTOL, Moscow, Russia) in 0.2-0.4 μmol/L final concentrations were set up in duplicates. Structures of the oligonucleotides with corresponding target symbols and descriptions are given in Table 1. Real-time PCR was carried out in DT-96 Real-Time PCR Cycler (DNA-Technology JSC, Moscow, Russia) at 95 ℃ 15 s, 62 ℃ for 10 s + reading, 72 ℃ for 20 s. The relative expression values were calculated by approach originally introduced (Pfaffl MW[10], 2001) with modifications (Vandesompele J *et al*[11], 2002) using *actb*, *b2m*, and *gapdh* (Table 1) as reference targets.

Statistical analysis

The data were analyzed using SigmaStat 3.5 (Systat Software Inc., Chicago, IL, United States). Proportion values were compared by 2-sample *z*-test; relative gene expression values were compared by the Mann-Whitney *U* test; more-than-two-groups comparisons were done using ANOVA on ranks; *P*-values < 0.05 were considered significant.

RESULTS

Animal survival

Some of the animals died within 3 d after the surgery, but none of them died after the 3 d timepoint, animal survival in the comparison group (72.1% ± 6.8%, $n = 43$) was significantly higher than in the MSC transplantation group (47.5% ± 7.8%, $n = 40$).

Liver mass recovery

MSC transplantation stimulated compensatory growth of residual hepatic tissues, which was expressed in more rapid liver mass recovery (Table 2). In the MSC transplantation group, the liver-to-body ratio returned to normal values by day 10 after the surgery, while in the comparison group it still did not reach the initial level (as measured for the intact control animals).

Hepatocyte proliferation dynamics

Mitotic index of hepatocytes was significantly higher in the MSC transplantation group than in the comparison group on day 3 as well as on day 10 after the surgery (Figure 1A, C, D, F and G). On day 7 after the surgery, however, this index was significantly lower in the MSC transplantation group than in the comparison group (Figure 1B, E and G).

A similar tendency was revealed by using the Ki67 immunostaining (Figure 2). Although no significant differences in the Ki67 proliferation index between the groups were observed on day 3 after the surgery (Figure 2A, D and G), this index for the MSC transplantation group on day 7 was significantly lower (Figure 2B, E and G), and on day 10 significantly higher, than for the comparison group (Figure 2C, F and G).

Differentiation of transplanted MSCs

The transplanted cells expressed neither CK18 or CK19 (Figure 3A and B), nor α-SMA (Figure 3D). Solitary CD31-expressing PKH26-labeled cells were observed at the site of injection on days 3 and 10 after the surgery (Figure 3C).

Macrophage polarization profiles of regenerating liver

It turned out, that on day 7 after the surgery the animals of the MSC transplantation group had significantly decreased numbers of total liver macrophages defined as CD68+ cells (Figure 4A, B and E). At the same time, calculated numbers of M2 macrophages (defined as CD206+ cells) in regenerating livers on day 7 after the surgery were higher in the MSC transplantation group than in the comparison group (Figure 4C, D and F).

Transplantation-dependent changes in gene expression

No transplantation-dependent changes in gene expression at the site of transplantation have been revealed in this study, despite that such changes were extensively sought for. We analyzed expression dynamics for a set of genes, which reportedly participate in regulation of mammalian liver recovery, and found no changes that could be related

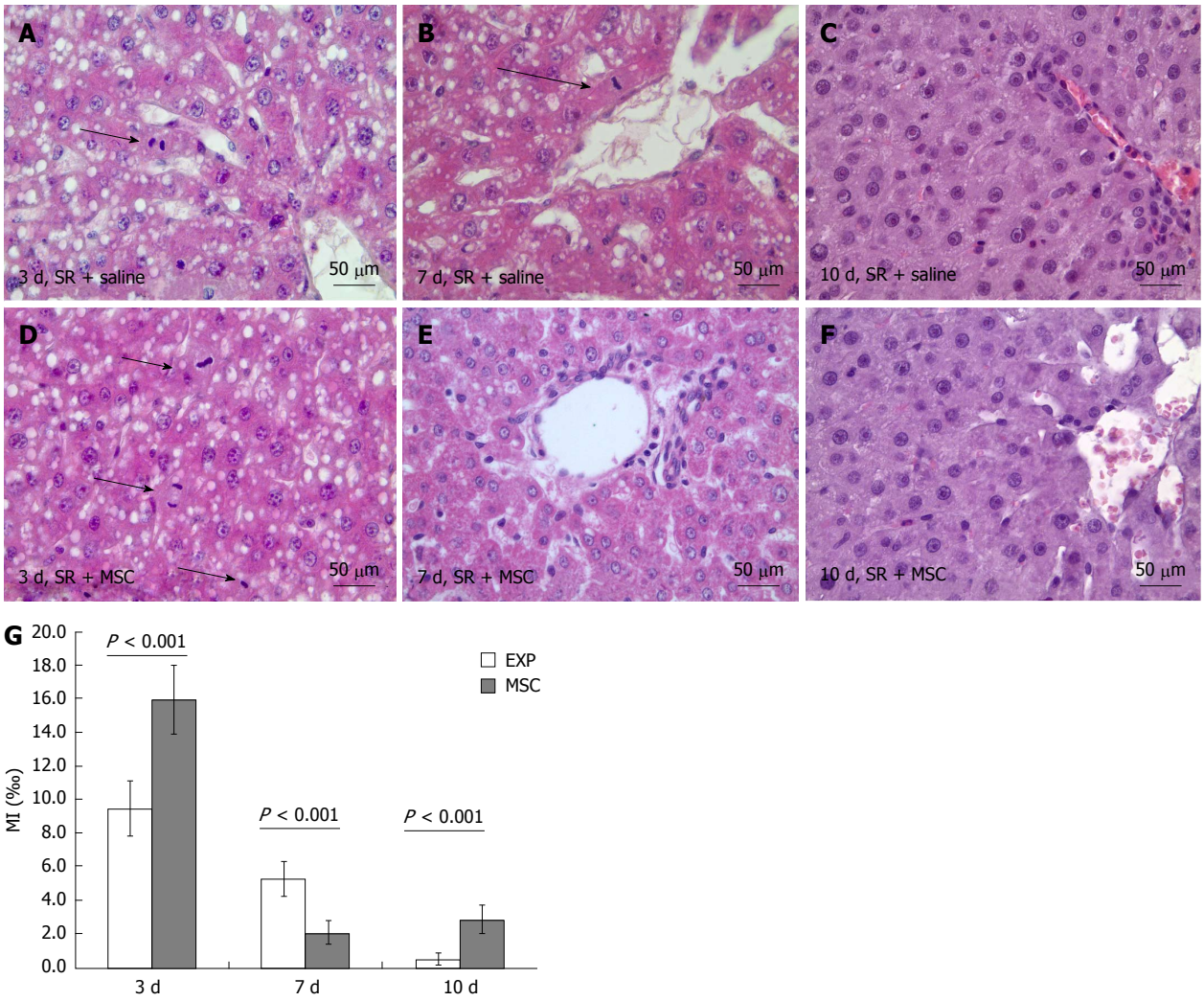

Figure 1 Influence of the multipotent stromal cells transplantation on mitotic activity of hepatocytes; Mitotic activity of hepatocytes in the residual livers at 3 (A, D), 7 (B, E) and 10 d (C, F) after the surgery; Mitotic index of hepatocytes is plotted against time after the surgery (G); HE staining. Arrowheads indicate mitotic figures in hepatocytes (A, B, D); Mitotic index of hepatocytes: Horizontal axis represents time elapsed after the surgery, vertical axis represents mitotic index of hepatocytes, ‰; White columns represent hepatocyte proliferation in the comparison group, gray columns represent hepatocyte proliferation in the multipotent stromal cells transplantation group; The data is presented as mean values with confidence intervals. SR: Subtotal resection; MSC: Multipotent stromal cells.

to the MSC transplantation (Figure 5).

DISCUSSION

In this study we observed a stimulating effect of the umbilical cord-derived MSCs on liver regeneration after subtotal resection in rats, manifested as increased survival of the operated animals, increased rates of liver mass recovery, and increased proliferation activity of hepatocytes.

The obtained evidence of the effect of MSCs on hepatocyte proliferation is consistent with the results of other studies[4]. It should be noted that the stimulating effect MSCs on hepatocyte proliferation is, apparently, due to accelerated passage of the cell cycle phases. This is indicated by the fact that on day 3 after the subtotal liver resection combined with MSC administration (mimicked by sham injection of saline in the comparison group), the Ki67 index did not differ between the groups indicating similar extent of hepatocyte engagement in cell cycle for both groups. At the same time, the proportion of hepatocytes undergoing mitosis per second was significantly higher in animals which received the MSCs, that is, their hepatocytes passed G1, S, and G2 phases more rapidly, to be able to divide by day 3 after the surgery. Such acceleration of cell cycles apparently led to their synchronization resulting in waves of hepatocyte proliferation with a temporary decrease in the mitotic activity of in between[12]. It is possible, that the increased rates of cell cycling in the early postoperative period were the cause of significantly better survival of animals in the MSC transplantation group.

Despite the clearly demonstrated regeneration-stimulating effect of the umbilical cord-derived MSC transplantation, exact mechanisms of this stimulation remained obscure.

However, the obtained data indicate that the replacement mechanism of stimulation of regeneration in this case is not the leading one.

The transplanted MSCs did not differentiate into

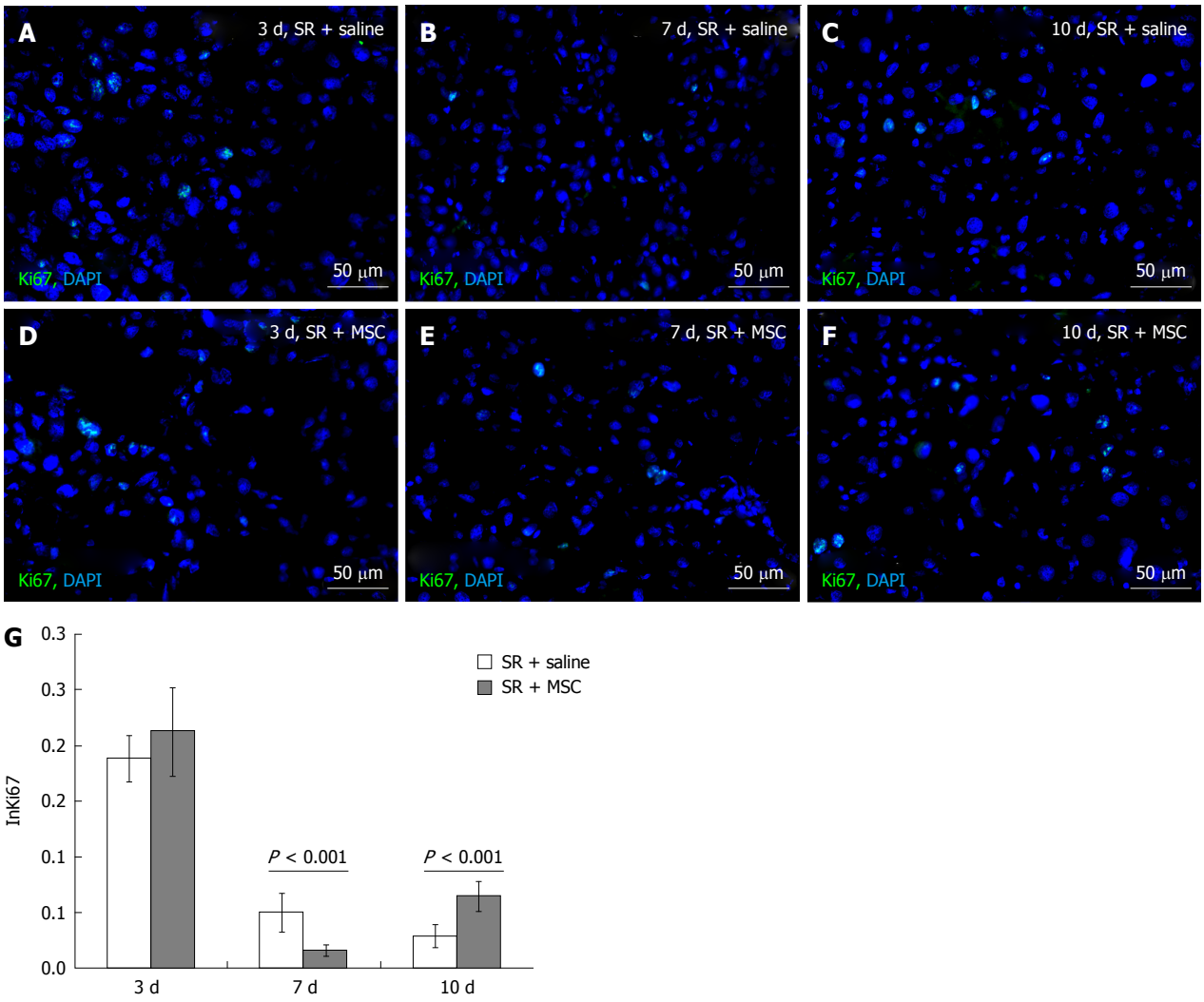

Figure 2 Influence of the multipotent stromal cells transplantation on Ki67 index of hepatocytes. Ki67 expression in the residual livers at 3 (A, D), 7 (B, E) and 10 d (C, F) after the surgery; cell nuclei are counterstained DAPI (blue); Index of Ki67+ hepatocytes is plotted against time after the surgery (G): Horizontal axis represents time elapsed after the surgery, vertical axis represents Ki67 proliferation index (lnKi67) of hepatocytes; White columns represent hepatocyte proliferation in the comparison group, gray columns represent hepatocyte proliferation in the multipotent stromal cells transplantation group; The data is presented as mean values with confidence intervals. SR: Subtotal resection; MSC: Multipotent stromal cells.

hepatocytes, cholangiocytes, smooth muscle cells, active fibroblasts, or myofibroblasts. Solitary PKH26-labeled MSCs at the site of transplantation were positive for the endothelial cell marker CD31. It is known that endothelial differentiation of MSCs is stimulated by VEGF and supported the endothelium-derived extracellular matrix; in combination they make MSCs to express the endothelium-specific markers[13]. It is also known, that the umbilical cord-derived MSCs give a stronger response to endothelial induction than the bone marrow-derived MSCs[14]. Despite that, only a minor fraction of transplanted cells could possibly differentiate into endothelial cells upon transplantation. This is consistent with our previously reported data on rapid elimination of transplanted MSCs by host macrophages. In particular, about 90% of transplanted cells in spleen and regenerating liver were destroyed by association with CD68+ cells[15].

We found no direct evidence for the paracrine effect of MSCs on liver regeneration after the subtotal liver resection in rats. Although MSCs show the ability to synthesize a rich set of biologically active molecules[2], the paracrine effect provided by MSCs is dose-dependent and typically short-term, and apparently difficult to study *in vivo*. Besides, several other studies demonstrating the positive effect of MSCs on liver regeneration show that repeated administration of MSCs, as well as an "in advance" transplantation of the cells before damage, gives a stronger effect, which may have a stronger paracrine component[16,17].

However, the paracrine mechanism of the therapeutic activity of transplanted MSC is indirectly indicated by a decrease in the total number of CD68 + macrophages and an increase in the proportion of M2 pro-repair macrophages in the regenerating liver as compared to animals in which the transplantation was only mimicked. It is known that the effect of MSC on inflammation is mediated by a local increase in IL-4, IL-13, TSG-6 and a decrease in TNFα, IL-6[5] influencing the immune system cells, especially macrophages[6,7]. It has been shown

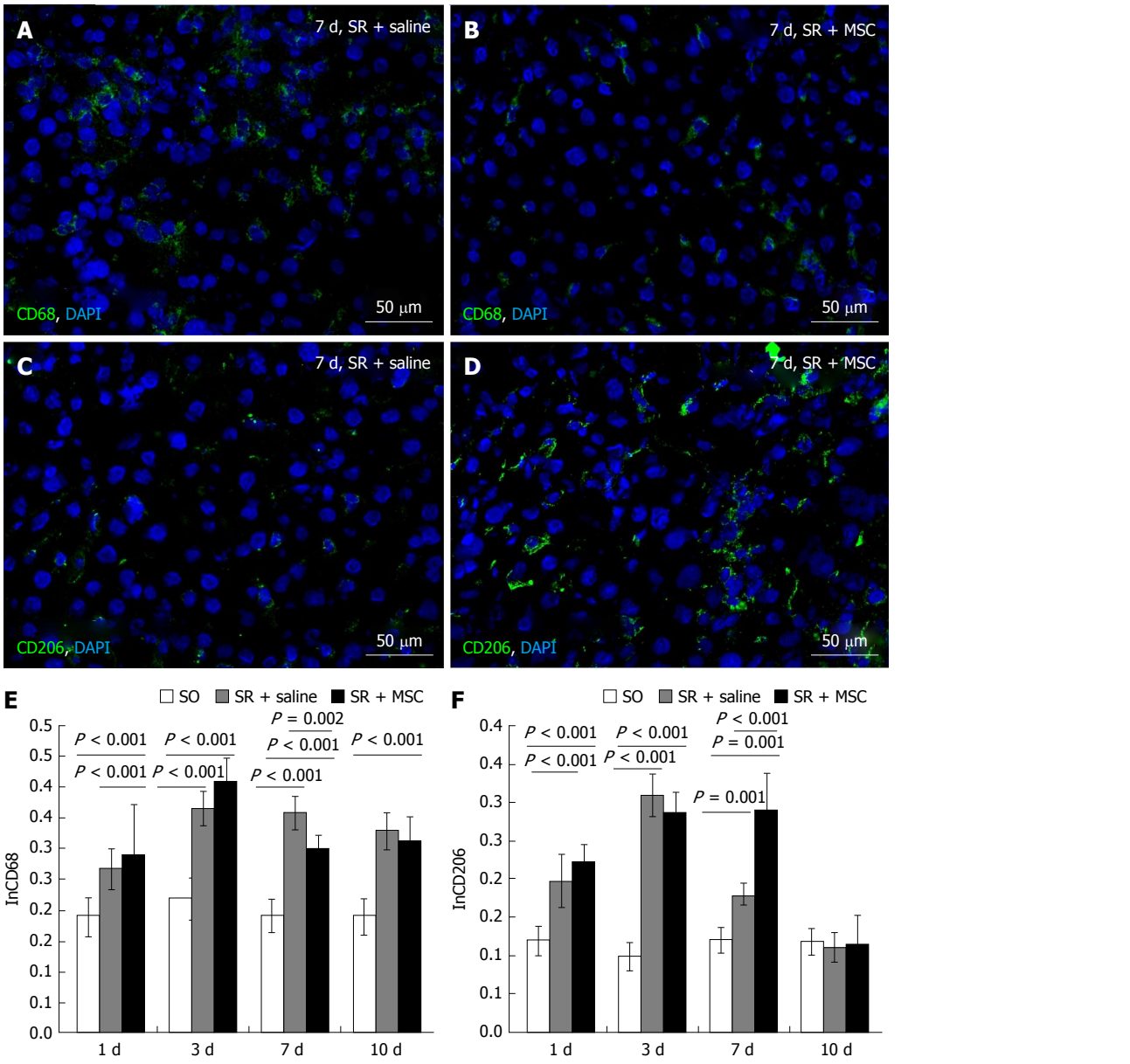

Figure 3 Influence of the transplantation on macrophage polarization profiles. CD68+ cells (green) in the liver in the comparison group (A) and in the residual liver in the MSC transplantation group (B) on day 7 after the surgery. The diagram shows dynamic changes in the CD68+ cell content liver in the comparison group and in the multipotent stromal cells (MSC) transplantation group (E); Relative quantities of CD206+ cells (green) in the liver in the comparison group (C) and in the residual liver in the MSC transplantation group (D) on day 7 after the surgery; The diagram shows dynamic changes in the CD206+ cell content in the course of regeneration; cell nuclei are counterstained with 4',6-Diamidino-2-phenylindole (blue). In E and F, white columns represent the sham-operated animals, gray columns represent the comparison group, black columns represent the MSC transplantation group, horizontal axis represents time elapsed after the surgery. SR: Subtotal resection; SO: Sham-operated animals; MSC: Multipotent stromal cells.

in many studies on various models that after MSC transplantation the number of activated macrophages of the alternative M2 phenotype (which have pro-repair and anti-inflammatory properties) increases, and the number of pro-inflammatory M1 macrophages decreases[18,19]. This is consistent with the data obtained by us on the model of liver regeneration after its subtotal resection in rats.

Exact molecular mechanisms of the paracrine effect of MSC transplantation on hepatocyte proliferation and liver macrophage behavior in the aftermath of subtotal liver resection remain unclear. Further studies in this direction are very desirable, since any possibility of controlling hepatocyte proliferation and/or liver macrophage polarization could be of great therapeutic importance, especially in severe liver injuries.

ARTICLE HIGHLIGHTS

Research background

The resulting post-hepatectomy liver failure is a major manifestation of the small-for-size liver remnant syndrome, when the critically reduced liver mass is insufficient to maintain normal liver function. The problem can be solved by some specific controllable stimulation of compensatory growth combined with intensive liver care and management for acute hepatic failure. Multipotent stromal cells may therefore represent a reasonable choice not only in cases of extensive hepatectomy but also for-other types of severe liver damage (*e.g.*

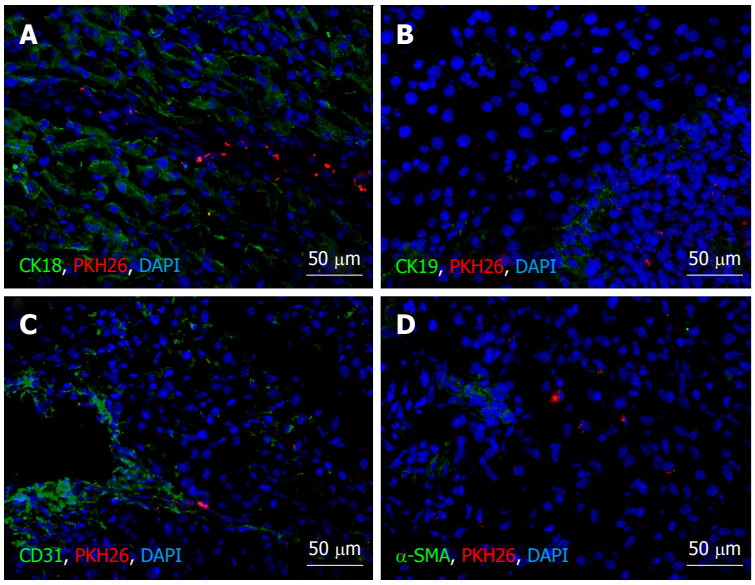

Figure 4 Immunochemical analysis of differentiation of the transplanted multipotent stromal cells on day 10 after the surgery. Immunostaining of cell type-specific proteins, A: CK18 (hepatocytes); B: CK19 (cholangiocytes); C: CD31 (endotheliocytes); D: αSMA (smooth muscle cells). Fluorescent microscopy images display immunostaining (green), PKH26 label (red), and cell nuclei counterstained with 4', 6-Diamidino-2-phenylindole (blue).

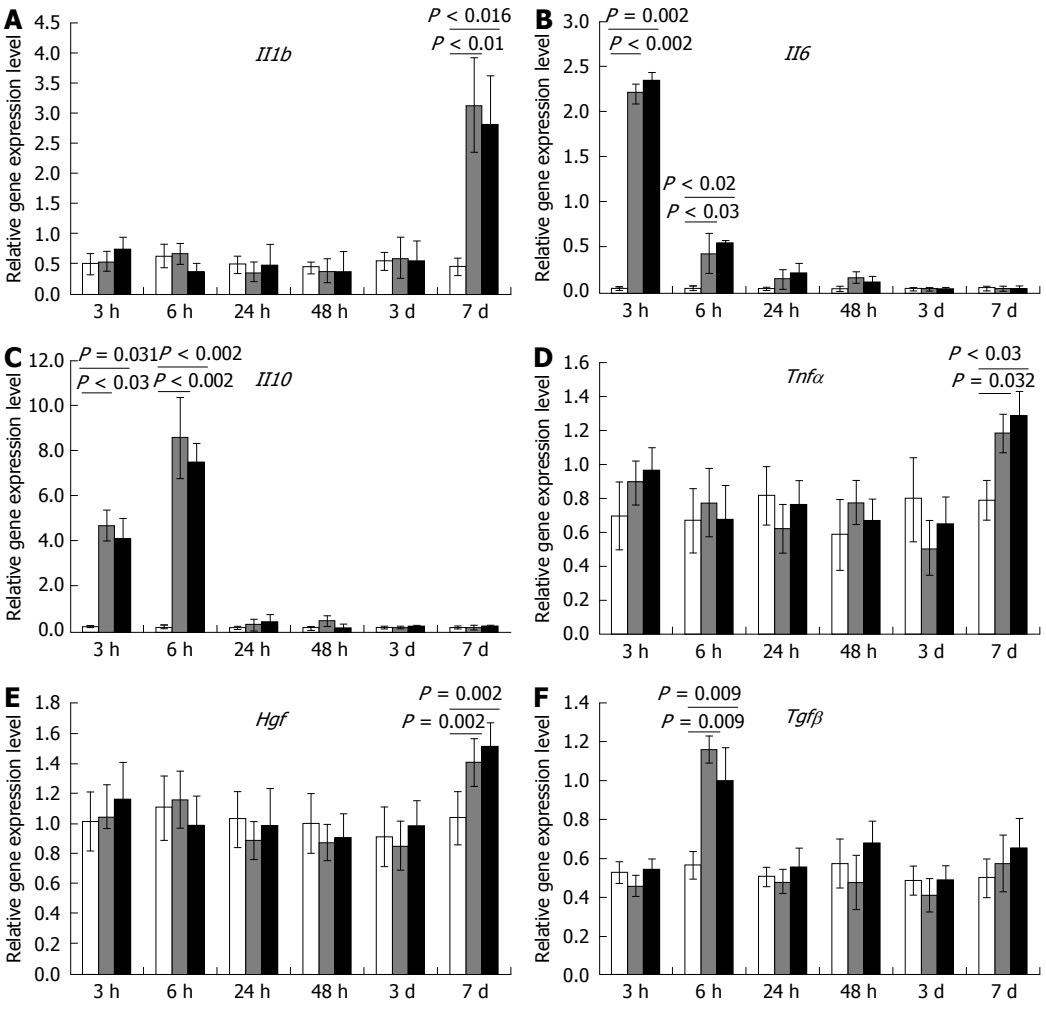

Figure 5 Influence of the transplantation on cytokine and growth factor gene expression within regenerating livers. A: *Il1b*; B: *Il6*; C: *Il10*; D: *Tnfα*; E: *Hgf*; F: *Tgfβ*. White columns correspond to gene expression values (in relative units) for the sham-operated animals, gray columns represent the values for the comparison group, black columns represent the values for the Multipotent stromal cells transplantation group, horizontal axis represents time elapsed after the surgery; The data is presented as mean values with confidence intervals. SR: Subtotal resection; SO: Sham-operated animals; MSC: Multipotent stromal cells.

cirrhosis). Multipotent stromal cells are found to be a helpful supplement in various repair processes in mammals, particularly in solid organs. These cells are considered as a promising tool of regenerative medicine. Their ability to stimulate reparative regeneration of damaged liver has been confirmed, but the mechanisms remain uncertain.

Research motivation

The experimentally proven enhancement of liver regeneration by multipotent stromal cells (MSCs) must be essentially paracrine, because their transplantation to residual livers causes an increase in concentrations of HGF and several other growth factors within the regenerating tissues. Therapeutic activity of MSCs is apparently related to their anti-inflammatory properties, which also represent a sort of paracrine regulation, as manifested by a local increase in IL-4, IL-13, and TSG-6 production paralleled by relative shortage of TNFα and IL-6. Modulation of inflammatory reactions may be implemented *via* influence of these, or similar, paracrine factors on immune cells, especially on macrophages. Several studies, however, confirm the ability of MSCs to differentiate into hepatocytes, which leads to quite a different explanation for the positive influence of MSCs on liver repair.

Research objectives

In our experiments we found that intrasplenic allogeneic transplantation of the umbilical cord-derived multipotent stromal cells stimulated hepatocyte proliferation and organ mass recovery after subtotal resection. These effects may result from positive paracrine influence of the transplanted cells on polarization of the liver resident macrophages to M2 phenotype.

Research methods

The MSCs were obtained from the intervascular tissue of umbilical cords, dissected from rat fetuses, by the explant culture technique. The vital labeling of MSCs with PKH26 was carried out on the 3rd passage. The subtotal resection was performed on male Sprague-Dawley rats. The experimental group animals received a transplant 106 MSCs infused into the spleen. Hepatocyte proliferation was assessed by counting of either mitotic figures or Ki67-positive cells in microscopic images. MSC differentiation was assessed with antibodies to hepatocyte-specific marker cytokeratin 18 (CK18), cholangiocyte-specific protein CK19, smooth muscle cell-specific protein α-SMA, the endothelial cell marker CD31, or the active fibroblast marker FAPα. Total macrophages of the liver were selectively stained in cryosections incubated with anti-CD68 antibodies (1:100, Abcam), while the M2a and M2c macrophage populations were selectively stained with anti-CD206 antibodies. Expression of interleukin and growth factor genes was evaluated with PCR-RT.

Research results

Intrasplenic allogeneic transplantation of the umbilical cord-derived multipotent stromal cells stimulates reparative processes within the residual liver tissue after subtotal resection (removal of 80% of the organ mass), as indicated by increased rates of hepatocyte proliferation and accelerated organ mass recovery. These effects may result from paracrine influence of the transplanted cells on the resident macrophage population of the liver. The transplantation favors polarization of macrophages to M2 phenotype (the M2-polarized macrophages specifically express CD206; they are known to suppress inflammation and support tissue repair). No differentiation of the transplanted cells into any of the liver cell types have been observed in the study.

Research conclusions

In this study we observed a stimulating effect of the umbilical cord-derived MSCs on liver regeneration after subtotal resection in rats, manifested as increased survival of the operated animals, increased rates of liver mass recovery, and increased proliferation activity of hepatocytes. We found no direct evidence for the paracrine effect of MSCs on liver regeneration after the subtotal liver resection in rats. However, the paracrine mechanism of the therapeutic activity of transplanted MSC is indirectly indicated by a decrease in the total number of CD68 + macrophages and an increase in the proportion of M2 pro-repair macrophages in the regenerating liver as compared to animals in which the transplantation was only mimicked.

Research perspectives

Exact molecular mechanisms of the paracrine effect of MSC transplantation on hepatocyte proliferation and liver macrophage behavior in the aftermath of subtotal liver resection remain unclear. Further studies in this direction are very desirable, since any possibility of controlling hepatocyte proliferation and/or liver macrophage polarization could be of great therapeutic importance, especially in severe liver injuries.

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ORIGINAL ARTICLE

Basic Study

Morphological and biochemical effects of weekend alcohol consumption in rats: Role of concentration and gender

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Abstract

AIM

To examine the association between weekend alcohol consumption and the biochemical and histological alterations at two different concentrations of alcohol in both genders in rats.

METHODS

Wistar rats weighing 170-200 g were divided into groups as follows: (1) Control groups; and (2) weekend alcohol-consumption group: 2 d/weekly per 12 wk, at two different concentrations: (1) Group of males or females with a consumption of a solution of alcohol at 40%; and (2) group of males or females with a consumption of a solution of alcohol at 5%. At the end of the experiment, serum and liver samples were obtained. The following enzymes and metabolites were determined in serum: Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Lactate Dehydrogenase, and Gamma-Glutamyltransferase, and glucose, triglycerides, cholesterol, bilirubin, and albumin. Liver samples from each group were employed to analyze morphological abnormalities by light microscopy.

RESULTS

In all of the weekend alcohol-consumption groups, AST activity presented a significant, 10-fold rise. Regarding ALT activity, the groups with weekend alcohol consumption presented a significant increase that was six times greater. Bilirubin levels increased significantly in both groups of females. We observed a significant increase in the parameters of fatty change and inflammation due to weekend alcohol consumption. Only the group of females that consumed alcohol at 40% presented slight hepatocellular disorganization

CONCLUSION

The results obtained herein provide solid evidence that weekend alcohol consumption gives rise to liver damage, demonstrated by biochemical and histological alterations, first manifested acutely, and prolonged weekend alcohol consumption can cause greater, irreversible damage.

Key words: Weekend alcohol consumption; Liver morphology; Transaminases; Damage

Core tip: At present, it is considered that the main weekend alcohol consumers comprises the young population, due to the gratifying effect of alcohol, this being a very important social and health problem. Our findings demonstrate an effect of the damage that is caused by weekend alcohol consumption, regardless of gender or the concentration of alcohol. Even more so, greater damage can be observed in females, and the metabolism of ethanol probably participates, specifically due to its first-pass metabolism, which is carried out in the stomach.

Morales-González JA, Sernas-Morales ML, Morales-González Á, González-López LL, Madrigal-Santillán EO, Vargas-Mendoza N, Fregoso-Aguilar TA, Anguiano-Robledo L, Madrigal-Bujaidar E, Álvarez-González I, Chamorro-Cevallos G. Morphological and biochemical effects of weekend alcohol consumption in rats: Role of concentration and gender. *World J Hepatol* 2018; 10(2): 297-307 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/297.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.297

INTRODUCTION

Various reports have demonstrated the adverse effects to health caused by the consumption of alcohol[1-3]. Similarly, it is well known that in Mexico, the main alcoholic beverages consumed are beer and distilled beverages (brandy, tequila, rum, whisky, cognac, vodka, *etc*.)[3], which contain approximately 5% and 36%-40% of alcohol, respectively[1]. It has been reported that alcohol consumption presents various patterns that are associated with gender, age, socioeconomic situation, consumption type (regular drinkers, intense or weekend drinkers), and, the alcoholic-beverage type (wine, mixed drinks)[4]. On the other hand, according to what has been reported in ENCODAT 2016, young people exhibit the tendency toward alcohol consumption in total population in Mexico (12-65 years of age), with daily alcohol consumption at 2.9% and habitual consumption (weekend) at 8.5%. Likewise, it was found that, although males consume more alcohol, women present an important index of alcohol consumption[3].

Weekend alcohol consumption for young people is becoming an important social and familial problem, but also a considerable health problem[5], and it can be due to the increase of Allopregnanolone (the testosterone metabolite that participates in the gratifying effect of alcohol)[6]. Various reports have associated the harmful effect to health engendered by weekend alcohol consumption, such as the following: reports on and the increase in deaths during Fridays,

Saturdays, and Sundays[7]; the greater neurocognitive and neurobehavioral deterioration, which is similar in many aspects to that observed in chronic alcohol drinkers[5]; deaths caused by ischemic heart disease[8,9], or the idiopathic arrhythmias that initiate during weekends, a risk factor for producing visual alterations (dyschromatopsia)[10].

On the other hand, some biochemical alterations have been reported as being caused by weekend alcohol consumption. Stranges *et al*[11] reported that there are modifications at the level of the enzymes released by the liver into the blood Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Gamma-Glutamyltransferase (GGT), and these authors concluded that these modifications were caused by alcohol consumption, finding that the highest elevated enzyme is GGT, identifying differences by gender: In males, daily alcohol drinkers exhibited highest GGT levels, while in females, highest GGT was observed in weekend alcohol drinkers. The authors concluded that, in addition to the amount of alcohol consumed, the pattern of consumption can affect liver function and that there are gender differences with respect to liver function and possible damage to this organ[11]. Hojnacki *et al*[12] compared the effect of the daily alcohol-consumption pattern (moderate, 12%) and weekend alcohol consumption (concentrated, 20%) utilizing squirrel monkeys. These authors found that moderate-daily alcohol consumption causes a moderate diminution in Body Weight (BW) and produces increases in the profile of coronary protective lipoproteins [HDL2/HDL3 cholesterol increases and low density lipoprotein (LDL) cholesterol decreases]; in contrast, in concentrated alcohol consumption, unfavorable alterations are produced in the lipoproteins (LDL cholesterol increases and Apolipoprotein B increases), together with weight loss and body fat depletion[12]. Rocha *et al*[13], on employing a weekend alcohol-consumption model in rat (alcohol in the drinking fountain at 30% during 3 d/wk per 70 d), reported an average alcohol consumption of 4.56 g per day, which gave rise to a dyslipedemic profile, an increase in energy expenditure, and hepatic metabolic changes similar to those associated with chronic ethanol consumption[13]. Finally, Liu *et al*[14] compared the effect of the damage that two alcohol-consumption patterns cause: Moderate-daily consumption 0.8 g/kg/7 d/wk *vs* weekend alcohol consumption 2.8 g/kg/2 d/wk, with a weekly consumption in both groups of 5.6 g/kg. The authors found an increase in the levels of total cholesterol and of HDL-C in both groups; on the other hand, a diminution was observed in the LDL-C levels of the moderate-daily consumption pattern, the latter significantly raised in weekend drinkers. Blood concentrations of alcohol as well as BW gain were greater in weekend alcohol consumers. These findings of weekend alcohol consumption (4 wk) favored the development of atherosclerotic plaque (increase in the plaque, diminution in the lux lumen, *etc.*), while precisely the contrary took place with moderate-daily alcohol consumption[14]. Some reports describe the histologic damage that alcohol causes to the liver[15-17] but, to our knowledge, there are no reports on the histologic changes caused by weekend alcohol consumption to the liver.

There are numerous studies on the effects of chronic weekend alcohol consumption; however, few reports exist, to our knowledge, on the association between weekend alcohol consumption and the damage produced in the liver. The objective of this study was to examine the association between weekend alcohol consumption and the biochemical and histological alterations at two different concentrations of alcohol in both genders in Wistar rats.

MATERIALS AND METHODS

Reagents

All chemical reagents were obtained from Merck (Merck de México, S.A.) and were of the best quality available.

Animals

We utilized male Wistar rats with an initial Body Weight (BW) of 170-200 g that were obtained from the Escuela Superior de Medicina (ESM) Bioterium of the Instituto Politécnico Nacional (IPN) in Mexico City. The rats were housed in cages at the ESM Bioterium. They were maintained at a temperature of 22 ℃ with 12-h/12-h light-dark cycles and received standard rat-pellet food (Purina de México, S.A.) and water *ad libitum* prior to the treatments. After 14 d of adaptation, the procedure was initiated. The protocol and the experimental procedures were conducted according to the Mexican Official Norm for the Use and Care of Laboratory Animals (NOM-062-ZOO-1999, México)[18]. The protocol was authorized by the Comité Interno del Cuidado y Uso de los Animales de Laboratorio (CICUAL), with registry number ESM.CICUAL-12/23-06-2017, and by the Research Committee with registry number ESM. CI-01/13-06-2017, both committees of the ESM of the IPN.

Experimental design

The animals were randomly divided into six groups in the following manner: (1) Control groups (males and females); and (2) weekend alcohol-consumption group: 2 d/weekly per 12 wk at two different concentrations: (1) Group of males with consumption of a solution of alcohol at 40%; (2) group of females with consumption of a solution of alcohol at 40%; (3) group of males with a consumption of a solution of alcohol at 5%; and (4) group of females with a consumption of a solution of alcohol at 5%. The control groups (females and males) had access to food and water *ad libitum* at all times. Daily alcohol consumption was quantified, reported in g/kg of weight/day, and BW gain weekly was reported in g.

Serum samples

At the end of the experiment, the animals were sacrificed by decapitation after being previously

anesthetized with pentobarbital sodium (at an overdose of 40 mg/kg BW). Blood samples were obtained and centrifuged in a clinical centrifuge to obtain the sera, which were frozen at -70 ℃ for later use.

Liver histology

Hepatic samples from each group Sere employed for light microscopy. Samples were fixed with formaldehyde (10% in isotonic solution), embedded in paraffin, and stained with Hematoxylin and Eosin. Biopsy specimens were coded and read blindly without knowledge of the other data by independent observers at two different laboratories (MLS-M and JB-R). The criteria utilized to analyze the morphological abnormalities were the same as those reported by Morales-González *et al*[15] as follows: Fatty infiltration (+, mild; ++, moderate; +++, severe, and ++++, very severe); inflammation (+, zonal localization, focal inflammatory cells; ++, moderate, not restricted to any one zone of the acinus, and +++, diffuse), and hepatocellular disorganization (+, isolated foci in zone 3 of the liver acinus; ++, more widespread, and +++, definitively diffused in the hepatic acini); apoptosis (+, mild; ++ moderate; +++ severe, and ++++ very severe), and mitosis(+ mild; ++ moderate; +++ severe, and ++++ very severe).

Determination of enzymes and metabolites in serum

The activities of serum Alanine Aminotransferase [ALT; Expansion Coefficient (EC) 2.6.1.2], Aspartate Aminotransferase (AST, EC 2.6.1.1), Lactate Dehydrogenase (LDH, EC 1.1.1.27), and Gamma-Glutamyltransferase (GGT, EC 2.3.2.2) were measured colorimetrically utilizing diagnostic kits (Spinreact de México, SA de CV), following the manufacturer's instructions; the results are reported in units/L.

Serum concentrations of glucose, triglycerides, cholesterol, bilirubin, and albumin were determined by spectrophotometric techniques using diagnostic kits (Spinreact de México, SA de CV) following the instructions provided by the manufacturer; the results are reported in mg/dL, except for albumin, which is reported in g/dL.

Statistical analysis

The results were analyzed using the SigmaPlot ver. 12.3 statistical software program. The results are expressed as the mean ± SE of the mean (SEM), as required. We carried out a statistical analysis using Student *t* test and/or Analysis of Variance (ANOVA) and the Student-Newman-Keuls method as *post-hoc* evaluation for multiple comparisons. We considered differences among the groups to be statistically significant when $P < 0.05$.

RESULTS

Effect of weekend alcohol consumption on weight gain

Average alcohol consumption per group was as follows: In females, at 5% of 0.83 g/kg per day; in the male group at 5% of 1.63 g/kg per day, and, in groups of females and males, at 40%, this was 5.52 and 2.26 g/kg per day, respectively (Table 1).

All of the weekend alcohol-consumption groups presented a significant weight gain in comparison with the control group. The group of females as well as in that of males with 40% alcohol consumption presented an increase in weight gain similar to that of 121.3 and 127.8 g, respectively. Surprisingly, the group of males at 5% exhibited a very important weight gain of 214 g; however, this was not so in the group of females with 5% consumption (98 g) (Table 1).

Activity of ALT, AST, LDH and GGT in serum after weekend alcohol consumption

The effect of weekend alcohol consumption was evaluated by determining the activity of ALT, AST, LDH, and GGT, because these enzymes classically reflect liver function, which is dependent on morphofunctional integrity.

In Figure 1, we observe the AST activity (upper panel) in the diverse experimental groups. In all of the weekend alcohol-consumption groups, AST activity presented a significant rise of 10 times in comparison with the control (23.8 U/L). Regarding ALT activity, the control presented ALT activity of 36.5 U/L, and the remaining groups with weekend alcohol consumption presented a significant increase that was 6-fold greater, independently of the alcohol concentration (Figure 1, lower panel).

The activity of the LDH enzymes (upper panel) and of the GGT enzymes (lower panel) can be observed in Figure 2. LDH activity in the control group was 147 U/L, noting that weekend alcohol consumption favored an increase in all groups of between 18 and 20 times greater in comparison with the control group, not finding differences among these in terms of the weekend alcohol-consumption groups with regard to of the activity of this (LDH) enzyme. On the other hand, with respect to the activity of the GGT enzyme, differences were not found in any weekend alcohol-consumption group in comparison with the control (22.6 U/L), or among the alcohol-treated groups.

Effects of weekend alcohol consumption on serum concentrations of glucose, triglycerides, and cholesterol

In the weekend alcohol-consumption groups (Table 2), we quantified serum metabolite modifications, which depend on the hepatic metabolism, such as glucose, cholesterol, and triglycerides. Regarding glucose levels, it may be observed in Table 2 that weekend alcohol consumption in all groups favored the increase of the serum levels of this metabolite significantly with a $P < 0.05$ *vs* control. The serum concentration of cholesterol in the control group was 58 mg/dL, while in the groups with weekend alcohol consumption, the following was found: in the group of females at 5% (71.83 mg/dL); males at 5% (70.67 mg/dL), and in females at 40% (68.33 mg/dL), this was statistically significant ($P < 0.05$) in all alcohol-consumption groups *vs* control. The

Table 1 General characteristics of rats: weight gain and alcohol consumption

Group	Initial body weight (g)	Final body weight (g)	Body weight gain (g) (%)	Average alcohol consumption (g/kg/d)
Control	176.50 ± 3.3	255.00 ± 23	78.5 ± 22 (43)	0
Females 5%	171.00 ± 10.57	269.00 ± 15.16	98.0 ± 20.71[a] (57)	0.83 ± 0.04
Females 40%	172.92 ± 4.61	294.25 ± 13.39	121.3 ± 13.91[a] (70)	5.52 ± 1.85
Males 5%	198.00 ± 3.21	412.00 ± 8.74	214.0 ± 11.53[a] (108)	1.63 ± 0.01
Males 40%	172.80 ± 9.17	300.00 ± 85.43	127.8 ± 41.29[a] (73)	2.26 ± 0.61

Values are expressed as the mean ± SE in each experimental group (n = 3-6). [a]P < 0.05 *vs* control group.

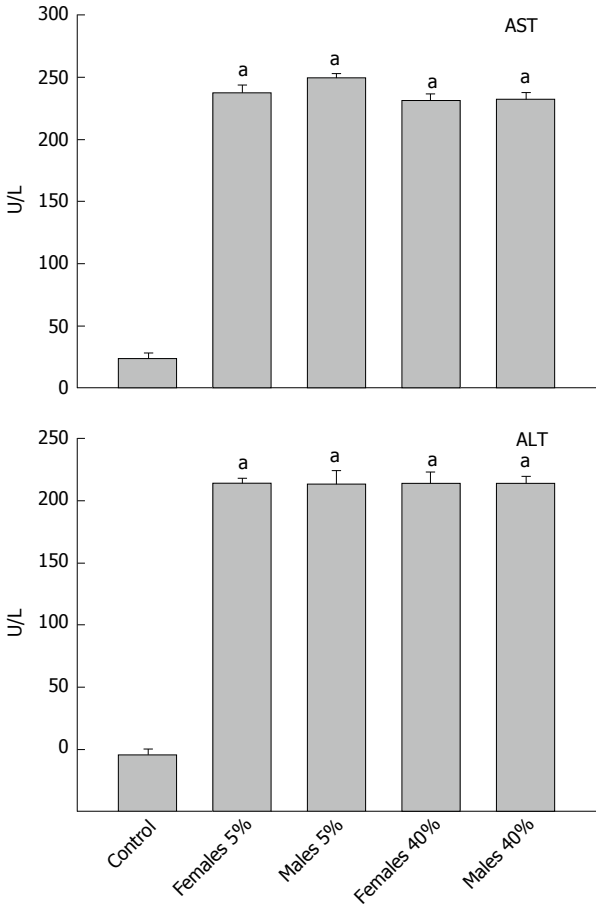

Figure 1 Activities of serum Aspartate and Alanine Aminotransferases after weekend alcohol consumption. Values are expressed as the mean ± SEM in each experimental group (n = 3-6). [a]P < 0.05 *vs* the control group. AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase.

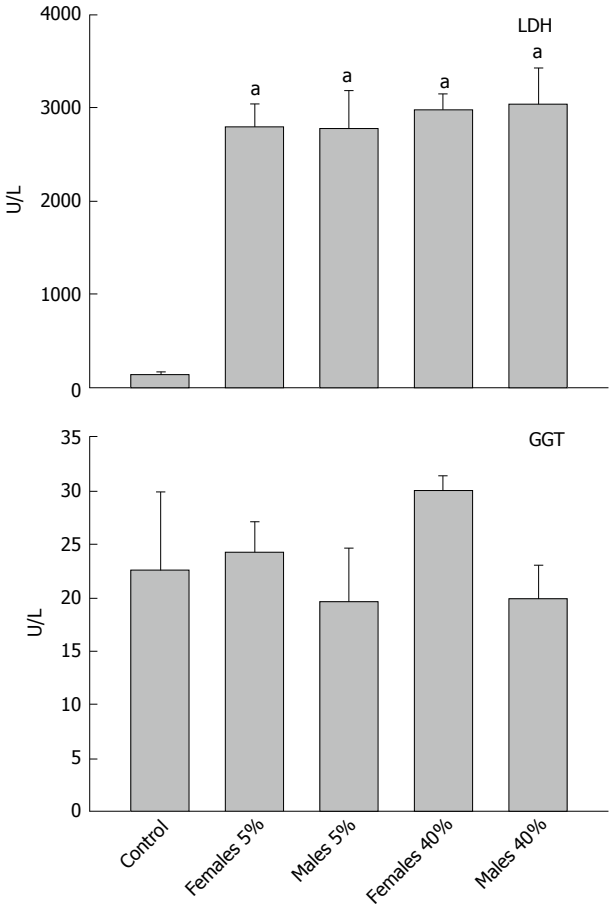

Figure 2 Activities of serum Lactate Dehydrogenase and Gamma-Glutamyltransferase after weekend alcohol consumption. Values are expressed as the mean ± SEM in each experimental group (n = 3-6). [a]P < 0.05 *vs* the control group. LDH: Lactate Dehydrogenase; GGT: Gamma-Glutamyltransferase.

serum levels of triglycerides were raised due to alcohol consumption (Table 2).

Effects of treatment with weekend alcohol consumption on serum concentrations of albumin and bilirubin

The results of the metabolic integrity of the liver are presented in Table 3. Bilirubin levels increased significantly in both groups of females (0.26 mg/dL), in comparison with the control group (0.15 mg/dL; P < 0.05). Regarding the groups of males with weekend alcohol consumption at 5% and 40%, their bilirubin levels were found to be 0.15 and 0.18 mg/dL, respectively, the latter not significant in comparison with the control group (0.15 mg/dL). Surprisingly, the levels of albumin in serum were not affected in any weekend alcohol-consumption group in comparison with the control group (Table 3).

Effect of weekend alcohol consumption on histological indicators (fatty change, inflammation, hepatocellular disorganization, necrosis, and apoptosis)

In Table 4, we can observe the effect exerted by weekend alcohol consumption on histological changes. We noted a significant increase in the parameters of fatty change and inflammation due to weekend alcohol consumption compared with the control group (Table 4). In Figure 3, we are able to observe representative images of the female group with alcohol consumption

Table 2 Effects weekend alcohol consumption on serum glucose, cholesterol, and triacylglycerols

Group	Glucose (mg/dL)	Cholesterol (mg/dL)	Triacylglycerols (mg/dL)
Control	85 ± 3.89	58 ± 1.5	90 ± 2.4
Females 5%	157.33 ± 6.88[a]	71.83 ± 2.79[a]	104.5 ± 8.70
Males 5%	166.33 ± 9.13[a]	70.67 ± 4.37[a]	111.33 ± 5.69[a]
Females 40%	145.5 ± 11.04[a]	68.33 ± 2.455[a]	104.167 ± 3.5[a]
Males 40%	153 ± 7.09[a]	66 ± 2.98	105.8 ± 4.92[a]

Metabolites are expressed as mean ± SE (n = 3-6). [a]$P < 0.05$ *vs* control.

Table 3 Effects weekend alcohol consumption on serum albumin and bilirubin

Group	Bilirubin (mg/dL)	Albumin (g/dL)
Control	0.15 ± 0.01	2.70 ± 0.11
Females 5%	0.26 ± 0.05[a]	2.87 ± 0.09
Males 5%	0.15 ± 0.02	2.90 ± 0.20
Females 40%	0.26 ± 0.03[a]	2.82 ± 0.08
Males 40%	0.18 ± 0.02	2.80 ± 0.21

Metabolites are expressed as mean ± SE (n = 3-6). [a]$P < 0.05$ *vs* control.

at 5%, where inflammation and steatosis are observed, as well as slight periportal fibrosis. In Figure 4, we may observe slight inflammation, steatosis, and slight periportal fibrosis in the group of males with alcohol consumption at 5%, while the groups of males and females with alcohol consumption at 40% were those with greatest histological changes in terms of the parameters of fatty change and inflammation, respectively (Table 4). Only the group of females that consumed alcohol at 40% presented slight hepatocellular disorganization (Table 4). Figure 5 presents the images of the group of females with alcohol consumption of 40%, where steatosis is observed, and inflammation, although this was more marked in comparison with the group of females with alcohol consumption at 5%. The latter can be noted, because it surpasses the limiting plaque and the periportal fibrosis is more evident (which can be observed as the loss of the amount of the periportal cell, replaced by fibrotic tissue). In Figure 6, moderate inflammation with leukocytes is observed that surpasses the limiting plaque, and also, the degree of periportal fibrosis is very important; likewise, apoptosis was only present in the group of males with alcohol consumption at 40% (Table 4). Last, the necrosis parameter is present in all of the groups that consumed alcohol on weekends, this being greater in the groups that consumed alcohol on weekends at 40% (females and males) (Table 4).

DISCUSSION

It is known that chronic alcohol consumption is a risk factor for various diseases, such as diabetes, cancer, gastritis, and gastric ulcers, and that it is especially related to liver damage[1]. In recent years, attention has been focused on weekend alcohol consumption as a cause of cognitive-intellectual alterations[5], a risk factor for homicides[7], cardiovascular diseases such as arrythmias or infarct[8,9], and even of visual alterations[10]. At present, it is considered that main weekend alcohol consumers comprise the young population, due to the gratifying effect of alcohol[6], this being a very important social and health problem.

Rocha *et al*[13], on utilizing the weekend alcohol consumption model for 10 wk in male rats and an alcohol concentration in the drinking fountain of 30%, reported an average alcohol consumption of 4.56 g/d, and that alcohol favors the increase of BW by approximately 30%, diminution in glucose levels, the rise of triglycerides and of ALT, and normal levels of total protein and cholesterol. In our study, alcohol consumption at a concentration similar to that reported by Rocha was 40%, finding similarity in average daily consumption of alcohol, in addition to an increase in BW in females (70%) as well as in males (73%) in comparison with the control (43%) (Table 1), the latter analogous to that reported previously by Rocha. In the same manner, we, like Rocha, found neither changes in albumin levels nor in those of total proteins, respectively (Table 3), which is probably due to that the damage is not yet chronic and severe. The weight gained is probably due to the increase in lipids, which may be observed in their elevation in blood (Table 2), as well as in hepatic tissue (Table 4) (Figures 3-6), caused by weekend alcohol consumption. Other reports demonstrate that weekend alcohol consumption gives rise to alterations in BW that are greater than those compared with moderate-daily alcohol consumption, and that this moderate consumption produces an increase in the protective lipoprotein profile. In contrast, concentrated alcohol consumption produces unfavorable alterations in the lipoproteins[12,14].

Surprisingly, alcohol consumption at 5% caused the greatest BW increase (108%) (Table 1). This can be explained by the metabolism of ethanol, in that, at different concentrations, the metabolism of the first pass of ethanol is modified, which is predominantly gastric[19] and is supplied mainly by the activity of the gastric ADH[20]. Previously, Roine *et al*[21] demonstrated that consumption of a concentrated solution of alcohol (40%) results in low levels of alcohol in blood in comparison with a diluted solution (4%), and that this effect is associated with first-pass metabolism and with lesser bioavailability with high concentrations of ethanol. Dohmen *et al*[22] demonstrated that, on administering a

Table 4 Histopathological changes induced by the consumption of weekend ethanol at two concentrations

Group	Fatty change	Inflammation	Hepatocellular disorganization	Necrosis	Apoptosis
Control	0	0	0	0	0
Females 5%	+/++	+/++	0	0/+	0
Males 5%	+/++	0/++	0	0/+	0
Females 40%	+/+++	+	0/+	0/++	0
Males 40%	+/++	+/+++	0	0/++	0/++

Histopathological parameters were evaluated as described in the Material and Methods.

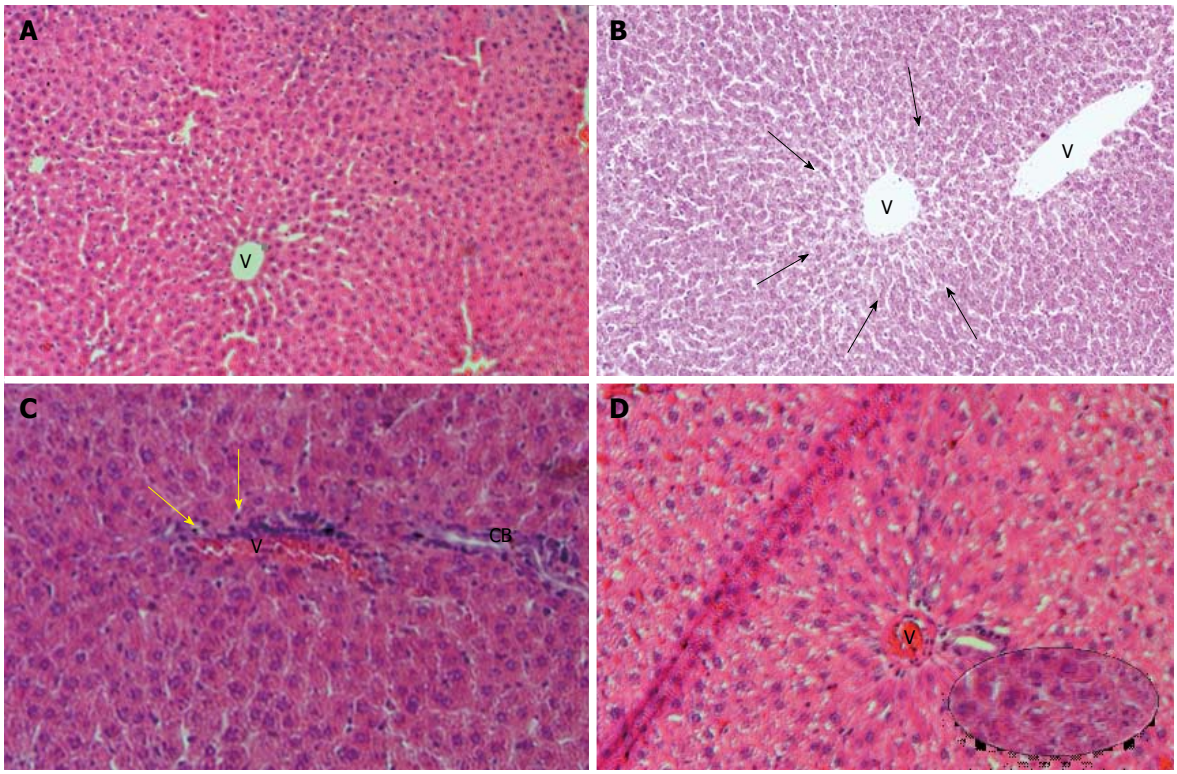

Figure 3 Hepatic histology of the group of females with weekend alcohol consumption at 5%. A: Image of the control group; B-D: Images of the group of females with alcohol consumption at 5%. A: Hepatocytes were observed as formed in a line (40 ×); B: A zone of less pigmentation is observed, marked with black arrows, corresponding to periportal necrosis (40 ×); C: Slight inflammation with blue-colored cells (leukocytes) around the portal vein, which do not surpass the limiting plaque (yellow arrows) (60 ×); D: In the lower left part, steatosis is observed (60 ×). V: Portal vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

solution of alcohol at 5%, first-pass metabolism is low, while with a 40% ethanol solution, first-pass metabolism is high and is furnished by the activity of the gastric dehydrogenase alcohol. It is probable that, in the groups of weekend alcohol consumption with a concentration of 40%, the subjects had initial alcohol metabolism in the stomach, and low ethanol concentration. In contrast, the group of males with a consumption of 5% had highest ethanol concentration, which probably modified the metabolites involved in the accumulation of body fat, such as glucose, triglycerides, and cholesterol, as may be observed in Table 2, where these metabolites exhibit a slight increase in comparison with the remaining groups. Our data are in agreement with those reported by Rocha *et al*[13] where, after 10 wk of alcohol consumption (3 d per week at 30% ad libitum), the authors found that food consumption was very similar to that exhibited between the control group and the group with alcohol consumption. Nonetheless, the group that consumed alcohol had a greater weight gain than the control. Thus, we think that it is the alcohol and its metabolism that is the cause of the weight gain, because it alters the fat level in the liver (Table 4), which we have previously reported[2,15,16]. Baraona *et al*[23] found that the first metabolic pass of the alcohol is found to be diminished in women due to low activity of gastric ADH; the authors concluded that the latter can increase the vulnerability of women to the effects of ethanol. In our study, we found that the levels of bilirubin were higher in both groups of females, regardless of the concentration of alcohol administered (Table 3). Likewise, greater histological changes in fatty change and inflammation (Table 4) and in alcohol consumption levels were lower in the group of females at 5% (0.83 ± 0.04) (Table 1).

On the other hand, the transaminase enzymes are indicators for the diagnosis of liver diseases. For

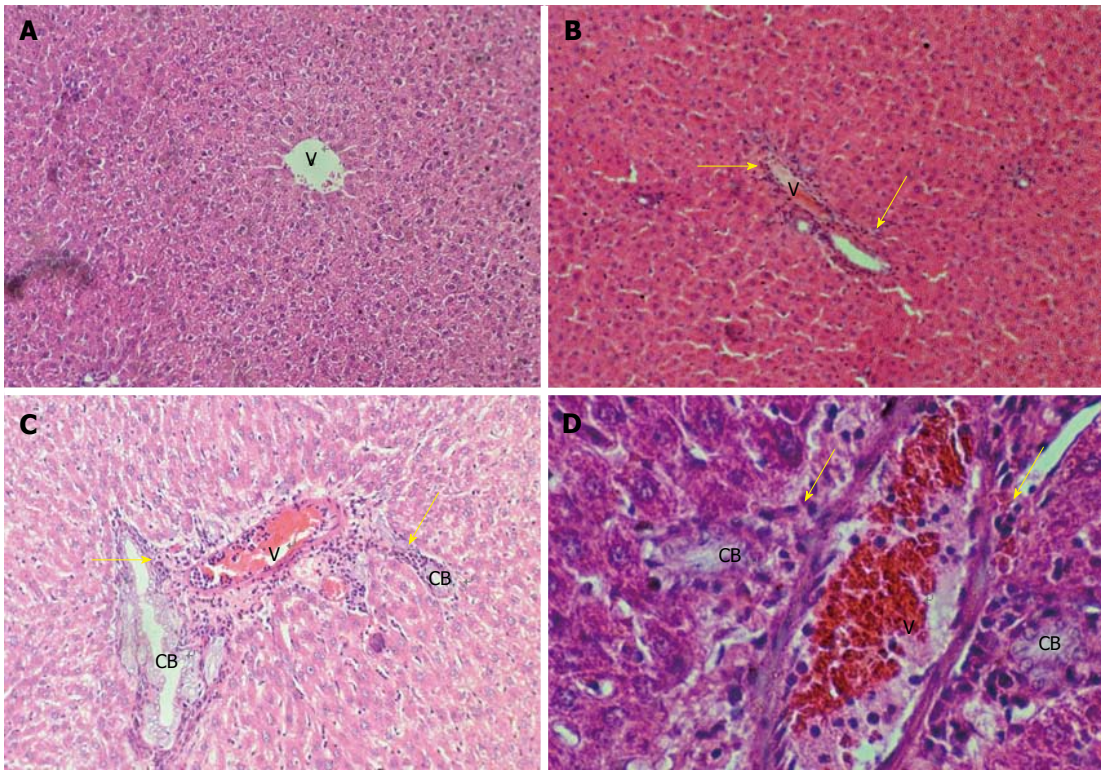

Figure 4 Hepatic histology of the group of males with weekend alcohol consumption at 5%. A: Image of the control group; B-D: Images of the group of males with alcohol consumption at 5%. A: The uniformity of the structures is observed to be conserved (40 ×); B: Presence of fine and thick steatosis (pigmentation-diminution zone in the cytoplasm), inflammation present with leukocytes in single file around the portal vein (yellow arrows) (40 ×); C: Important inflammation is observed, represented by leukocytes around the portal vein and in the Bile Canaliculus (BC) (yellow arrows) (40 ×); D: Fine as well as thick steatosis is observed (pigmentation-diminution zone in the cytoplasm), as well as leukocyte infiltrate (yellow arrow) (100 ×). V: Portal Vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

example, ALT and AST are released into the bloodstream at high concentrations, in which there is a membrane alteration in the hepatocyte; however, hepatocellular necrosis is not a requirement for the release of these enzymes, which gives rise to low correlation between the level of aminotransferases and liver damage[15]. This could be explained by the early release of hepatocytes, which initiate a proliferative process such as a sign, due to that it has been reported that liver regeneration is linked by selective enzyme release[15]. We previously reported that these comprise an alteration in the levels of transaminases depending on time of exposure to alcohol. Parra-Vizuet *et al*[24] reported a diminution in the serum levels of AST and ALT 24 h after the administration of a unique dose of alcohol of 1.5 g/kg. On the other hand, Morales-González *et al*[15] and Ramírez-Farías *et al*[25] reported an increase in the serum levels of the transaminases (ALT, AST, LDH, GDH and OTC) 7 d after the administration of ethanol. Similarly, Morales-González *et al*[26] reported a diminution in the activity of transaminase enzymes in hepatic tissue 24 h after ethanol administration (5 g/kg). Rocha *et al*[13] reported a rise in ALT during weekend alcohol consumption (4.5 g/d). Our results demonstrate that weekend alcohol consumption produces an increase in the serum of transaminases (AST, ALT and LDH) regardless of gender or of the concentration of alcohol (Figures 1 and 2). Nonetheless, surprisingly, alcohol consumption does not affect the serum levels of GGT (Figure 2). On the other hand, Stranges *et al*[11] reported that the principal transaminase affected by alcohol consumption is GGT. We consider that, in our study, there was no elevation of GGT, due to that weekend alcohol consumption was of 12 wk, and probably, sufficiently severe damage did not exist for it to be reflected by alteration of GGT, or by the levels of albumin (Table 3). This coincides with that reported by Rocha *et al*[13] in terms of protein levels in blood not being affected on consuming alcohol.

It has been reported that ethanol favors hepatic steatosis, probably because of the increase of lipogenesis, diminution of lipid transport of the liver, alteration of oxygenation of fatty acids[27], and even infiltration of monocytes, macrophages, *etc*., which are fundamental for pathogenic activity after acute or chronic hepatic injury[27]. The latter can explain in part the histological findings encountered in our study, where fatty change as well as inflammation comprised the most important changes in weekend alcohol consumption in all of the groups (Table 4), probably due to that they are related with the acute damage caused by ethanol to the liver[16-24]. Likewise, it was reported that acute treatment with ethanol in rats induced hepatic steatosis accompanied by an increase in the production of neutral fats (triglycerides). Thus, the serum levels of the triglycerides may not only reflect the production of the liver, but also the equilibrium between the production

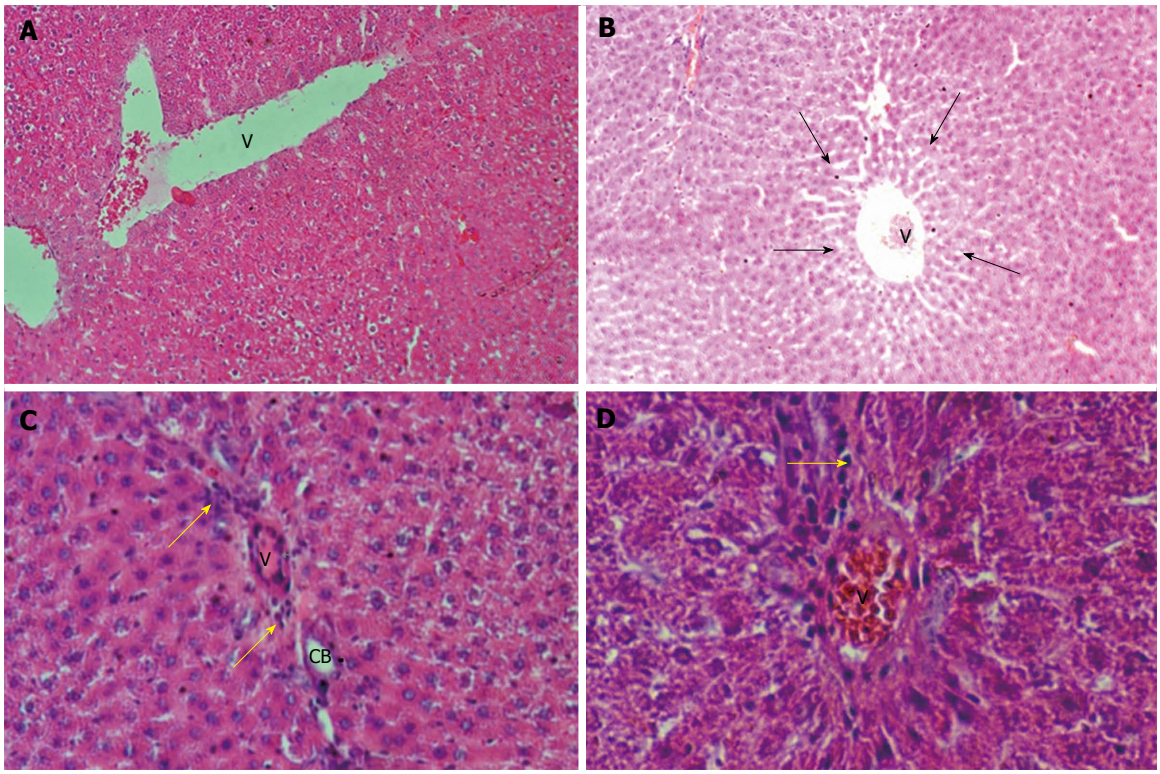

Figure 5 Hepatic histology of the group of females with weekend alcohol consumption at 40%. A: Image of the control group; B-D: Images of the group of females with alcohol consumption at 40%. A: The uniformity of the structures is observed to be conserved (40 ×); B: Periportal fibrosis, loss of cells around the portal vein (black arrows) (40 ×); C: Fine and thick steatosis and leukocytes in single file around the portal vein and some that emerge from the limiting plaque (yellow arrows) (60 ×); D: Both fine and thick steatosis and single-file leukocytes are observed (100 ×). V: Portal Vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

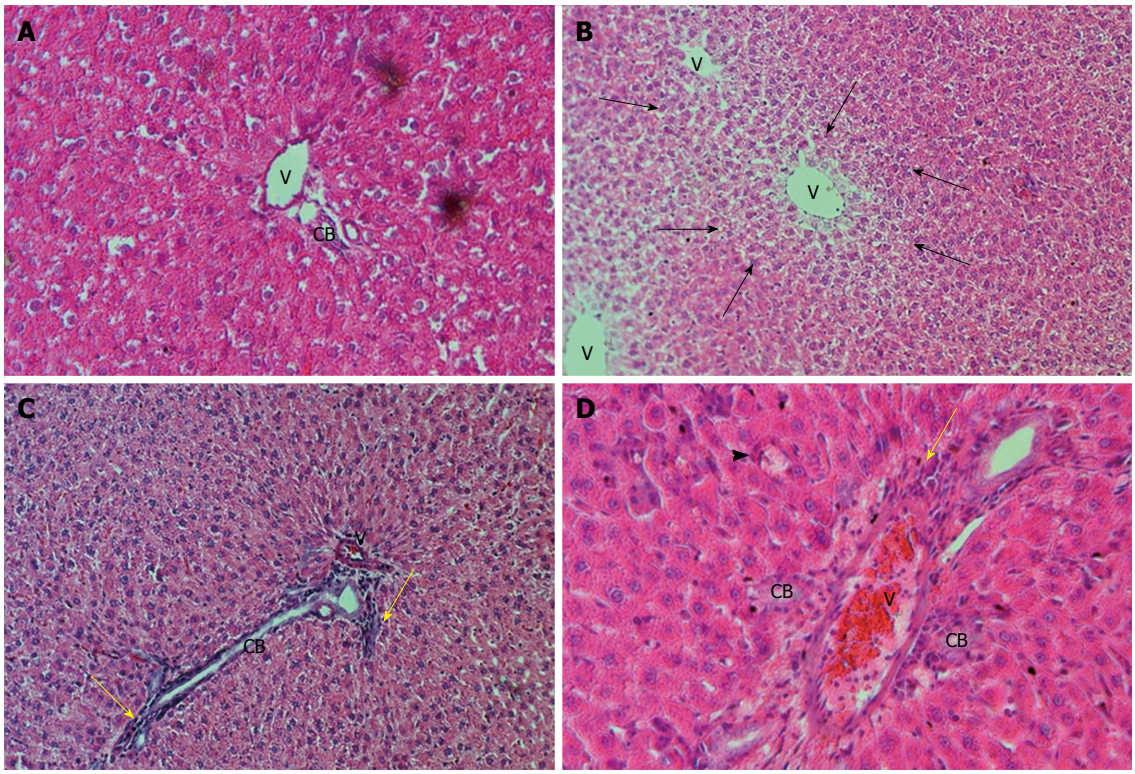

Figure 6 Hepatic histology of the group of males of 40%. A: Image of the control group; B-D: Images of the group of males with alcohol consumption at 40%. A: The conserved, uniformized structures of the form and size of the hepatocytes are observed (40 ×); B: Periportal fibrosis is observed (black arrows) (40 ×); C: Leukocytes are observed in a cited single file (yellow arrows) (40 ×); D: Fine and thick steatosis is observed (zone with less pigment inside the cellular cytoplasm of the hepatocyte), leukocytes (yellow arrow), and the apoptotic cell (point of the black arrow) (60 ×). V: Portal Vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

and utilization of neutral fats[28]. Our data reveal that weekend alcohol consumption produces fatty liver (Table 4) and the mobilization of neutral fats (Table 2), which is more evident when alcohol is consumed at 40%. Notwithstanding this, the group of males that consumed alcohol at 5%, a lesser amount than the groups at 40%, exhibited a pattern that was very similar in terms of levels of triglycerides and steatosis.

In conclusions, our findings demonstrate an effect of the damage that is caused by weekend alcohol consumption, regardless of gender or the concentration of the alcohol. Even more so, greater damage can be observed in females, and the metabolism of ethanol probably participates, specifically due to its first-pass metabolism, which is carried out in the stomach. Finally, the results obtained herein provide solid evidence that weekend alcohol consumption gives rise to liver damage, demonstrated by the biochemical and histological alterations, first manifested acutely, and their prolonged consumption can cause greater, irreversible damage.

ARTICLE HIGHLIGHTS

Research background

It is known that alcohol consumption is a risk factor for various diseases, specifically liver diseases. Due to that alcohol consumption, especially weekend alcohol consumption, has Increased, in recent years the effect has been studied of the damage that this pattern of alcohol consumption can cause to various organs, for example, to the heart and the liver. This study contributes, to our knowledge for the first time, solid evidences of the damage caused by weekend alcohol consumption to the liver, as well as of the relationship that gender and the concentration of alcohol maintain in generating liver damage.

Research motivation

The consumption of alcohol is a problem of prime magnitude at the worldwide level that gives rise to a great number of medical (gastritis, cirrhosis, cancer, infarcts, *etc.*), as well as non-medical problems (automobile accidents, homicides, absenteeism from work, *etc.*). Therefore, carrying out investigations to understand the mechanisms of cellular damage and identifying patterns of alcohol consumption that are on the rise, such as weekend alcohol consumption, and how these patterns come to damage the liver at different magnitudes, are of utmost importance.

Research objectives

The majority of studies that investigate alcohol-associated liver damage address chronic or acute alcohol consumption. Few experimental studies inquire into the liver damage that is caused by weekend alcohol consumption. To our knowledge, this is the first report that describes the histological alterations caused by weekend alcohol consumption. It is of utmost importance to ascertain the mechanisms of liver damage given rise to by weekend alcohol consumption, due to the growing number of young people who acquire this consumption pattern, and to find a therapeutic window.

Research methods

While the histological study is well known worldwide and has been employed in an infinite number of investigations, it can newly be a very important tool to find mechanisms of cellular damage caused by alcohol that are complemented with biochemical assays, in this manner taking the first steps in utilizing other investigative tools, such as electronic microscopy, molecular biology assays, *etc.* The most important and novel in this is the experimental design.

Research results

Histological and biochemical findings demonstrate that weekend alcohol consumption causes liver damage, irrespective of gender or the concentration of the alcohol. The contribution of this work resides in that a probable mechanism of damage to the liver due to weekend alcohol consumption comprises the metabolism of the first pass of the alcohol, which is carried out in the stomach. Lacking is the study in this model of variables such as age, food consumption, doses of alcohol, the consumption of antioxidants, *etc.*

Research conclusions

As conclusions of the investigation, this is the first report, to our knowledge, that describes the histological alterations caused by weekend alcohol consumption that, in addition to the biochemical assays, provides solid evidence on the damage caused by weekend alcohol consumption, which initially can be acute and reversible, but that probably can become irreversible. We employed a known technique, histology, but with the experimental design being novel. This type of study, in which the mechanisms of damage are investigated, can open a therapeutic window in future clinical practice.

Research perspectives

These perspectives include, in the first place, considering weekend alcohol consumption as a health problem of utmost importance, and even the same as chronic alcohol consumption. In second place, we conducted the investigation of the mechanisms of liver damage in terms of weekend alcohol consumption, and third, we found that this would be novel in the design of experimental models, in this manner utilizing techniques such as histology and biochemical assays, which comprise the first step in terms of orientation to the mechanisms of damage caused by alcohol, and these can be confirmed with molecular biology techniques. The questions to solve include knowing the following in terms of this model: How is the activity in the hepatic and gastric dehydrogenase alcohol enzyme found? Does the Nrf2 factor participate as cytoprotector?, and can the consumption of antioxidants prevent the alterations that weekend alcohol consumption cause?

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ORIGINAL ARTICLE

Retrospective Cohort Study

Survival outcomes of liver transplantation for hepatocellular carcinoma in patients with normal, high and very high preoperative alpha-fetoprotein levels

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Abstract

AIM

To investigate the impact of alpha-fetoprotein (AFP) on long-term recurrence rate and overall survival and we also aimed to define the level of AFP leading to a higher risk of disease recurrence and affecting patient survival.

METHODS

Data of adult patients who received liver transplant (LT) for hepatocellular carcinoma (HCC) at our hospital from January 2000 to December 2013 were reviewed. Reviewed data included demographic characteristics, preoperative AFP level, operative details, follow-up details, and survival outcomes. Patients were mostly listed for LT based on Milan or UCSF criteria. For the purpose of this study, normal AFP level was defined as AFP value < 10 ng/mL, high AFP level was defined as AFP value ⩾ 10 to < 400 ng/mL, and very high

AFP level was defined as AFP ⩾ 400 ng/mL. The patients were divided into these 3 groups accordingly. Survival rates were plotted as Kaplan-Meier curves and compared by log-rank analysis. Continuous variables were expressed as median (interquartile range). Categorical variables were compared by Spearman's test. Discriminative analysis was used to define the lowest value of AFP that could affect the overall survival in study population. Statistical significance was defined by a *P* value of < 0.05.

RESULTS

Totally 250 adult patients underwent LT for HCC in the study period. Eight-four of them received deceased-donor LT and 166 had living-donor LT. The patients were divided into 3 groups: Group A, AFP < 10 ng/mL (*n* = 83); Group B, AFP ⩾ 10 to < 400 ng/mL (*n* = 131); Group C, AFP ⩾ 400 ng/mL (*n* = 36). The commonest etiology was hepatitis-B-related cirrhosis. The Model for End-stage Liver Disease scores in these groups were similar (median, 13 *vs* 13 *vs* 12; *P* = 0.745). The time to operation in Group A was longer (median, 94 *vs* 31 *vs* 35 d; *P* = 0.001). The groups were similar in hospital mortality (*P* = 0.626) and postoperative complication (*P* = 0.702). Pathology of explants showed that the 3 groups had similar numbers of tumor nodules, but the tumors in Group C were larger (A: 2.5 cm, B: 3.0 cm, C: 4.0 cm; *P* = 0.003). Group C had a bigger proportion of patients who were beyond Milan criteria (*P* = 0.010). Poor differentiation and vascular permeation were also more common in this group (*P* = 0.017 and *P* = 0.003 respectively). It also had poorer 5-year survival (A: 85.5%, B: 82.4%, C: 66%; *P* = 0.029). The 5-year disease-free survival was 84.3% in Group A, 80.1% in Group B, and 61.1% in Group C. Receiver operating characteristic area under the curve for AFP in predicting tumor recurrence was 0.685. The selected cut-off value was 54 ng/mL for AFP (C-index 0.685; 95%CI: 0.592-0.779; sensitivity 0.595; specificity 0.687). On discriminative analysis, AFP value of 105 ng/mL was shown to affect the overall survival of the patients.

CONCLUSION

HCC patients with a high preoperative AFP level had inferior survival after LT. AFP level of 54 ng/mL was associated with disease recurrence, and AFP level of 105 ng/mL was found to be the cut-off value for overall survival difference.

Key words: Alpha-fetoprotein; Liver transplantation; Recurrence; Survival

Core tip: Various established criteria have been used to identify patients with hepatocellular carcinoma who would benefit from liver transplant with reasonable survival. Alpha-fetoprotein (AFP) level has been identified as an important factor associated with suboptimal survival with high recurrence rate. This study demonstrated that AFP level correlated well with the pathological findings of tumor differentiation and microvascular invasion, which are usually confirmed in explant pathology. In this set of data, AFP level of 54 ng/mL was associated with disease recurrence, and AFP level of 105 ng/mL was found to be the cut-off value for overall survival difference.

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INTRODUCTION

Liver transplant (LT) is the best treatment option for hepatocellular carcinoma (HCC) as it removes both the tumor and the cirrhotic liver. The Milan criteria have been well adopted worldwide as a set of guidelines for listing patients for LT. Patients within the Milan criteria have a 5-year post-LT survival of 65%-80%, with a recurrence risk of 8%-15%[1]. However, the Milan criteria are criticized for being too stringent, since many patients beyond the criteria could still have reasonable post-LT survival[2-8]. Therefore, in additional to morphological consideration of tumor, the adoption of biological markers such as alpha-fetoprotein (AFP), response to therapy and evolution after therapy[9] is advocated.

AFP has been used as a tumor marker for HCC, and a high AFP level has been shown to be associated with poorer outcomes[10,11]. In previous studies, suboptimal results with high recurrence rates were seen in patients who had received LT with an AFP level of > 1000 ng/mL[12-14]. Such a level is considered a contraindication to LT. This level is applied not only to extended criteria but also to patients within the Milan criteria. Unfortunately, the exact consensual cut-off value remains undefined.

In this study, we investigated the impact of AFP on long-term recurrence rate and overall survival. We also aimed to define the level of AFP leading to a higher risk of disease recurrence and affecting patient survival.

MATERIALS AND METHODS

Prospectively collected data of adult patients who received deceased-donor LT (DDLT) or living-donor LT (LDLT) for HCC at our hospital in the period from January 2000 to December 2013 were reviewed and analyzed. These data included demographic characteristics, preoperative AFP level, operative details, follow-up details, and survival outcomes. Institutional review board approval was not required for this study because it was a retrospective analysis of anonymous data. Patient treatments were not affected by this study.

Patient selection for LT

The strategies adopted for selection of patients with known HCC for LT have been described elsewhere[15,16]. In brief, tumor evaluation was done with computed tomography of the abdomen and thorax, in addition to radionuclide bone scan at initial diagnosis. In recent years, dual-tracer (11C-acetate and 18 F-fluoro-deoxyglucose) positron emission tomography (PET) was performed to exclude extrahepatic metastasis. Patients who were 65 years old or younger and not eligible for partial hepatectomy or local ablation were considered for LT. The age limit as a selection criterion was getting relatively loose as long as the patient was physically fit. The Milan criteria[1] and the UCSF criteria[14] were used for selection of patients for listing. Patients who had recurrent HCC after hepatectomy would still be considered for LT if their disease was still within selection criteria. There was no mandatory waiting period prior to LT, and bridging therapy with transarterial chemoembolization was offered to LT candidates with reasonable liver function. From October 2009 onwards, an arbitrary Model for end-stage liver disease (MELD) score of 18 points were given to DDLT candidates with HCC remaining at stage 2 six months after radiological confirmation of their stage-2 disease. Two MELD points were added every three months as long as their disease remained at stage 2 or below[17].

Patients who were beyond the Milan and UCSF criteria because they had slightly larger tumors or slightly more tumors were not eligible for DDLT but could be considered for LDLT if they had no portal or hepatic vein invasion.

Treatments

Surgery was performed using standard techniques. Cell-saver device was not used. Explants were examined by pathologists for tumor size and number, differentiation, and presence of microscopic vascular invasion. Tumors found on explant examination were regarded as incidental tumors. Neither medical nor radiation adjuvant treatment was given to any patient after LT. The patients were monitored regularly by measurement of serum AFP level, chest radiography and abdominal and chest computed tomography every 3 mo. Recurrences suspected on clinical grounds were confirmed by histological examination as far as possible.

Donor and recipient operations were performed as described elsewhere[18]. The decision to use left-lobe graft *vs* right-lobe graft was based on a number of donor and recipient factors, the most important of which were the ratio of graft weight to standard liver volume, the ratio of graft weight to recipient weight, MELD score, and donor liver anatomy. The Urata formula [Liver volume (mL) = Body surface area (m^2) × 706.2 + 2.4] was used to calculate standard liver volume[19]. Implantation process and techniques were similar for left and right lobe grafts[18,20]. The immunosuppression and prophylaxis regimens prescribed have been described earlier[21].

Statistical analysis

For the purpose of this study, normal AFP level was defined as AFP value < 10 ng/mL, high AFP level was defined as AFP value ⩾ 10 to < 400 ng/mL, and very high AFP level was defined as AFP ⩾ 400 ng/mL. The patients were divided into these 3 groups accordingly.

Receiver operating characteristic (ROC) analysis was used to evaluate the ability of AFP to predict postoperative recurrence and to choose the optimal cut-off value for subsequent analysis. For indication for LDLT, high specificity was essential for avoiding excluding a large number of patients who would not develop recurrence.

Clinical profiles and outcomes of patients were compared on the basis of AFP level. Comparisons were made on short- and long-term outcomes, including graft function, graft survival, patient survival, and incidence of biliary complication. Continuous variables were expressed as median (range), and the Mann-Whitney *U* test was used for subgroup comparison. Categorical variables were compared by χ^2 test or Fisher's exact test. The cumulative probability of recurrence and survival was estimated by the life-table method and compared by the log-rank test. Deaths from all causes were included in the calculation of survival. Patients without recurrence were regarded as censored observations in the calculation of cumulative recurrence rates. Variables related to graft, tumor and tumor treatment before LT were analyzed for prognostic significance. The Kaplan-Meier method was used for survival analysis and the log-rank test was used for survival comparison. Discriminative analysis was used to define the lowest value of AFP that could affect the overall survival in the study population. Statistical significance was defined by a *P* value of < 0.05. The computer software SPSS, version 20.0 (SPSS Inc., Chicago, IL, United States), was used for all statistical calculations.

RESULTS

From January 2000 to December 2013, 250 adult patients underwent LT for HCC. Eight-four of them received DDLT and 166 had LDLT. The patients were divided into 3 main groups according to their preoperative AFP level: Group A, AFP < 10 ng/mL (normal); Group B, AFP ⩾ 10 to < 400 ng/mL (high); Group C, AFP ⩾ 400 ng/mL (very high) (Table 1). Patients in Group C were significantly younger (*P* = 0.037). The 3 groups has similar distribution of sex (*P* = 0.492). The commonest etiology was hepatitis-B-related cirrhosis. The median MELD scores in the 3 groups were similar (*P* = 0.745). The median time to operation in Group A was significantly longer (*P* = 0.001).

There were no differences in terms of blood transfusion amount, operation time, cold ischemic time or warm ischemic time among the groups, suggesting that the operative procedures were similar in the groups. Moreover, no differences were found in intensive care

Table 1 Comparison of Group A, Group B and Group C

	Group A (n = 83)	Group B (n = 131)	Group C (n = 36)	P value
Age (yr)	56 (38-65)	55 (3-72)	51.5 (11-66)	0.037
Male/Female	67/16	113/18	29/7	0.492
Diagnosis:				
Cirrhosis				
Cryptogenic	2	2	1	
Hepatitis B	67	89	25	
Hepatitis C	4	22	3	
Alcoholic	0	1	1	
Hepatitis B + C	1	2	0	
Alcoholic + hepatitis C	1	0	1	
Alcoholic + hepatitis B	0	1	0	
Autoimmune	1	0	0	
Wilson's disease	0	0	0	
Preoperative MELD score	13 (6-35)	12 (6-35)	12 (8-43)	0.745
Waiting time (d)	94 (1-2735)	31 (1-1874)	35 (1-1473)	0.001
Blood transfusion (units)	4.2 (0-32)	2 (0-56)	4 (0-31)	0.128
Fresh frozen plasma transfusion (units)	8 (0-24)	6 (0-30)	6 (0-22)	0.609
Platelet transfusion (units)	8 (0-26)	6 (0-32)	8 (0-22)	0.978
Operation time (min)	650 (370-1105)	678 (333-1110)	707 (300-1273)	0.598
Cold ischemic time (min)	182 (62-652)	125 (60-633)	133 (70-500)	0.206
Warm ischemic time (min)	49.5 (25-102)	52 (26-108)	55.5 (30-93)	0.209
Hospital stay (d)	1.7 (8-132)	15 (0-83)	15 (7-47)	0.251
Intensive care unit stay (d)	3 (1-42)	3 (0-30)	3 (2-16)	0.283
Follow-up (mo)	82.4 (0.59-204.9)	89.1 (0-210.82)	68.2 (5.95-204.24)	0.242
Hospital mortality	2 (2.4%)	2 (1.5%)	0	0.626
LDLT:DDLT	45:38	93:38	28:8	0.012
Explant Milan Within:Beyond	56:23	84:46	15:21	0.010
Explant UCSF Within:Beyond	63:16	98:32	23:13	0.188
No. of tumor in explant	1 (1-multiple)	2 (1-multiple)	1 (1-20)	0.272
Largest size of tumor in explant (cm)	2.5 (0.90-7.00)	3.0 (0.25-9.00)	4.0 (1.5-19.5)	0.003
Differentiation:				0.017
Well	26	41	3	
Moderate	41	66	25	
Poor	2	8	6	
Undifferentiated	0	2	0	
Unknown	10	13	2	
Vascular permeation:				0.003
No	60	85	14	
Yes	18	40	21	
Unknown	1	5	1	
Graft loss	18 (21.7%)	28 (21.4%)	15 (41.7%)	0.033
Patient status Alive:Dead	65:18	104:27	21:15	0.027
Graft survival, yr				0.038
1	96.40%	93.10%	97.20%	
3	89.20%	84.70%	80.60%	
5	85.50%	81.60%	66.00%	
Patient survival, yr				0.029
1	96.40%	94.70%	97.20%	
3	89.20%	85.50%	80.60%	
5	85.50%	82.40%	66.00%	
Disease-free survival, yr				0.007
1	92.80%	89.30%	80.60%	
3	88.00%	81.70%	72.20%	
5	84.30%	80.10%	61.10%	
Postoperative early complication by Clavien grading:				0.702
No	40	68	20	
Ⅰ	19	24	9	
Ⅱ	5	13	2	
ⅢA	11	11	3	
ⅢB	6	6	2	
ⅣA	1	7	0	
ⅣB	0	0	0	
Ⅴ	1	2	0	

Group A-AFP < 10 ng/mL; Group B-AFP ⩾ 10 to < 400 ng/mL; Group C, AFP ⩾ 400 ng/mL. MELD: Model for end-stage liver disease; DDLT: Deceased-donor liver transplant; LDLT: Living-donor liver transplant.

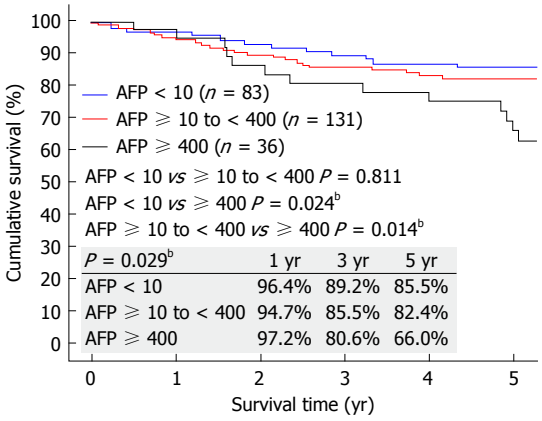

Figure 1 Overall survival of patients with preoperative AFP < 10 ng/mL, ⩾ 10 to < 400 ng/mL, and ⩾ 400 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

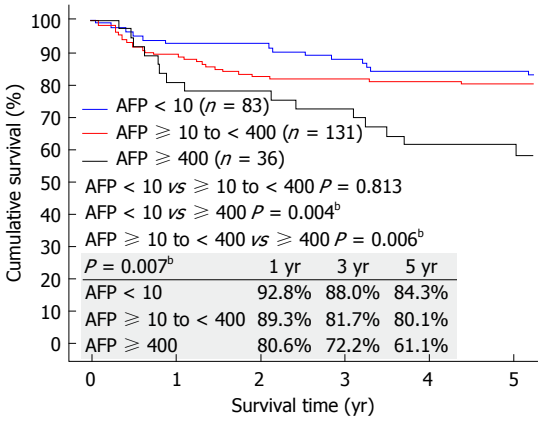

Figure 2 Disease-free survival of patients with preoperative AFP < 10 ng/mL, ⩾ 10 to < 400 ng/mL, and ⩾ 400 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

unit stay (*P* = 0.283), hospital stay (*P* = 0.251), hospital mortality (*P* = 0.626), or postoperative complication (*P* = 0.702). Pathology of explants showed that the 3 groups had similar numbers of tumor nodules but the tumors in Group C were larger (*P* = 0.003), and thus more patients in Group C were beyond the Milan criteria (*P* = 0.010). Furthermore, Group C had more cases of poor differentiation (*P* = 0.017) and vascular permeation (*P* = 0.003).

No patients were lost to follow-up in the study period. The 3 groups had similar follow-up period (*P* = 0.242). More patients in Group C had graft loss (*P* = 0.033), and hence this group had poorer 5-year graft survival (*P* = 0.038) and 5-year patient survival (*P* = 0.029) (Figure 1). Most patients died of recurrent HCC. The 5 year-disease-free survival was 84.3% in Group A, 80.1% in Group B, and 61.1% in Group C. Disease-free survival was similar in Groups A and B (*P* = 0.813) but significantly different between Groups A and C (*P* = 0.004) and between Groups B and C (*P* = 0.006) (Figure 2).

Disease recurrence and ROC curve analysis

Recurrence of HCC was identified in 42 patients (42/250 = 16.8%). The ability of preoperative AFP to predict

Area	Standard error	Asymptotic 95% confidence interval	
		Lower bound	Upper bound
0.685	0.048	0.592	0.779

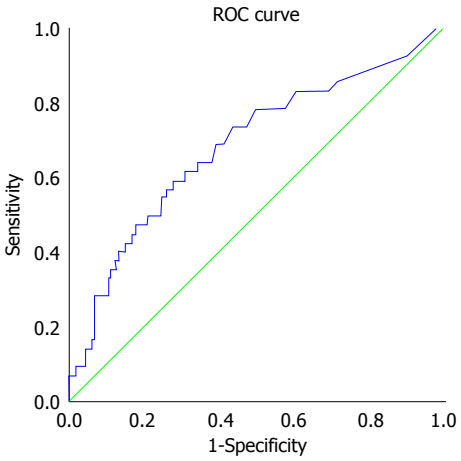

Figure 3 Receiver operating characteristic curve for alpha-fetoprotein in predicting hepatocellular carcinoma recurrence after liver transplant.

HCC recurrence was analyzed by ROC curve. Area under the curve for AFP was 0.685. Among the cut-off values with sufficient specificity, the cut-off point with the highest C-index was chosen as the optimal cut-off value for subsequent analysis. The selected cut-off value was 54 ng/mL for AFP (C-index 0.685; 95% confidence interval 0.592-0.779; sensitivity 0.595; specificity 0.687) (Figure 3).

Further analysis was performed to identify the lowest AFP level that could affect patient survival. On discriminative analysis, AFP value of 105 ng/mL was identified as the level that could affect the overall survival of the patients. Patients with AFP ⩽ 105 ng/mL (Group D) were compared with patients with AFP >105 ng/mL (Group E) (Table 2). Patients in Group E were younger (*P* = 0.017). When it comes to preoperative comorbidity, underlying cause of cirrhosis, MELD score, operative details, postoperative complication and hospital stay, no significant differences were seen. However, Group E had poorer 5-year graft survival (*P* = 0.024), patient survival (*P* = 0.045) (Figure 4), and disease-free survival (*P* = 0.006) (Figure 5). Looking into the details of the pathological results of the 2 groups, it was clear that the tumors in Group E had worse pathology. Group E had larger tumors (*P* = 0.017) and fewer cases of well differentiation (15.71% *vs* 32.78%; *P* = 0.001), while vascular permeation was more common in this group (44.29% *vs* 26.67%; *P* = 0.014) (Table 2).

DISCUSSION

AFP has been used as a tumor marker for HCC. Its elevation depends on pathological characteristics, including tumor size and degree of differentiation of tumor cells. It is a well-established surrogate of tumor biology, as it correlates with histological grading and

Table 2 Comparison of the two subgroups, Group D and Group E

	Group D (n = 180)	Group E (n = 70)	P value
Age (yr)	56 (38-67)	53.5 (3-72)	0.017
Male:Female	150:30	59:11	0.855
Diagnosis:			
Cirrhosis			
Cryptogenic	4	1	
Hepatitis B	136	45	
Hepatitis C	19	10	
Alcoholic	1	1	
Hepatitis B + C	1	2	
Alcoholic + hepatitis C	1	1	
Alcoholic + hepatitis B	1	0	
Autoimmune	1	0	
Wilson's disease	0	0	
Preoperative MELD score	12 (6-35)	12 (6-43)	0.972
Waiting time (d)	58.5 (1-2735)	32 (1-1874)	0.183
Blood transfusion (units)	3 (0-56)	4(0-32)	0.988
Fresh frozen plasma transfusion (units)	6 (0-30)	6 (0-22)	0.798
Platelet transfusion (units)	6 (0-30)	8 (0-32)	0.708
Operation time (min)	654 (333-1110)	716.5 (300-1273)	0.151
Cold ischemic time (min)	137.5 (60-652)	127.5 (66-633)	0.195
Warm ischemic time (min)	51 (25-108)	53 (28-93)	0.308
Hospital stay (d)	15.5 (0-132)	16 (7-48)	0.497
Intensive care unit stay (d)	3 (0-42)	3 (2-30)	0.806
Follow-up (mo)	87.2 (0.0-210.8)	75.4 (3.8-206.8)	0.173
Hospital mortality	4 (2.2%)	0	0.486
LDLT: DDLT	112:68	54:16	0.025
Explant Milan Within:Beyond	116:60	39:30	0.170
Explant UCSF Within:Beyond	135:41	49:20	0.354
No. of tumor in explant	1.5 (1-multiple)	1 (1-20)	0.551
Largest size of tumor in explant (cm)	2.85 (0.90-7.00)	3.5 (0.25-19.5)	0.017
Differentiation			0.0001
Well	59	11	
Moderate	91	41	
Poor	6	10	
Undifferentiated	0	2	
Unknown	20	5	
Vascular permeation			0.014
No	124	35	
Yes	48	31	
Unknown	4	3	
Graft loss	37 (20.6%)	24 (34.3%)	0.023
Patient status Alive:Dead	143:37	47:23	0.041
Graft survival, yr			0.024
1	95.00%	94.30%	
3	87.20%	81.40%	
5	83.80%	72.30%	
Patient survival, yr			0.045
1	95.60%	95.70%	
3	87.20%	82.90%	
5	83.80%	73.80%	
Disease-free survival, yr			0.006
1	91.10%	84.30%	
3	85.00%	75.70%	
5	82.20%	70.00%	
Postoperative early complication by Clavien grading			0.798
No	90	38	
Ⅰ	35	17	
Ⅱ	16	4	
ⅢA	19	6	
ⅢB	11	3	
ⅣA	6	2	
ⅣB	0	0	
Ⅴ	3	0	

Group D-AFP ⩽ 105 ng/mL; Group E-AFP >105 ng/mL. MELD: Model for end-stage liver disease; DDLT: Deceased-donor liver transplant; LDLT: Living-donor liver transplant.

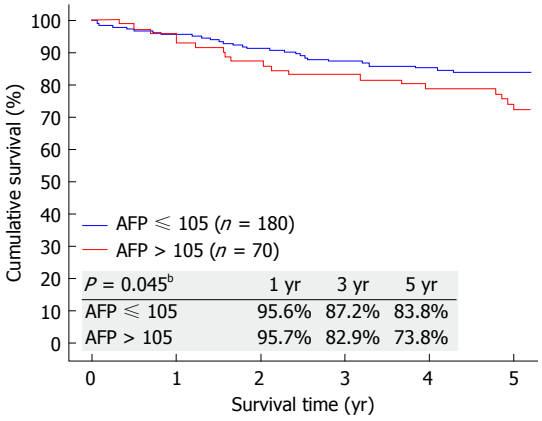

Figure 4 Overall survival of patients with preoperative alpha-fetoprotein ⩽ 105 ng/mL and > 105 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

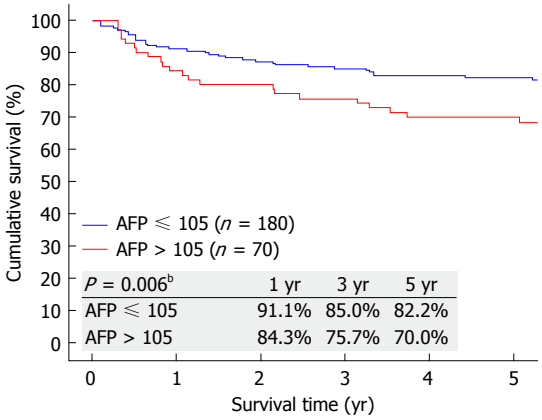

Figure 5 Disease-free survival of patients with preoperative alpha-fetoprotein ⩽ 105 ng/mL and > 105 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

vascular invasion[12,13,22,23]. The presence of microvascular invasion and poor differentiation of tumor cells are associated with recurrence in patients within and beyond various transplant criteria[24-29]. However, most of the time, preoperative histological results are not available, and therefore prediction of disease recurrence and overall survival cannot be made preoperatively.

AFP is an oncogene protein produced by HCC. Currently, an AFP level of > 400 ng/mL together with a liver mass with characteristic features is a diagnostic feature of HCC. Serum AFP is a well-established prognostic marker of increased tumor virulence in HCC[12,13,30-35]. It has also been shown to be associated with increased risk of waitlist dropout[13,36,37] and post-LT recurrence[12,30-35,38-40]. AFP level has been integrated into a number of transplant criteria, including the Hangzhou criteria[41], the extended Toronto criteria[42], the "total tumor volume"[43], and the Kyoto criteria[44].

Regarding LT for HCC, disease recurrence is a major concern. Extrahepatic metastasis is a clear contraindication to LT as it represents systemic disease, which cannot be cured by LT. The presence of macrovascular invasion has been shown to be an independent risk factor for recurrence and associated with worsened survival[25,45], and therefore is considered a contraindication to LT in the Milan, UCSF and "up-to-seven" criteria[1,5,14]. Poor tumor biology (poor cellular differentiation and presence of microvascular invasion) is associated with an increased risk of tumor recurrence. However, most of the time, the tumor biology cannot be known before operation. Liver biopsy of the target lesion can be an important tool for identifying tumor differentiation and microvascular invasion. It has been proposed that liver biopsy should be included into the Toronto criteria for any number and any size of HCC lesion[42]. While AFP is known to be a well-established surrogate of tumor biology for its correlation with histological grading and vascular invasion[12,13,22,23], it has been used for risk stratification, with elevation of AFP associated with a higher incidence of disease recurrence. Unfortunately, there is no exact cut-off value as an absolute value for contraindication to LT.

AFP is associated with vascular invasion and intrahepatic metastasis is not expressed in well-differentiated HCC[46]. AFP is considered an independent prognostic factor which correlates with histological differentiation[12,47]. This was also reflected in our patients. In Group A, the median time to operation was longer and the outcome was better, suggesting that this group had more favorable tumor biology. Microvascular invasion has been proven to be a strong predictor of outcome (liver resection or LT) for HCC patients[48-51]. Unfortunately, tumor differentiation and microvascular status require histological proof, and most of the time they can only be known after operation. Our patients were divided into 3 main groups, with normal, high and very high preoperative AFP levels. Results showed that higher AFP level was associated with tumor recurrence and correlated with tumor differentiation and microvascular invasion. It was apparent that when the AFP value was lower, the chance of poor differentiation and vascular permeation was also lower. However, this could have been affected by the fact that Group C had more patients beyond the Milan criteria. ROC analysis was performed to assess the ability of preoperative AFP in predicting HCC recurrence after LT. According to C-index analysis based on ROC, the optimal cut-off value was set at 54 ng/mL. However, on further discriminative analysis, 105 ng/mL was set as the cut-off value and it demonstrated significant survival difference despite same amounts of patients who were within the Milan and UCSF criteria. This suggested the importance of using AFP as one of the preoperative surrogate markers to evaluate LT candidates, as it represents additional information on identifying high-risk patients preoperatively so as to predict the risk of recurrence and to let patients have realistic anticipation regarding their long-term outcomes.

HCC patients within the Milan criteria consistently have a 10%-15% risk of disease recurrence after LT[1]. However, strictly following the rules would turn down a substantial number of patients who could benefit from LT and get a cure, even with reasonable disease-

free and overall survival. Therefore, allowance is given mainly based on tumor number and size[5,35,52]. It is hoped that further developed selection tools with expanded criteria can identify patients with low risk of recurrence. Applying AFP cut-off could have resulted in the exclusion of certain patients who could have reasonable survival but were at higher risk of tumor recurrence as compared with patients with a lower AFP level. However, using AFP alone to predict the subsequent disease course would result in bias in selection of patients for LT, since patients with the same AFP level can have similar disease-free survival as well as overall survival. Therefore, in listing patients for LT, other factors should also be considered, such as established criteria and tumor status assessed by PET. Fluorine-18-fluorodeoxyglucose PET is able to pick up poorly differentiated HCC[53], while 11C-choline PET has strong avidity for HCC, particularly well differentiated and moderately differentiated tumors[54-56]. Both ways provide preoperative information without the need for biopsy of the liver tumor. For patients who have poor liver function that precludes liver resection, ablative therapy and transarterial chemoembolization, palliative treatment would be the only option if they are denied LT. For these patients, LT should not be ruled out if a relatively inferior survival outcome is acceptable to them. Since LDLT is almost exclusively performed among family members, oftentimes they would accept a relatively inferior survival outcome of the recipient, given a reasonable donor operative outcome.

The findings of this study may not be universally applicable, as AFP 105 ng/mL is a value calculated from our cohort of 250 patients. Moreover, this is a retrospective cohort study with inevitable selection bias. Furthermore, the different levels of AFP (normal, high and very high) were arbitrarily defined. Before adopting AFP as a decision-making tool based on current selection criteria, we have to balance the risk of disease recurrence (hence overall survival) and the patients' expectation. Still, it is hoped that this study can shed some light on the importance of adding AFP to the armamentarium of assessment tools for LT listing.

HCC patients with a high preoperative AFP level had inferior survival after LT. AFP level of 54 ng/mL was associated with disease recurrence, and AFP level of 105 ng/mL was found to be the cut-off value for overall survival difference.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation is the best treatment option for hepatocellular carcinoma. However, only patients' tumor criteria should fit the current adopted selection criteria. Most of the criteria are morphological descriptions, including size and number, with the recently added alpha-fetoprotein in some of the updated criteria.

Research motivation

We hoped to identify the cutoff value of alpha-fetoprotein in predicting disease recurrence and overall survival. Apart from using size and number as the selection criteria for liver transplantation, the additional use of alpha-fetoprotein might be able to give practical prediction of disease recurrence.

Research objectives

The objective of this study is to investigate the impact of alpha-fetoprotein on the long-term recurrence rate and overall survival of recipients of liver transplantation for hepatocellular carcinoma.

Research methods

Data of adult patients who received liver transplantation for hepatocellular carcinoma at our hospital from January 2000 to December 2013 were reviewed. Data of included patients were analyzed. We defined the different levels of alpha-fetoprotein as normal (< 10 ng/mL), high (≥ 10 to < 400 ng/mL) and very high (≥ 400 ng/mL). The patients were divided into these 3 groups accordingly. Group comparison was then made.

Research results

Alpha-fetoprotein level was normal in 83 patients, high in 131 patients, and very high in 36 patients. The commonest etiology was hepatitis-B-related cirrhosis. The Model for End-stage Liver Disease scores in these groups were similar (median, 13 *vs* 13 *vs* 12; $P = 0.745$). Patients with normal alpha-fetoprotein level had longer time to operation (median, 94 *vs* 31 *vs* 35 d; $P = 0.001$). The groups were similar in hospital mortality ($P = 0.626$) and postoperative complication ($P = 0.702$). Pathology of explants showed that the 3 groups had similar numbers of tumor nodules, but patients with very high alpha-fetoprotein level had bigger tumors ($P = 0.003$). This group also had a bigger proportion of patients who were beyond Milan criteria ($P = 0.010$). Poor differentiation and vascular permeation were commoner in this group ($P = 0.017$ and $P = 0.003$ respectively). It also had poorer 5-year overall survival ($P = 0.029$) and disease-free survival ($P = 0.007$). Receiver operating characteristic area under the curve for alpha-fetoprotein in predicting tumor recurrence was 0.685. The selected cut-off value was 54 ng/mL (C-index 0.685; 95%CI: 0.592-0.779; sensitivity 0.595; specificity 0.687). On discriminative analysis, alpha-fetoprotein value of 105 ng/mL was shown to affect the overall survival of the patients.

Research conclusions

This study showed that patients with high preoperative alpha-fetoprotein levels had poorer post-transplant survival. An alpha-fetoprotein level of 54 ng/mL was associated with disease recurrence, and 105 ng/mL was found to be the cutoff value for overall survival difference. These findings would be useful when considering liver transplantation for patients with a high alpha-fetoprotein level. Currently, there is no definite cutoff value of alpha-fetoprotein for ideal oncological outcomes in hepatocellular carcinoma. With the above alpha-fetoprotein values, superior long-term disease-free and overall survival will be achievable if liver transplantation is offered to patients with a lower preoperative alpha-fetoprotein level. The additional use of alpha-fetoprotein will allow better prediction of the long-term survival outcome, and hence affect the future practice in selection of patients for liver transplantation.

Research perspectives

Selection of patients for liver transplantation should not be based on morphological criteria alone. Other biomarkers such as alpha-fetoprotein should be added to the criteria currently used.

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ORIGINAL ARTICLE

Clinical Practice Study

Hepatitis C virus knowledge improves hepatitis C virus screening practices among primary care physicians

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Informed consent statement: The HSIRB considered the return of the anonymous survey as deemed voluntary consent to participate in the study and therefore did not require an individual consent form from each participant.

Conflict-of-interest statement: Martinez AD has served as a speaker, a consultant and an advisory board member for Gilead, Intercept, Salix, Bayer, BMS, and Abbvie, and has received research funding from Abbvie, Gilead, Merck, Tobira, and Intercept; Talal AH has served as a speaker, a consultant and an advisory board member for Abbott Laboratories, Merck, and Abbvie, and has received research funding from Merck, Gilead, Abbott Laboratories, Abbvie, Intercept and Conatus; None of the other authors have any financial disclosures.

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Abstract

AIM

To understand the role of knowledge as a promoter of hepatitis C virus (HCV) screening among primary care physicians (PCP).

METHODS

A 45-item online questionnaire assessing knowledge of HCV natural history, risk factors, and treatment was distributed to 163 PCP. Logistic regression, adjusted for survey responses, assessed associations between PCP knowledge of HCV natural history and treatment and birth cohort (*i.e.*, birth between 1945 and 1965) screening. Response stratification and weighting were used to account for nonresponse and to permit extension of responses to the entire survey population. Associations between various predictors including demographic characteristics, level of training, and HCV treatment experience and HCV knowledge were assessed.

RESULTS

Ninety-one individuals (55.8%) responded. Abnormal liver enzymes (49.4%), assessment of HCV-related risk factors (30.6%), and birth cohort membership (20%) were the leading HCV screening indications. Most PCP (64.7%) felt that the combination of risk-factor and birth cohort screening utilizing a self-administered survey while awaiting the physician (55.3%) were the most efficient screening practices. Implementation of birth cohort screening was associated with awareness of the recommendations (*P*-value = 0.01), knowledge of HCV natural history (*P*-value < 0.01), and prior management of HCV patients (*P*-value < 0.01). PCP with knowledge of HCV treatment was also knowledgeable about HCV natural history (*P*-value < 0.01). Similarly, awareness of age-based screening recommendations was associated with HCV treatment knowledge (*P*-value = 0.03).

CONCLUSION

Comprehensive knowledge of HCV is critical to motivate HCV screening. PCP-targeted educational interventions are required to expand the HCV workforce and linkage-to-care opportunities as we seek global HCV eradication.

Key words: Viral hepatitis; Hepatitis C virus global eradication; Hepatitis C virus diagnosis; Hepatitis C virus surveillance; Knowledge of hepatitis C virus

Core tip: Many hepatitis C virus (HCV)-infected patients worldwide are unaware of their infection status. The key to increasing HCV detection and linkage-to-care is augmentation of virus screening by primary care physicians (PCP). Understanding factors that promote HCV screening among PCP is crucial to its eradication. We assessed PCP knowledge of HCV natural history and treatment and awareness of screening recommendations. PCP knowledge of HCV natural history and prior management of HCV patients were important predictors of implementation of HCV screening. Comprehensive HCV education targeted to PCP, including screening recommendations, is critical to increase HCV detection and linkage-to-care to obtain global eradication.

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INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of cirrhosis that can ultimately result in end-stage liver disease, hepatocellular carcinoma[1], and liver transplantation[2,3]. An estimated 5.2 million individuals in the United States are HCV-infected[4]. HCV mortality continues to increase, and it now surpasses the combined mortality of 60 notifiable infectious diseases[5]. Simultaneously, direct acting antivirals (DAAs) have improved HCV treatment tremendously; all oral, highly efficacious agents with minimal side effects and short treatment duration. HCV elimination in the United States, an often cited goal, is substantially hampered since 40% of HCV-infected patients are unaware of their infection status[6], and the 20000 hepatologists and infectious diseases physicians[7] in the United States are insufficient to care for up to 5 million HCV-infected individuals[4].

Historically, primary care physicians (PCP) had a role in HCV detection and counseling, but HCV treatment was considered beyond their practice scope[8], a notion that has recently been challenged[9,10]. Many PCP, the gatekeepers of the healthcare system, have limited HCV knowledge[11,12]. A systematic review identified significant knowledge gaps among PCP related to HCV natural history, diagnostic approaches, and treatment[12]. The rapid change in the HCV therapeutic landscape has only magnified the need for HCV education. Indeed, a recent survey indicated that the vast majority (84%) of PCP desired additional HCV training[13]. Delivering HCV education to resident physicians may effectively increase HCV knowledge among PCP. Indeed, most Family Medicine residency training program directors believe that chronic HCV is a significant primary care problem and PCP should be involved in building capacity for HCV management[14]. PCP education on HCV natural history and treatment has also been shown to expedite HCV treatment, adherence, and viral eradication[15,16].

Strategies have also been implemented to increase identification of HCV-infected individuals who remain undiagnosed. In 2012, the Centers for Disease Control and Prevention (CDC) and the US Preventive Services Task Force (USPSTF) promoted the recommendation that all individuals born between 1945 and 1965 (*i.e.,* birth cohort) should have a one-time HCV screening test[17,18], since 75% of undiagnosed HCV–infected individuals are birth cohort members[19]. Unfortunately, however, limited implementation of birth cohort

screening has diminished its originally anticipated impact with screening rates that are substantially reduced compared to those originally proposed and that vary widely from institution to institution[20,21]. Limited PCP knowledge of birth cohort screening recommendations may partially account for diminished impact. Indeed, systematic reviews have established the pre-eminent role of provider education in successful implementation of quality of care clinical guidelines[22]. Education should target uncertainties in physician knowledge and should be modified over time in order to ensure continued guideline application[23].

In consideration of PCP's expanding role in HCV treatment[9,10,14] combined with the need for knowledge on HCV natural history, screening, and treatment[12], we assessed knowledge of HCV natural history and treatment on implementation of birth cohort screening recommendations. We surveyed 91 PCP affiliated with an academic medical center. We sought to provide insight into topics for provider education, particularly to physicians in training, as these are important considerations in order to expand the HCV workforce.

MATERIALS AND METHODS

Study population and eligibility

The University at Buffalo (UB)-affiliated primary care clinics is a network of 9 clinics catering to urban and suburban patients in and around the City of Buffalo, New York. Eligible participants were PCP working in UB-affiliated clinics as supervising physicians or residents and who had experience with HCV treatment. Participant's scope of practice was General Internal Medicine, Family Medicine or a related combination of the two in such disciplines as Pediatrics or Social and Preventive Medicine. Medical students and physician extenders (*i.e.*, nurse practitioners or physician assistants) were excluded from the study. The study was deemed exempt from review by UB's Health Sciences Institutional Review Board, and it considered the return of the anonymous survey as deemed voluntary consent.

Hypothesis, questionnaire development and administration

The primary objective of this study was to evaluate the association of PCP knowledge of natural history and treatment of HCV on the implementation of birth cohort-based HCV screening. As a secondary objective, we sought to evaluate PCP related factors that could influence implementation of birth cohort-based HCV screening. We hypothesized that PCP with greater knowledge of HCV natural history and treatment would be more likely to implement birth cohort-based screening for HCV. The study design was a prospective questionnaire-based single-site study. Over a six month period, eligible PCP were distributed an anonymous, web-based 45 question survey that contained 18 knowledge questions that assessed HCV natural history and 19 that assessed knowledge of HCV treatment. Survey completion took approximately 30 min. Physicians who did not respond to the initial request or who partially completed the initial survey were sent follow up completion reminders weekly. No gifts or incentives were offered for survey completion. The survey instrument also inquired about general information concerning PCP practice locations and specialties.

Testing for internal validity and data analysis

After initial questionnaire development by subject matter experts, the survey was pretested among 5 providers. Based upon responses received, changes were made to the survey lay-out and format. The final version of the questionnaire was then distributed for completion. The internal validity of the survey was evaluated by including questions with similar meaning and by checking for agreement in the responses. We found that agreement between questions was moderate (kappa statistic estimate: 0.536; $P < 0.01$).

Statistical analysis was performed using R (http://www.r-project.org/). Categorical variables are summarized as counts and/or percentages, while continuous variables are summarized by their mean/median and standard deviation/interquartile range, as appropriate. Kappa statistic was used to evaluate agreement between paired dichotomous data. Knowledge of HCV natural history and treatment were evaluated as the number of correctly answered questions. To estimate the density of the scores, we used kernel density estimation methods with a Gaussian kernel and the corresponding bandwidth parameter was automatically selected *via* the R function "density". Logistic regression was used to assess the effect of patients' characteristics on the birth cohort based screening. Linear regression was used to evaluate the effect of patient characteristics on the knowledge of HCV natural history and treatment. Post-stratification was used to compensate for the fact that physicians with certain characteristics are not as likely to respond to the survey. We use weighting to adjust the regression results with weights being the percentages of the levels of the variable "primary care location" (the response rates of the three levels of this variable are significantly different) thereby extending the results from the responders to the entire population. In linear regression, Box-Cox transformation was used to achieve normality of knowledge of HCV natural history and treatment. The significance level in all tests (2-sided) was set to = 0.05. Predictors that were evaluated for HCV knowledge (both for natural history and treatment) were gender, prior experience in evaluating patients with HCV infection (*i.e.*, at least one HCV-infected patient evaluated in the past two years), clinical practice locations and level of medical training among those currently in medical training.

RESULTS

Study participants

A total of 163 surveys were distributed to PCP who were randomly selected from the population satisfying the

Table 1 Information about the entire population invited to complete the survey (*n* = 163)

Variable	Total	Level	*n*	Percent
Completed survey	163	No	72	44.2
		Yes	91	55.8
Gender	163	Female	80	49.1
		Male	83	50.9
Primary practice location	163	Buffalo general medical center	33	20.3
		Erie county medical center	56	34.4
		Others	74	45.4
Role in primary care clinic	163	Resident in training	134	82.2
		Supervising physician/attending	29	17.8
Level of training[1]	143	Resident PGY1	48	33.6
		Resident PGY2	44	30.8
		Resident PGY3	40	28.0
		Resident PGY4 and above	11	7.7

[1]Twenty subjects had missing values the variable "level of training". PGY: Post-graduate year.

Table 2 Baseline characteristics of those individuals who responded to the survey (*n* = 91) from among the entire population invited to complete the survey (*n* = 163)

Variable	Total[1]	Level	Count/Mean	Percent/SD
Gender	91	Male	50	55.0
		Female	41	45.1
Specialty of practice	91	Family medicine	9	9.9
		Others	82	90.1
Primary practice location	91	Erie county medical center	36	39.6
		Buffalo general medical center	29	31.9
		Others	26	28.6
Evaluated at least one HCV patient in past 2 yr	90	Yes	47	52.2
		No	31	34.4
		Not Sure	12	13.3
Role in primary care clinic	91	Supervising physician/attending	15	16.5
		Resident in training	76	83.5
Level of training	85	Resident PGY1	25	29.4
		Resident PGY2	30	35.3
		Resident PGY3 or above	30	35.3
Awareness of age-based rule for screening	85	Yes	49	57.6
		No	36	42.4
Implementation of age-based rule for screening	85	Yes	34	40.0
		No	51	60.0
Knowledge of HCV natural history	85	Scores from 0 to 18	10.6	4.7
Knowledge of HCV treatment	82	Scores from 0 to 19	11.0	2.9

[1]Missing values account for difference between number of responses recorded and the total number of survey respondents (*n* = 91). HCV: Hepatitis C virus; PGY: Post-graduate year.

eligibility criteria, and 91 (55.8%) responded. Baseline characteristics extrapolated to the entire population to whom the survey was distributed and those of the responders are illustrated (Tables 1 and 2, respectively). The survey was distributed to an approximately equal percentage of males and females, most of whom were in training (82.2%), and who had their primary practice location at one of the two principal UB-affiliated hospitals. Among the respondents, 54.9% were male, 45.1% female and 90.1% practiced internal medicine or its combined tracks. Residents in training comprised of 83.5% of the respondents, and the remaining were attending/supervising physicians. Practice location was predominantly a university-affiliated county hospital-based primary care clinic (39.6%). Electronic medical charting was used by 80.2% of the respondents.

PCP knowledge of HCV natural history and treatment

The distribution of scores indicating the number of correctly answered questions associated with the 18 items that assessed PCP knowledge of HCV natural history is illustrated in Supplementary Figure 1. Figure 1A illustrates the corresponding kernel density of the scores computed using a Gaussian kernel and a bandwidth parameter equal to 1.405. The density plot indicates that knowledge of HCV natural history is spread among three groups: (1) A group with "low" knowledge of HCV natural history; (2) a group constituting the majority of respondents has "moderate" knowledge, and (3) a smaller group of PCP with high knowledge.

The distribution of the scores associated with the 19 questions that assessed PCP knowledge of HCV

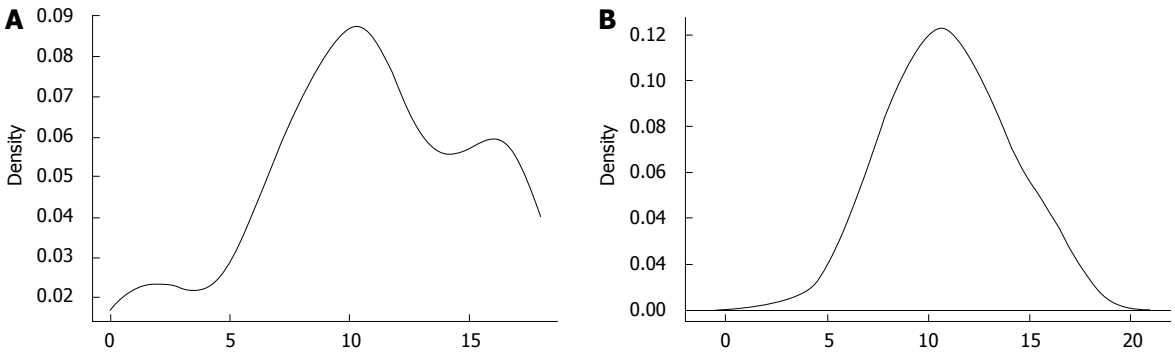

Figure 1 Distribution of responses for knowledge of hepatitis C virus natural history and treatment. A: Plot of the density of scores for the variable "Knowledge of hepatitis C virus (HCV) Natural History". The density estimation uses a Gaussian kernel with bandwidth 1.405. The data illustrate that HCV natural history knowledge is spread among three separate groups: those with low knowledge, the majority that has moderate knowledge, and a smaller group with vast knowledge; B: Plot of the density of scores for the variable "Knowledge of HCV Treatment". The density estimation uses a Gaussian Kernel with bandwidth 1.394. The plot illustrates the distribution of scores for primary care physicians (PCP) knowledge of HCV treatment. Out of a total of 19 possible points, most PCP knowledge scores were greater than 10 with knowledge symmetrically distributed around a score of 11.

Table 3 Regression analysis for Box-Cox transformed knowledge of hepatitis C virus natural history

Variable	Level	Estimation	SD	*P* value
Intercept		-24.90	1.39	0.001
Knowledge of HCV treatment		5.93	0.10	0.001
Gender	Male		Reference level	
	Female	0.04	0.54	0.94
Primary practice location	Erie county medical center		Reference level	
	Buffalo general medical center	0.54	0.77	0.49
	Others	-0.58	0.61	0.35
At least one HCV patient in past 2 yr	Yes		Reference level	
	No/not Sure	-0.54	0.65	0.41
Level of training	Resident PGY 1		Reference level	
	Resident PGY 2	-0.98	0.83	0.24
	Resident PGY 3 and above	0.06	0.78	0.94
Awareness of age-based rule for screening	Yes		Reference level	
	No	-0.92	0.65	0.17
Implementation of age-based rule for screening	Yes		Reference level	
	No	-0.07	0.65	0.91

The table illustrates those factors significantly associated with knowledge of HCV natural history. HCV: Hepatitis C virus; PGY: Post-graduate year.

treatment is illustrated in Supplementary Figure 2. Scores are calculated as previously, that is, each question that is answered correctly receives 1 point, for a total of 19 points. In contrast to the results obtained for HCV natural history knowledge, using a Gaussian kernel with bandwidth parameter 1.394, the corresponding density plot (Figure 1B) illustrates that most PCP have knowledge scores greater than 10 with general knowledge symmetrically distributed around a score of 11.

Predictors of PCP knowledge of HCV natural history and treatment: We next utilized linear regression to evaluate the association between various predictors and two principle outcome measures, knowledge of HCV natural history and of HCV treatment. Both outcome variables are represented by the total scores obtained in answering the relevant questions and are of interval scale. To satisfy the assumption of normality, we use the Box-Cox transformation with $\lambda = 1.116$ for the scores corresponding to knowledge of HCV treatment and with $\lambda = 1.75$ for the scores corresponding to knowledge of HCV natural history. Further, the percentages from the primary care location variable corresponding to the population surveyed (20.25%, 34.35%, and 45.40%) are treated as weights for the observations. In this way, we adjust the regression results to also include those who did not respond to the survey.

We found that knowledge of HCV natural history is significantly associated with knowledge of HCV treatment (*P*-value < 0.01), after adjusting for all relevant predictors (Table 3). That is, PCP with higher knowledge of HCV treatment tended to have higher knowledge of HCV natural history. Also, we found that knowledge of HCV treatment is significantly associated with awareness of the age-based screening recommendation (*P*-value = 0.03, Table 4), indicating that PCP who are aware of the age-based screening recommendation for HCV tend to have higher knowledge of HCV treatment. These findings suggest that knowledge of HCV natural history is a prerequisite for knowledge about HCV treatment. Similarly, those who were aware of age-based HCV screening recommendations also

Table 4 Regression analysis for Box-Cox transformed knowledge of hepatitis C virus treatment

Variable	Level	Estimation	SD	*P* value
Intercept		11.98	2.12	0.001
Knowledge of HCV natural history	score < 6		Reference level	
	6 ⩽ score < 15	-0.68	1.38	0.62
	15 ⩽ score	-2.42	1.64	0.14
Gender	Male		Reference level	
	Female	0.31	0.86	0.72
Primary practice location	Erie county medical center		Reference level	
	Buffalo general medical center	0.27	1.20	0.82
	Others	-1.44	0.95	0.13
At least one HCV patient in past 2 yr	Yes		Reference level	
	No/not sure	0.88	1.02	0.39
Level of training	Resident PGY 1		Reference level	
	Resident PGY 2	1.51	1.30	0.25
	Resident PGY 3 and above	1.76	1.25	0.16
Awareness of age-based rule for screening	Yes		Reference level	
	No	-2.21	1.00	0.03
Implementation of age-based rule for screening	Yes		Reference level	
	No	1.01	1.01	0.32

The table illustrates those factors significantly associated with knowledge of HCV treatment. HCV: Hepatitis C virus; PGY: Post-graduate year.

Table 5 Regression analysis for implementation of birth cohort screening recommendations

Variable	Level	Estimate	SD	*P* value
Intercept		-4.48	2.19	0.04
Knowledge of HCV treatment		-0.02	0.11	0.86
Knowledge of HCV natural history		0.27	0.08	0.002
Gender	Male		Reference level	
	Female	-0.94	0.68	0.17
Primary practice location	Erie county medical center		Reference level	
	Buffalo general medical center	0.77	0.81	0.34
	Others	-0.36	0.72	0.62
At least one HCV patient in past 2 yr	Yes		Reference level	
	No/not sure	2.43	0.90	0.001
Level of training	Resident PGY1		Reference level	
	Resident PGY2	1.57	1.18	0.19
	Resident PGY3 or above	1.62	1.15	0.16
Awareness of age-based rule for screening	Yes		Reference level	
	No	-2.32	0.88	0.01

The table illustrates those factors significantly associated with implementation of birth cohort screening recommendations. HCV: Hepatitis C virus; PGY: Post-graduate year.

scored better on the HCV knowledge assessment.

Predictors for implementation of birth cohort-based screening: Next we focused on implementation of the age-based screening recommendation. As this is the primary outcome variable of interest, we used logistic regression to identify potential significant associations between this variable and a number of predictor variables. As above, we treat the percentages from the primary care location variable as weights for the observations to adjust the results for those invited but who did not complete the survey. We found that the implementation of age-based screening for HCV is significantly associated with knowledge of HCV natural history (*P*-value < 0.01), with awareness of birth cohort based screening (*P*-value = 0.01), and with whether the PCP had seen HCV patients previously (*P*-value < 0.01) (Table 5). Therefore, PCP who have higher HCV natural history knowledge levels, who have not seen HCV patients in the past two years, and who are aware of birth cohort recommendations for HCV are more likely to implement age-based screening in their practices.

Analysis of reasons for and appropriateness of obtaining an HCV screening test: At the time of the survey, the majority of the PCP had ordered an HCV screening test in the recent past (88.2%) (Table 6). Of the reasons that PCP decided to pursue HCV screening, the leading reason (49.4%) was abnormal liver function or other biochemical tests while 30.6% of the PCP cited HCV risk factors, and 20% cited membership in the "birth cohort" as the leading reasons to screen for HCV. In terms of PCP ordering the appropriate screening test, 55.3% of the respondents identified the correct initial screening test as the HCV antibody. In addition, 28.2% of the PCP indicated that they do not follow any society

Table 6 Primary care physician screening practices for hepatitis C virus infection

Question	Total	Option	Count	Percent
In the past 2 yr, have you ordered a test with an intention to screen for HCV?	85	Yes	75	88.2
		No or Not Sure	10	11.8
What is the strongest indication to screen for HCV?	85	Risk factor identified on patient encounter	26	30.6
		Patients born between 1945-1965	17	20.0
		Abnormal liver enzymes	42	49.4
How have you screened for hepatitis C?	85	HCV antibody	47	55.3
		Anti HCV antibody and HCV RNA PCR	11	12.9
		Other combinations of Anti HCV antibody, HCV RNA, liver function tests, and "let the lab choose"	27	31.8
Do you follow professional society guidelines for HCV screening?	85	Yes	61	71.8
		No	24	28.2

HCV: Hepatitis C virus.

Table 7 Primary care physician practice patterns for hepatitis C virus screening

Question	Total	Option	Count	Percent
How often are HCV risk factors assessed during a clinic visit?	85	Always	14	16.5
		Often	30	35.3
		Sometimes	25	29.4
		Rarely or never	16	18.8
Do you order an HCV screening test after identifying at least one risk factor?	85	Always	28	32.9
		Often	30	35.3
		Sometimes or rarely	27	31.8
Do you document HCV screening discussion/risk factor assessment in the health maintenance section of the patient's chart?	85	Always or often	20	23.5
		Sometimes	24	28.2
		Rarely	29	34.1
		Never	12	14.1

HCV: Hepatitis C virus.

guidelines when ordering an HCV screening test.

We also assessed PCP HCV screening practice patterns including how often PCP assessed HCV-related risk factors (Table 7). A total of 81.2% of providers assessed for HCV risk factors at least sometimes. In terms of how the knowledge of the presence of HCV risk factors was utilized, 68.2% of PCP frequently or always ordered an HCV screening test after identification of at least one HCV risk factor. Documentation of a discussion with the patient concerning screening and risk assessment was performed always or often by 23.5% of PCP, whereas 14.1% never documented the screening discussions and risk assessment in the patient's chart.

PCP perceptions to screening for HCV: With regards to HCV screening, only 30.6% of PCP was satisfied with the existing screening approaches utilized at their practice site (Table 8). The most effective strategy to screen patients in the clinic was incorporation of both risk-based and birth cohort-based screening. We also evaluated the PCP's perception as to the most effective way to initiate HCV screening, to which 55.3% suggested that having the patient complete a screening questionnaire during the waiting period prior to the evaluation was the most effective strategy.

PCP barriers to screening for HCV: We next evaluated PCP-identified barriers in their practice location for effective screening for HCV (Table 9). Constraints in the allotted time with the patient to obtain all risk factors were cited as a barrier by 14.1% of participants. An additional 14.1% mentioned that unawareness of screening guidelines among PCPs was a barrier to HCV screening. Lack of a pre-set health maintenance evaluation protocol for the clinic location, such as automatic stop prompts and screening alerts, in combination with the above two reasons were cited as barriers by 16.4% of participants. Other reasons were mentioned by 55.3% of respondents and are described in the last column of Table 9.

DISCUSSION

The development of DAAs has resulted in the need for significant expansion in the number of providers who can treat HCV. While PCP have always had a role in detection and counseling for HCV[8], their scope of practice is expanding to include HCV treatment. Several recent studies have documented equivalent HCV eradication rates whether patients were treated by PCP, hepatologists, or infectious diseases physicians[9,10]. Recent investigation has also documented that family medicine training program directors believe that HCV is a significant problem for family physicians and that HCV

Table 8 Primary care physician perceptions toward screening for hepatitis C virus

Question	Total	Option	Count	Percent
Satisfied with the screening approach in the clinic	85	Yes	26	30.6
		No	25	29.4
		Not Sure	34	40.0
What is the most effective strategy in screening HCV in your clinic	85	Incorporate risk based screening	19	22.4
		Incorporate birth cohort based screening	11	12.9
		Incorporate both risk based and birth cohort screening	55	64.7
Most effect way to initiate screening during a clinic visit	85	Have patient fill out a screening questionnaire during wait period	47	55.3
		Incorporate mandatory screening questions into EMR	19	22.4
		Facilitate screening by use of posters in patient rooms	9	10.6
		Printed patient handout about screening	10	11.7

HCV: Hepatitis C virus; EMR: Electronic medical record.

Table 9 Primary care physician identified barriers to screening for hepatitis C virus (n = 85)

Option	Count	Percent
Inconsistency in offering HCV screening as a part of pre-set health maintenance protocol, time constraints in obtaining all HCV risk factors, unawareness of screening guidelines	14	16.5
Time constraints in obtaining all HCV risk factors	12	14.1
Unawareness of screening guidelines	12	14.1
Other combinations of inconsistency in offering HCV screening as a part of pre-set health maintenance protocol, time constraints in obtaining all HCV risk factors, taboo in asking confidential and personal information as outlined in the screening questionnaire, and unawareness of screening guidelines	47	55.3

HCV: Hepatitis C virus.

education should be part of their training curriculum[14]. As limited data exist on PCP knowledge of HCV natural history, screening and treatment and the effect of this knowledge on implementation of age-based screening, we performed this investigation primarily to understand the factors associated with implementation of HCV screening recommendations and to identify gaps in HCV-related knowledge among PCP.

Broadly, we found that knowledge of HCV natural history is a prerequisite for implementation of HCV screening. More specifically, we found that knowledge of particular recommendations, in this case those associated with birth cohort screening, were necessary for their implementation. We also observed that knowledge of HCV treatment was significantly associated with knowledge of HCV natural history, indicating the need to educate PCP about the entire HCV disease process. Among our survey respondents, laboratory abnormalities were the single most important indication to screen for HCV as opposed to screening based upon guideline implementation, and most PCP endorsed the combination of risk-factor and birth cohort screening utilizing a self-administered survey. These findings indicate that HCV educational interventions targeted toward PCP should be comprehensive covering all aspects of the infection. Furthermore, they should emphasize guideline-based screening recommendations.

We found that PCP knowledge of the natural history of HCV positively impacts the implementation of birth cohort based screening by PCP. Increasing HCV screening is required to identify the 40% of HCV-infected individuals who are unaware of their infection status[6], so that the stated national goal of HCV elimination can be realized[24]. Substantial HCV-related knowledge and familiarity with HCV screening may enable PCP to offer HCV treatment thereby providing additional linkage-to-care opportunities[25]. A recent study reported that only 22% of PCP believed they should treat HCV. PCP who managed a high proportion of HCV-infected patients and practices that actively managed a variety of related conditions (HIV, mental health and substance use disorders) were factors significantly associated with a higher likelihood of offering HCV treatment[13]. HCV education targeted to PCP is likely to play an important role in increasing the number of treating physicians.

Ongoing HCV education to PCP is also required given the rapid progress in our understanding of HCV natural history and recent therapeutic advances. For example, recent data have confirmed all-cause and liver-specific mortality reductions as a result of HCV eradication[26-30]. Similarly, further PCP education is needed not only on when to screen, *i.e.,* basing screening on guidelines instead of on clinical abnormalities, but how to screen. Indeed, we would encourage pharmaceutical companies to continue to invest in PCP education through sponsorship of educational programs targeted to PCP. Our survey revealed that many PCP continue inappropriate and ineffective HCV screening strategies, such as obtaining aminotransferase or HCV RNA levels, instead of obtaining an initial HCV antibody assessment.

Physician engagement is crucial to successful guideline implementation, and an initial crucial step is guideline awareness[31]. Our study illustrates that physician knowledge of HCV natural history, as well as awareness of birth cohort screening recommendations, were associated with age-based screening implementation. PCP knowledge of HCV treatment did not predict implementation of birth cohort screening, but was associated with knowledge of natural history of HCV. PCP knowledge gaps have been cited as obstacles to liver disease screening for hepatocellular carcinoma surveillance among cirrhotic patients[32] and in hepatitis B[33] infection. Education targeted to patients may also improve screening and linkage to care. Consequently, education to both providers and to patients is extremely important toward achieving HCV elimination. Linking HCV screening with treatment programs will also be tremendously important toward achieving the goal of eradication.

Study strengths include a reasonable number of survey responses obtained from physicians in training, an important population for HCV treatment workforce expansion. Furthermore, we obtained a reasonable response rate obtaining responses from one half of those to whom the survey was distributed. Additionally, weighting PCP responses permitted inference to those physicians who did not complete the survey, and we also assessed the instrument's internal validity. Study limitations include responses largely from PCP in training at an academic medical center, which may affect generalizability to community-based PCP. Additional limitations include a relatively small sample size and responses obtained *via* a self-administered online questionnaire. Future investigation should endeavor to include additional respondents at other academic centers.

HCV-related knowledge gaps among PCP must be addressed in order to increase the HCV workforce leading to increased opportunities for HCV screening and engagement into care. Our study illustrates how PCP knowledge of HCV natural history and treatment can influence birth cohort-based screening practices. It also provides insights into PCP attitudes and barriers toward HCV screening in the primary care setting. As PCP engagement is paramount to successful intervention implementation, our study highlights topics needed for provider-based educational interventions designed to optimize HCV screening in clinical practice.

ARTICLE HIGHLIGHTS

Research background

In order to achieve global hepatitis C virus (HCV) eradication, it is crucial to increase HCV diagnosis and linkage-to-care.

Research motivation

In many countries, primary care physicians (PCP) care for those who are HCV-infected yet undiagnosed. Increasing PCP willingness to screen and to treat HCV is crucial to its global eradication. Understanding promotors or barriers to HCV screening and linkage-to-care among PCP, especially the role of knowledge of the infection and screening guidelines, is crucial to expansion of the HCV workforce.

Research objectives

We sought to assess PCP knowledge about HCV natural history and treatment as well as with regard to implementation of birth cohort screening recommendations.

Research methods

We administered a 45-item survey to 163 PCP, 82% of whom were in training in internal or family medicine.

Research results

PCP knowledge of HCV natural history and prior management of HCV patients were important predictors of implementation of HCV screening. Clinical abnormalities remained the leading indication for ordering an HCV screening test.

Research conclusions

Comprehensive HCV education targeted to PCP, including screening recommendations, is critical to increase HCV detection and linkage-to-care to obtain global eradication. Familiarity with HCV management increased the likelihood that PCP would care for HCV-infected patients.

Research perspectives

Increasing physician education should lead to increased HCV screening. Linking HCV screening to treatment is crucial to obtain global HCV eradication.

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ORIGINAL ARTICLE

Observational Study

Outcomes assessment of hepatitis C virus-positive psoriatic patients treated using pegylated interferon in combination with ribavirin compared to new Direct-Acting Antiviral agents

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Author contributions: Damiani G and Pigatto P designed the research; Franchi C, Pigatto P and Pacifico A performed the research; Damiani G analyzed the data; Altomare A and Pace MC supervised the manuscript; Petrou S revised the manuscript in order to improve and polish language; Damiani G, Leone S and Fiore M wrote the paper.

Institutional review board statement: This study was approved by the Institutional Review Board of I.R.C.C.S. Istituto Ortopedico Galeazzi (Milan, Italia).

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Abstract

AIM

To evaluate the outcomes in biological treatment and quality of life of psoriatic patients with chronic hepatitis C (CHC) treated with new Direct-Acting Antiviral agents (DAAs) compared to pegylated interferon-2α plus ribavirin (P/R) therapy.

METHODS

This is a retrospective study involving psoriatic patients in biological therapy who underwent anti-hepatitis C virus (HCV) treatment at the Department of Dermatology Galeazzi Orthopaedic Institute Milan, Italy from January 2010 to November 2017. The patients were divided into two groups: patients that underwent therapy with DAAs and patients that underwent HCV treatment with P/R. Patients were assessed by a dermatologist for psoriasis symptoms, collecting Psoriasis Area Severity Index (PASI) scores and the Dermatology Quality of Life Index (DLQI). PASI and DLQI scores were evaluated 24 wk after the end of HCV treatment and were assumed as an outcome of the progression of psoriasis. Switching to a different bDMARD was considered as an inadequate response to biological therapy. The dropout of HCV therapy and sustained virological response (SVR) were considered as outcomes of HCV therapy.

RESULTS

Fifty-nine psoriatic patients in biological therapy underwent antiviral therapy for CHC. Of this, 27 patients were treated with DAAs and 32 with P/R. After 24 wk post treatment, the DLQI and the PASI scores were significantly lower ($P < 0.001$ and $P < 0.005$, respectively) in the DAAs group compared with P/R group. None of the patients in the DAAs group (0/27) compared to 8 patients of the P/R group (8/32) needed a shift in biological treatment.

CONCLUSION

DAAs seem to be more effective and safe than P/R in HCV-positive psoriatic patients on biological treatment. Fewer dermatological adverse events may be due to interferon-free therapy.

Key words: Hepatitis C virus; New Direct-Acting Antiviral agents; Psoriasis; Biological disease modifying drugs

Core tip: Psoriasis is a chronic inflammatory disease affecting approximately the 2% of population in Europe and North America. The hepatitis C virus (HCV) infection affects approximately the 3% of the world population with an estimated prevalence of 5 million people in the United States. Up to 0.06% of people in the United States suffer from both psoriasis and HCV. Psoriatic patients with HCV are excluded by randomized controlled clinical trials. Therefore, no data is currently available concerning the concomitant administration of biological disease modifying drugs and the new Direct-Acting Antiviral agents (DAAs) medications approved for the treatment of HCV infection. The aim of this study is to evaluate the outcomes in biological treatment and quality of life of psoriatic patients with HCV infection treated with DAAs compared to the previous standard therapy of Pegylated Interferon plus Ribavirin.

Damiani G, Franchi C, Pigatto P, Altomare A, Pacifico A, Petrou S, Leone S, Pace MC, Fiore M. Outcomes assessment of hepatitis C virus-positive psoriatic patients treated using pegylated interferon in combination with ribavirin compared to new Direct-Acting Antiviral agents. *World J Hepatol* 2018; 10(2): 329-336 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/329.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.329

INTRODUCTION

Psoriasis is a chronic systemic inflammatory disease affecting approximately 2% of the population in Europe and North America with approximately 7.5 million people in the United States[1,2]. Hepatitis C virus (HCV) is an infection affecting approximately 71 million people worldwide; the most affected regions being WHO Eastern Mediterranean and European Regions with a prevalence of 2.3% and 1.5%[3]. Prevalence of HCV infection in other WHO regions varies from 0.5% to 1.0%[3]. According to the Centers for Disease Control and Prevention, there are currently about 3.3 (2.7-3.9) million people with chronic hepatitis C (CHC) in the United States[4]. The estimated prevalence of both psoriasis and HCV in the United States is 0.02%-0.06% (55000-150000 persons)[5].

The pathogenesis of psoriasis remains unclear, however Th1/Th17 autoimmune involvement has been hypothesized[6]. Its association with HCV is still a matter of debate. Some evidence suggests that psoriasis may be initiated and maintained by both HCV and interferon-α[7]. In a large case-control study, including 12502 psoriatic patients and 24287 controls, the prevalence of HCV in psoriatic patients was increased compared to controls (1.03% *vs* 0.56%; $P < 0.001$)[8].

The therapeutic armamentarium available for psoriasis encompasses the conventional disease modifying drugs (cDMARDs) and biological DMARDs (bDMARDs). cDMARDS represent the first line therapy in high-need psoriatic patients, while bDMARDs are for those subjects in whom cDMARDs have either failed, were not tolerated, or were contraindicated[9]. While bDMARDs are generally safer than cDMARDs, there is one concern emerging from registries or long-term studies which is an increased risk of infection. When patients lose an adequate response to bDMARDs the possible options include increasing the dose, shortening the dosing interval, combination therapy with a topical or another systemic treatment, or switching to a different drug[10].

Recently, the standard of care for CHC has changed. The new Direct-Acting Antiviral agents (DAAs) such as Sofosbuvir, Daclatasvir and the Sofosbuvir/Ledipasvir

combination are now part of the preferred regimens in the WHO guidelines and can achieve cure rates above 95%[3]. These agents are much more effective, safer and better-tolerated than older therapies involving pegylated interferon and ribavirin (P/R) which nowadays play a very limited role for specific scenarios[3]. Of the 71 million persons living with CHC globally in 2015, 20% (14 million) knew their diagnosis and 7.4% of those diagnosed (1.1 million) were started on treatment[3].

Psoriatic patients with CHC are excluded by randomized controlled clinical trials. Data on psoriatic patients with HCV infection treated with bDMARDs is available solely on the reports of single cases or analyses of small groups of patients. Therefore, no data is currently available concerning the concomitant administration of bDMARDs for psoriasis and the new DAAs approved for the treatment of CHC[9].

The aim of this study is to evaluate the disease progression and outcomes in biological treatment and quality of life of HCV psoriatic patients on bDMARDs also treated with new DAAs compared to P/R therapy.

MATERIALS AND METHODS

Study population

This is an observational analysis performed by checking all psoriatic patients admitted to the Department of Dermatology and Venereology of I.R.C.C.S. Istituto Ortopedico Galeazzi (Milan, Italy) after 2007. Furthermore, in the present study we collected only HCV positive psoriatic patients admitted in our department from 2010 until now undergoing biological therapy. The study complies with the Declaration of Helsinki and all patients signed a standard consent regarding sensitive personal data treatment.

Study design

The present study is a spin-off observational retrospective study on HCV psoriatic patients, resulted as re-analysis of a previous study towards the re-circulation of CD4+ memory T-cells in psoriatic patients, approved by the Institutional Review Board (Comitato Etico dell'Ospedale San Raffaele, Milano)[11]. This previous study had as exclusion criterion the absence of acute and chronic systemic or cutaneous infections during sample collections[11], conversely we enrolled the previously discarded HCV psoriatic patients, focusing on their HCV eradication management. The subjects were divided into two groups: (1) Patients that underwent therapy with DAAs (DAA-group) (2) patients that underwent HCV treatment with P/R (P/R-group). Patients were assessed by a dermatologist for psoriasis symptoms, collecting Psoriasis Area Severity Index (PASI) and Dermatology Quality of Life Index (DLQI). They were also assessed by a hepatologist to determine liver function while collecting biochemical and clinical data in two times: Baseline (before HCV treatment) (T0) and 24 wk after the end of HCV treatment (T1). The data was extracted to monitor psoriasis biological therapy and HCV therapy[12,13].

Inclusion criteria

Inclusion criteria were age > 18 years old, signed consent forms, no previous transplantation, no pregnancy, no hereditary hepatic diseases, no drug addiction, and no alcohol abusers [Alcohol Use Disorders Identification Test (AUDIT) score < 7], negative results at screening tuberculosis, absence of acute and chronic systemic or cutaneous infections during sample collection. Psoriasis diagnosis was performed by a Dermatologist following the Psoriasis Italian Guidelines[10], and the diagnosis of CHC according the WHO guidelines[14].

Exclusion criteria

Exclusion criteria were age < 18 years old, pregnancy, drug addiction, alcohol abusers [Alcohol Use Disorders Identification Test (AUDIT) score > 7], HIV infection.

Outcomes of the study

PASI, DLQI were evaluated 24 wk after the end of HCV treatment (T1) and were assumed as an outcome of the progression of psoriasis. Switching to a different bDMARD was considered as inadequate responses to biological therapy. The dropout of HCV therapy, and sustained virological response (SVR) were considered as outcomes of HCV therapy.

Data collection and variables definitions

Baseline clinical characteristics, medical history, biochemical variables, and pharmacologic treatments employed during hospitalization were retrospectively collected and recorded on a computer database. SVR was defined as a confirmed undetectable serum HCV-RNA level 24 wk after the discontinuation of HCV therapy. Patients not fulfilling the SVR definition criteria were classified as non-SVR.

Statistical analysis

Continuous variables are presented as mean ± SD and categorical variables are presented as absolute values and percentages. Comparisons between continuous variables were performed using Mann–Whitney or Kruskall–Wallis tests and comparisons between categorical variables were performed using the χ^2 or Fisher's exact test. Statistical significance was defined as $P < 0.05$. Data analysis was performed using statistical software R-version 3.2.4.

RESULTS

A total of 59 psoriatic patients met the inclusion criteria (27 in the DAA-group and 32 in the P/R-group) and were included in this analysis. The patients' main characteristics are summarized in Table 1. All the patients in the P/R-group were treated with pegylated interferon-2α plus ribavirin; in the DAA-group 25 patients (92.6%) were treated with Sofosbuvir plus Daclatasvir, 1 patient was treated with Sofosbuvir + ribavirin: 1 (3.7%) and 1 patient (3.7%) was treated with Sofosbuvir plus simeprevir plus ribavirin. The median age was 56.7 ±

Table 1 Levels of sIL-2R, ALT, and HBV DNA in the sera of patients with chronic hepatitis B virus infection (mean ± SD)

	DAA-group	P/R-group
Age, yr, mean ± SD	56.7 ± 8.9	58.2 ± 6.7
Male/female, *n* (%)	18/9 (66.7/33.3)	24/8 (75/25)
BMI, mean ± SD, kg/m^2	24.3 ± 2.41	25 ± 1.86
Psoriasis duration, yr, mean ± SD	21.5 ± 8.6	18 ± 6.7
Current biological therapy, *n* (%)		
Etanercept	12 (44.4)	15 (46.9)
Adalimumab	9 (33.3)	10 (31.3)
Infliximab	-	-
Ustekinumab	5 (18.5)	7 (21.9)
Secukinumab	1(3.7)	-
Shifted biological drugs, mean ± SD	1.2 ± 0.3	1.5 ± 0.5
Psoriatic arthritis, *n* (%)	2 (7.4)	3 (9.4)
HCV		
Genotype, *n* (%)		
1	21 (77.8)	29 (90.6)
2	5 (18.5)	1 (3.1)
3	-	2 (6.3)
4	1 (3.7)	-
5/6	-	-
MELD score	9.3 ± 2.5	6,5 ± 0.8
HCV viral load (T0), IU/mL, mean ± SD	6.2 log10 ± 5.7 log10	6.1 log10 ± 6.0 log10
SVR, *n* (%)	27/27 (100)	12/32 (37.5)
Autoimmune comorbidities, *n* (%)		
Rheumatic arthritis	1 (3.7)	-
Ankylosing spondylitis	-	1 (3.1)
Systemic erythematosus lupus	1 (3.7)	-
Other comorbidities, *n* (%)		
Cardiovascular disease	6 (22.2)	3 (9.4)
Metabolic syndrome	2 (7.4)	1 (3.1)
COPD	5 (18.5)	2 (6.3)
Renal insufficiency	0 (0)	0 (0)
HBV	1 (3.7)	1 (3.1)
	0 (0)	0 (0)

Categorical variables are expressed as *n* (%). Numeric variables are expressed as median and SD. BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; DAA: New Direct-Acting Antiviral agent; HBV: Hepatitis B virus; HCV: Hepatitis C virus; P/R: Pegylated interferon-2α plus ribavirin; MELD: Model for end-stage liver disease; SVR: Sustained virological response.

8.9 years in the DAA-group and 58.2 ± 6.7 years in the P/R-group, respectively; 66.7% patients were men in the DAA-group and 75% patients were men in the P/R-group, respectively. The median body mass index (BMI) was 24.3 ± 2.41 in the DAA-group and 25 ± 1.86 in the P/R-group, respectively. The median psoriasis duration was 21.5 ± 8.6 years in the DAA-group and 18 ± 6.7 years in the P/R-group, respectively. In the DAA-group the bDMARD used, was Etanercept, Adalimumab, Ustekinumab and Secukinumab in 12 (44.4%), 9 (33.3%), 5 (18.5%), and 1 (0.04%) patients, respectively; in the P/R-group the bDMARD used, was Etanercept, Adalimumab and Ustekinumab in 15 (46.9%), 10 (31.3%) and 7 (21.9%) patients, respectively. Switching among biologic therapies, before hepatitis C treatment, was 1.2 ± 0.3 in DAA-group and 1.5 ± 0.5 in P/R-group. Two patients (7.4%) of the DAA-group and 3 patients (9.4%) of the P/R-group presented psoriatic arthritis, respectively. In the DAA-group the HCV genotypes were 1, 2 and 4 in 21 (77.8%), 5 (33.3%) and 1 (3.7%) patients, respectively; in the P/R-group the HCV genotypes were 1, 2 and 3 in 29 (90.6%), 1 (3.1%) and 2 (6.3%) patients, respectively. The MELD score was 9.3 ± 2.5 in DAA-group and 6.5 ± 0.8 in the P/R-group, respectively. The viral load at the baseline was 6.2 $\log_{10}$ ± 5.7 $\log_{10}$ UI/mL in the DAA-group and 6,1 $\log_{10}$ ± 6.0 $\log_{10}$ UI/mL in the P/R-group, respectively. All the 27 patients (100%) in the DAA-group obtained SVR, whereas only 37.5% (12/32) of patients in the P/R-group achieved a SVR. Two out of 27 patients of DAA-group had autoimmune comorbidities: 1 (3.7%) rheumatic arthritis and 1 (3.7%) systemic erythematosus lupus; 1 (3.1%) out of 32 patients of P/R-group had autoimmune comorbidity: ankylosing spondylitis. In the DAA-group other comorbidities were cardiovascular diseases, metabolic syndrome, chronic obstructive disease, and HBV in 6 (22.2%), 2 (7.4%), 5 (18.5), 1 (3.7%) patients, respectively. In the P/R-group other comorbidities were cardiovascular diseases, metabolic syndrome, chronic obstructive disease, and HBV in 3 (9.4%), 1 (3.1%), 2 (6.3%), 1 (3.1%) patients, respectively. None of the 59 psoriatic patients enrolled in the study had renal insufficiency.

The comparison between the patient scores and laboratories findings according to HCV eradication treatment is shown in Table 2: There were no differences in PASI scores between the 2 HCV treatment groups; after 24 wk to the end of the HCV treatment there

Table 2 Levels of sIL-2R, ALT, and HBV DNA in the sera of patients with chronic hepatitis B virus infection (mean ± SD)

	DAA-group	P/R-group	*P* value
PASI (T0), mean ± SD	11.6 ± 5.2	9.4 ± 3.5	-
PASI (T1), mean ± SD	5.2 ± 1.6	8.3 ± 4.5	< 0.005
Biological treatment shifts, *n* (%)	-	8 (25)	< 0.001
Topical treatments (T0), *n* (%)	27 (100)	32 (100)	-
Topical treatments (T1), *n* (%)	27 (100)	32 (100)	-
DLQI (T0), mean ± SD	13 ± 2.3	12 ± 3.1	-
DLQI (T1), mean ± SD	4.2 ± 2.3	11 ± 2.3	< 0.001
HCV treatment details, *n* (%)	Sofosbuvir + daclatasvir: 25 (92.6) Sofosbuvir + simeprevir + ribavirin: 1 (3.7) Sofosbuvir + ribavirin: 1 (3.7)	Pegylated interferon-2α + ribavirin 32 (100)	-
Laboratory tests, mean ± SD			
T0			
ALT	45.6 ± 16.5	43 ± 9.2	-
AST	54.6 ± 5.51	52.4 ± 12.5	-
GGT	42.0 ± 13.59	43.3 ± 14.6	-
T1			
ALT	42.21 ± 12.4	43 ± 8.5	-
AST	51.3 ± 4.2	51.9 ± 8.9	-
GGT	40.8 ± 12.2	42.9 ± 14.1	-
Dropout HCV-treatment, *n* (%)	0/27 (0)	9/32 (28.1)	< 0.005
SVR, *n* (%)	27/27 (100)	12/32 (37.5)	< 0.005

Categorical variables are expressed as *n* (%), and compared by χ^2 or Fisher's exact test. Numeric variables are expressed as median and SD, and compared by Mann–Whitney or Kruskall–Wallis tests. Statistical significance was considered as a *P*-value of < 0.05. DAA: New Direct-Acting Antiviral Agent; P/R: Pegylated interferon-2α plus ribavirin; PASI: Psoriasis Area Severity Index; T0: Baseline time (before starting the hepatitis C eradication treatment); T1: Six months after the end of hepatitis C eradication treatment; DLQI: Dermatology Quality of Life Index; HCV: Hepatitis C virus; SVR: Sustained virological response; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase.

was a significant reduction of PASI score in DAA-group when compared to P/R-group patients ($P < 0.005$). Patients in the P/R-group (8 patients, 25%) need a significantly ($P < 0.001$) frequent change of biological treatment compared to DAA-group (no patients). There was no difference in the use of topical treatments in the 2 groups at baseline (100% in both groups) and after 24 wk to the end of the HCV treatment (100% in both groups). Although there was no difference in the DLQI at the beginning of the treatment in the 2 groups, after 24 wk the DLQI was significantly improved in the DAA-group when compared to P/R-group patients ($P < 0.001$). HCV treatment was significantly better tolerated ($P < 0.005$) by DAA-group patients with no dropout; 9 out of 32 P/R-group patients (28.1%) dropped out of HCV treatment. HCV eradication was significantly higher ($P < 0.005$) in DAA-group compared to P/R-group: SVR was obtained in 100% (27/27) of patients treated with DAAs and in 37.5% (12/32) of patients treated with P/R. Three Adverse Drug Events (ADEs) were observed in DAA-group patients during HCV treatment. Two of the patients experienced fatigue and one experienced insomnia. Fifteen of the thirty-two patients of the P/R-group experienced ADEs during HCV treatment. Ten experienced fatigue with nausea, three diarrhea and two cefalea.

DISCUSSION

In our study, psoriatic patients of the DAA-group had a better prognosis compared to the P/R-group, both in progression of psoriasis (and consequent worsening of quality of life) and in eradication of HCV. Patients with a worse prognosis treated with P/R could be attributed to two possible causes: (1) Interferon "itself" has dermatological side effects[15], worsening psoriasis and (2) DAAs are more effective than interferon-based therapy increasing the rates of SVR (even up to 100%)[16], suggesting that HCV could promote psoriatic disease.

Eradication treatment of HCV with interferon has been described as the drug that can induce *de novo* psoriasis or flares in psoriatic patients[17-19]. The flare usually occurs one to 6 wk after starting interferon-α and may lead to its discontinuation[20].

There appears to be an intricate relationship between psoriasis and HCV infection as seen in previous reports. HCV in predisposed individuals upregulates cathelecidin, Toll like receptor 9 (TLR-9) and interferon (IFN)-γ which are all involved in the development of psoriasis plaques. Pegylated interferon may also initiate and maintain psoriasis inflammation by activating Th1 and Th17 *via* myeloid DCs[7,21]. Cathelecidin bonds self-DNA. It is released after various injury stimuli *via* TLR-9 plasmacytoid and DCs to produce type I interferons (α/β), which drives T-cell polarization towards Th1 and Th17 *via* myeloid DCs. This initiates the trigger and further maintenance of psoriasis[7]. Likewise, pegylated interferon may act directly *via* myeloid resident DCs on Th1 cells and also on Th17 cells at the same time by increasing their activation and release of IL-17. IL-17 is a chemo-attractant for neutrophils to the skin and IL-22 causes keratinocyte hyperproliferation[21]. Despite these preliminary hypotheses, data regarding the association of HCV and psoriasis remain unclear and a matter of

debate. Some studies show no association[22] while others show an increased prevalence of psoriasis among HCV-patients as reported by Cohen[8]. Furthermore, limited data is present in literature regarding psoriasis induction and exacerbation due to interferon in HCV-psoriatic patients[21].

Experimental studies have shown that an intradermal injection of IFN-γ, both on the non-lesional psoriatic and healthy skin, causes an elevation of inflammatory products such as TNF, IL-23 and inducible nitric oxide synthase which are characteristic of psoriatic plaques[23]. An association between HCV infection and psoriasis has been suggested. The prevalence of HCV in psoriatic patients was increased compared to controls (1.03% *vs* 0.56%; $P < 0.001$)[8]. In a single center cross-sectional study conducted in a Japanese university hospital, the frequency of HCV infection was significantly higher in psoriatic (7.5%) than in non psoriatic patients (3.3%) in overall ages[24]. Interestingly, when stratified by age at the first visit, HCV infection frequency was significantly higher in patients with psoriasis than in controls aged over 60 years (11.8% *vs* 6.6%, respectively, $P = 0.0215$) and 70 s (19.5% *vs* 7.3%, $P < 0.0001$)[24]. Psoriatic patients with CHC were significantly older at onset than non psoriatic CHC patients (median, 54 *vs* 39 years)[24]. There was also a stronger male predominance (male/female ratio, 4.4:1), similar family history of psoriasis, higher association of diabetes mellitus and hypertension, and significantly lower body mass index, in an age-stratified (≥ 40 years) analysis[24]. Psoriatic patients with CHC were less obese, but still had a higher frequency of diabetes mellitus and hypertension[24]. The authors hypothesized that psoriasis and HCV have pathophysiological factors in common with both mediated by proinflammatory cytokine tumor necrosis factor (TNF-α)[24]. In HCV infection, continuous inflammation mediated by TNF-α leads to liver cirrhosis and diabetes mellitus[24]. The link between psoriasis and HCV infection has been shown experimentally in a recent study of Chun *et al*[7]; the authors performed two 2 mm punch biopsies of lesional and nonlesional skin in 10 patients who were HCV-negative psoriatic and 7 HCV-positive psoriatic patients. The biopsies were used to measure cathelicidin, TLR9 and IFNc mRNA expression by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)[7]. The mRNA expression was calculated relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and demonstrated that the cutaneous levels of inflammatory genes in HCV positive psoriatic patients are higher than the levels in patients with only psoriasis[7]. The increased cutaneous levels of cathelicidin, TLR9 and IFNc of HCV-positive psoriatic patients as compared to HCV-negative psoriatics suggest that HCV infection may predispose patients to developing psoriasis[7]. These findings seem to be confirmed clinically due to the worse prognosis of psoriasis in HCV-positive patients[7,25]. The mean PASI score is significantly higher in cohorts of patients affected with hepatitis C than those with psoriasis alone[7,25].

In line with previous studies, our study confirmed that bDMARDs are safe in psoriatic patients with HCV infection[26-29]. In literature bDMARDs are safe either in psoriatic patients with HCV infection and past HBV infection[30]. Patients in the DAA-group had a significantly better response to bDMARDs compared to P/R-group, requiring a smaller transition to different bDMARDs[10]. This may be due to a more favorable outcome in psoriasis (due to interferon-free therapy and greater HCV eradication), but also due to intrinsic DAAs action[31]; Immune reconstitution occurs in patients with whom HCV was successfully eradicated *via* DAAs therapy[31]. Restoration of the CD4+ T-cell compartment in the peripheral blood and a re-differentiation of the T lymphocyte memory compartment resulted in a more effector memory T cell population and a reduction in expression of the co-inhibitory molecule TIGIT in bulk T lymphocytes[31]. Burchill *et al*[31] observed a partial reversal of the exhausted phenotype in HCV-specific CD8+ T cells and a dampening of the activation state in peripheral NK cells. Spaan *et al*[32] showed that viral load decline, as a consequence of DAAs therapy in patients with chronic hepatitis C infection, reduces serum levels of NK cell-stimulating cytokines and causes correction of the altered NK cell phenotype observed in chronic HCV patients. CHC is characterised by innate immune activation with increased interferon-stimulated gene expression and by an altered phenotype of interferon-responsive natural NK cells[33]. DAAs treatment could improve the pro-inflammatory status due both to psoriasis and to the HCV infection making bDMARDs actions more effective.

The current study contains some limitations that need to be taken into account. This is a retrospective observational study conducted in a single Italian centre, thus our conclusions should be interpreted with caution and cannot be generalized to all HCV-positive psoriatic patients. In particular, a general under-reporting of toxicity, as with all observational studies, is possible. Another limitation is the small sample size and the relatively short follow-up. This is the first study to compare DAAs to P/R for the management of HCV-positive psoriatic patients and unmeasured confounding variables could influence our findings. Thus, larger series with long-term follow-up are required to confirm this preliminary data.

In conclusion, new DAAs are more effective than P/R in the eradication of HCV and the control of symptoms in psoriatic patients with CHC. Future studies are needed to evaluate the effects of DAAs in this clinical setting, which may further aid in elucidating the etiologic and pathogenetic mechanism of psoriasis.

ARTICLE HIGHLIGHTS

Research background

Up to 0.06% of people suffer from both psoriasis and hepatitis C virus (HCV). Psoriatic patients with HCV are excluded by randomized controlled clinical trials.

Research motivation
No data is currently available concerning the concomitant administration of biological drugs and the medications approved for the treatment of HCV infection, as new Direct-Acting Antiviral agents (DAAs).

Research objectives
Evaluate the outcomes in biological treatment and quality of life of psoriatic patients with chronic hepatitis C (CHC) treated with new DAAs compared to pegylated interferon-2α plus ribavirin (P/R) therapy.

Research methods
Psoriatic patients, in biological therapy, who underwent anti-HCV treatment were retrospectively reviewed. The patients were divided into two groups: patients that underwent therapy with DAAs and patients that underwent HCV treatment with P/R. Patients were assessed for Psoriasis Area Severity Index (PASI) scores and the Dermatology Quality of Life Index (DLQI) switching to a different bDMARD, dropout of HCV therapy and sustained virological response (SVR).

Research results
Twenty-seven patients were treated with DAAs and thirty-two with P/R. At three months, after completion of antiviral therapy, the DLQI and the PASI scores were significantly lower ($P < 0.001$ and $P < 0.005$, respectively) in DAAs group compared with P/R group. None of the patients in the DAAs group compared to the eight patients of the P/R group needed a change in biological treatment.

Research conclusions
DAAs seem to be more effective and safe than P/R in HCV-positive psoriatic patients on biological treatment.

Research perspectives
This is the first study which evaluated the HCV treatment of psoriatic patients on biological agents. Future studies are needed to evaluate the effects of DAAs in this clinical setting, which may further aid in elucidating the etiologic and pathogenetic mechanism of psoriasis.

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META-ANALYSIS

Outcomes of kidney transplantation in patients with hepatitis B virus infection: A systematic review and meta-analysis

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Abstract

AIM

To assess outcomes of kidney transplantation including patient and allograft outcomes in recipients with hepatitis B virus (HBV) infection, and the trends of patient's outcomes overtime.

METHODS

A literature search was conducted using MEDLINE, EMBASE and Cochrane Database from inception through October 2017. Studies that reported odds ratios (OR) of mortality or renal allograft failure after

kidney transplantation in patients with HBV [defined as hepatitis B surface antigen (HBsAg) positive] were included. The comparison group consisted of HBsAg-negative kidney transplant recipients. Effect estimates from the individual study were extracted and combined using random-effect, generic inverse variance method of DerSimonian and Laird. The protocol for this meta-analysis is registered with PROSPERO (International Prospective Register of Systematic Reviews; no. CRD42017080657).

RESULTS

Ten observational studies with a total of 87623 kidney transplant patients were enrolled. Compared to HBsAg-negative recipients, HBsAg-positive status was significantly associated with increased risk of mortality after kidney transplantation (pooled OR = 2.48; 95%CI: 1.61-3.83). Meta-regression showed significant negative correlations between mortality risk after kidney transplantation in HBsAg-positive recipients and year of study (slopes = -0.062, *P* = 0.001). HBsAg-positive status was also associated with increased risk of renal allograft failure with pooled OR of 1.46 (95%CI: 1.08-1.96). There was also a significant negative correlation between year of study and risk of allograft failure (slopes = -0.018, *P* = 0.002). These associations existed in overall analysis as well as in limited cohort of hepatitis C virus-negative patients. We found no publication bias as assessed by the funnel plots and Egger's regression asymmetry test with *P* = 0.18 and 0.13 for the risks of mortality and allograft failure after kidney transplantation in HBsAg-positive recipients, respectively.

CONCLUSION

Among kidney transplant patients, there are significant associations between HBsAg-positive status and poor outcomes including mortality and allograft failure. However, there are potential improvements in patient and graft survivals in HBsAg-positive recipients overtime.

Key words: Hepatitis B; Kidney transplant; Kidney; Renal transplantation; Transplantation; Meta-analysis

Core tip: Hepatitis B is one of the most common infectious diseases worldwide. Despite advances in medicine, chronic hepatitis B virus (HBV) infection is currently incurable. In addition, clinical outcomes of kidney transplantation in HBV infected patients are still unclear. To further assess these outcomes, we conducted this systematic review and meta-analysis to assess patient and allograft outcomes after kidney transplantation in patients with HBV Infection. We found significant associations between HBV positive status and poor outcomes including 2.5-fold increased risk of mortality and 1.5-fold increased risk of allograft loss.

Thongprayoon C, Kaewput W, Sharma K, Wijarnpreecha K, Leeaphorn N, Ungprasert P, Sakhuja A, Cabeza Rivera FH, Cheungpasitporn W. Outcomes of kidney transplantation in patients with hepatitis B virus infection: A systematic review and meta-analysis. *World J Hepatol* 2018; 10(2): 337-346 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/337.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.337

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most common infectious diseases and major health problems worldwide[1-3]. In 2017, approximately 257 million people have chronic hepatitis B virus infection[1]. Despite advances in medicine which have resulted in a cure for hepatitis C infection in recent years[4], chronic HBV infection is still currently considered as an incurable disease[2,3,5], leading to significant mortality (887000 death in 2015) and morbidities including cirrhosis and hepatocellular carcinoma[1,2].

Advances in immunosuppression and kidney transplant techniques have led to significant improvements in short-term survival of the renal allograft[6]. Long-term graft survival, however, has remained relatively lagged behind and has now become one of the main problems in kidney transplantation[7-9]. Although HBV is preventable disease by HBV vaccine, HBV infection remains a challenge issue in patients with end-stage renal disease on dialysis, affecting from 1.3% up to 14.6% of chronic dialysis patients [hepatitis B surface antigen (HBsAg) – positive] depending on geographical regions[10-12], and, consequently leading to chronic HBV infection kidney transplant patients[13-31]. Among renal transplant patients with HBV (HBsAg positive), there have been reported cases of HBV reactivation[32], massive liver necrosis due to fulminant hepatitis, and severe cholestatic hepatitis after kidney transplantation[10,16,33-36]. In addition, chronic HBV infection may result in HBV-related membranous nephropathy after kidney transplantation[37-43].

Even though HBV is incurable, current more available antiviral agents against HBV effectively suppress viral replication[10]. Thus, these agents can prevent hepatic fibrosis[10] and potentially reduce significant hepatic and extra-hepatic complications related to chronic HBV. In spite of improvement of HBV care, outcomes of kidney transplantation including patient and allograft outcomes in recipients with HBV infection remain unclear. Thus, we conducted this meta-analysis to (1) Assess the risks of mortality and allograft failure in kidney transplant recipients with HBsAg-positive status; and (2) evaluate trends of patient's outcomes overtime.

MATERIALS AND METHODS

Literature review and search strategy

The protocol for this meta-analysis is registered

with PROSPERO (International Prospective Register of Systematic Reviews; no. CRD42017080657). A systematic literature search of MEDLINE, EMBASE, and the Cochrane Database of Systematic Reviews from database inception to October 2017 was conducted to identify studies assessing outcomes of kidney transplantation including patient and allograft outcomes in patients with HBV. The systematic literature review was undertaken independently by two investigators (C.T. and W.C.) applying the search approach that incorporated the terms of "hepatitis B" or "HBV", or "viral hepatitis" and "kidney transplantation" which is provided in online supplementary data 1. No language limitation was applied. A manual search for conceivably relevant studies using references of the included articles was also performed. This study was conducted by the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement[44] and previously published guidelines[45,46].

Selection criteria

Eligible studies must be randomized controlled trials or observational studies including cohort studies, case-control, or cross-sectional that assessed the risks of mortality and/or allograft loss after kidney transplantation in patients with HBV (HBsAg-positive). They must provide the effect estimates odds ratios (OR), relative risks (RR), or hazard ratios (HR) with 95%CI. The comparison group consisted of HBsAg-negative kidney transplant recipients. Retrieved articles were individually reviewed for their eligibility by the two investigators (C.T. and W.C.) noted previously. Discrepancies were discussed and resolved by mutual consensus. Newcastle-Ottawa quality assessment scale was used to appraise the quality of study for case-control study and outcome of interest for cohort study[47]. The modified Newcastle-Ottawa scale was used for cross-sectional study[48], as shown in Table 1.

Data abstraction

A structured data collecting form was used to derive the following information from each study including title, year of the study, name of the first author, publication year, country where the study was conducted, demographic and characteristic data, mean age, patient's sex, donor type, % of patients with HBeAg, % of patients with coexist patients infected with hepatitis C virus, and adjusted effect estimates with 95%CI and covariates that were adjusted in the multivariable analysis.

Statistical analysis

Comprehensive Meta-analysis (version 3; Biostat Inc) was used to analyze the data. Adjusted point estimates from each study were consolidated by the generic inverse variance approach of DerSimonian and Laird, which designated the weight of each study based on its variance[49]. Given the likelihood of increased inter-observation variance; a random-effect model was utilized to assess the pooled prevalence and pooled OR with 95%CI for the risks of atrial fibrillation with sleep duration, insomnia, and frequent awakening. Cochran's Q test and I^2 statistic were applied to determine the between-study heterogeneity. A value of I^2 of 0-25% represents insignificant heterogeneity, 26%-50% represents low heterogeneity, 51%-75% represents moderate heterogeneity, and > 75% represents high heterogeneity[50]. The presence of publication bias was evaluated *via* the Egger test[51].

RESULTS

A total of 1673 potentially eligible articles were identified using our search strategy. After the exclusion of 1639 articles based on the title and abstract for clearly not fulfilling inclusion criteria on the basis of the type of article, study design, population or outcome of interest, leaving 34 articles for full-length review. Eighteen of them were excluded from the full-length review as they did not report the outcome of interest while six articles were excluded because they were descriptive studies without comparative analysis. Thus, the final analysis included 10 cohort studies[18,21,31,36,52-57] with 87623 kidney transplant patients. The literature retrieval, review, and selection process are demonstrated in Figure 1. The characteristics and quality assessment of the included studies are presented in Table 1. Patients in most included studies used calcineurin inhibitor-based immunosuppression. Mean age was between the ages of 32-49.

Mortality after kidney transplantation in patients with HBsAg-positive vs HBsAg-negative status

Ten studies assessed the mortality risk after kidney transplantation in patients with HBV as shown in Table 1. Compared to *HBsAg-negative* patients, HBsAg-positive status was significantly associated with increased risk of mortality after kidney transplantation (pooled OR = 2.48; 95%CI: 1.61-3.83, I^2 = 82, Figure 2). When meta-analysis was limited only to non-HCV patient population, the pooled OR of mortality was 2.98 (95%CI: 1.47-6.07, I^2 = 89). When meta-analysis was limited only to studies with adjusted analysis for confounders[36,53,54], the pooled OR of mortality was 1.27 (95% CI: 1.06-1.51, I^2 = 85). Meta-regression showed significant negative correlations between mortality risk after kidney transplantation in HBsAg-positive patients and year of study (slopes = -0.062, P = 0.001, Figure 3). Meta-regression showed no significant impact of donor type on the association between HBsAg-positive status and increased risk of mortality after kidney transplantation (P = 0.11).

Impact of antiviral treatments on patient survival after kidney transplantation in patients with HBsAg-positive

Of 10 studies[18,21,31,36,52-57], 3 studies[18,21,54] provided data on prophylactic antiviral treatment for HBV. When meta-analysis was limited only to studies with HBsAg-positive

Table 1 Main characteristics of the studies assessing outcomes of kidney transplantation in patients with hepatitis B virus

Study	Lee *et al*[55]	Breitenfeldt *et al*[56]	Chan *et al*[21]	Morales *et al*[57]	Ridruejo *et al*[52]	Aroldi *et al*[53]	Yap *et al*[18]	Reddy *et al*[36]	Grenha *et al*[31]	Lee *et al*[54]
Country	Taiwan	Germany	Hong Kong	Spain	Argentina	Italy	Hong Kong	USA	Portugal	Korea
Study design	Cohort study	Cohort study	Cohort study	Cohort study	Cohort study	Cohort study	Cohort study	Cohort	Cohort study	Cohort study
Year	2001	2002	2002	2004	2004	2005	2010	2011	2015	2016
Total number	477	927	509	3365	231	541	126	75681	2284	3482
Age (yr)	38.6 ± 11.5	41.7	N/A	45.6 ± 13.0	38	31.7	49.2	N/A	44.3	40.6 ± 12.9
Male	280 (58.7%)	595 (64.2%)	N/A	2119 (63.0%)	136 (58.9%)	322 (59.5%)	90 (71.4%)	45249 (59.8%)	1524 (66.7%)	2084 (59.9%)
Living donor	N/A	19 (2.0%)	N/A	N/A	N/A	62 (11.5%)	41 (32.5%)	32096 (42.4%)	80 (3.5%)	2571 (73.8%)
HBsAg	62 (13.0%)	37 (4.0%)	67 (13.2%)	76 (2.2%)	17 (7.3%)	77 (14.2%)	63 (50%)	1346 (1.8%)	76 (3.3%)	160 (4.6%)
HBeAg in HbsAg (+) patients	N/A	11/37 (29.7%)	29/67 (43.3%)	N/A	N/A	34/77 (44.2%)	16/63 (25.4%)	N/A	N/A	N/A
HBV treatment	N/A	N/A	Lamivudine 26/67 (39%)	N/A	N/A	N/A	Lamivudine 38/63 (60%)	N/A	N/A	Not specified 129/160 (81%)
Anti-HCV	151 (31.7%)	130 (14.0%)	0 (0%)	513 (15.2%)	106 (45.9%)	244 (45.1%)	0 (0%)	0 (0%)	113 (4.9%)	55/3482 (1.6%)
Immunosuppression	Cyclosporine, steroid, azathioprine, MMF	N/A	Cyclosporine, steroid, azathioprine	Cyclosporine, steroid, azathioprine, MMF, FK506	Cyclosporine, steroid, azathioprine	Cyclosporine, steroid, azathioprine	Cyclosporine/ tacrolimus, steroid, MMF	Cyclosporine/ tacrolimus, steroid, azathioprine, MMF, mTOR	N/A	Cyclosporine/ tacrolimus, steroid, azathioprine/MMF
Follow-up after KTx	6.0 ± 7.0 yr	9.2 ± 4.4 yr	82 ± 58 mo	N/A	39.9 (1-10.4.2) mo	11 yr	140.1 mo	1098 d	10 yr	89.1 ± 54.1 mo
Mortality	Overall 2.72 (1.48-4.99) No HCV 4.61 (2.41-8.84)	Overall 4.08 (2.10-7.93) No HCV 3.60 (1.72-7.54)	No HCV 8.07 (3.65-17.86)	Overall 2.06 (1.24-3.40) No HCV 2.97 (1.66-5.33)	Overall 2.20 (0.57-8.34)	Overall 2.36 (1.50-3.70) No HCV 4.40 (2.06-9.41)	No HCV 11.70 (1.45-94.40)	Overall 1.07 (0.88-1.31) Living donor 0.98 (0.59-1.63) Deceased donor 1.09 (0.88-1.36)	Overall 1.33 (0.78-2.29) No HCV 1.02 (0.54-1.94)	2.37 (1.16-4.87)
Graft failure	Overall 1.84 (1.08-3.15) No HCV 3.56 (1.89-6.71)	Overall 2.07 (1.06-4.05) No HCV 1.48 (0.71-3.08)	No HCV 1.61 (0.86-3.03)	Overall 0.62 (0.37-1.02) No HCV 0.59 (0.32-1.09)	5.45 (1.95-15.23)	Overall 1.55 (1.12-2.14) No HCV 0.65 (0.27-1.54)	N/A	Overall 1.02 (0.81-1.28) Living donor 0.90 (0.62-1.30) Deceased donor 1.10 (0.82-1.47)	Overall 1.57 (0.99-2.49) No HCV 1.63 (0.98-2.71)	1.38 (0.55-3.50)
Confounder adjustment	None	None	None	None	None	Age, Hepatitis C status	none	Recipient age, gender, BMI, race, comorbid, dialysis duration, donor HBcAb, expanded criteria donor, HLA DR mismatch, cold ischemia time, induction therapy, immunosuppressants	none	Age, sex, DM, BMI, primary renal disease, donor type, hypertension, ischemic heart disease, immunosuppressive agents
New Castle-Ottawa score	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 1 O 3	S 4 C 0 O 3	S 4 C 2 O 3	S 4 C 0 O 3	S 4 C 2 O 3

FK506: Tacrolimus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HCV: Hepatitis C virus; KTx: Kidney transplantation; MMF: Mycophenolate mofetil; S, C, O: Selection, comparability, and outcome.

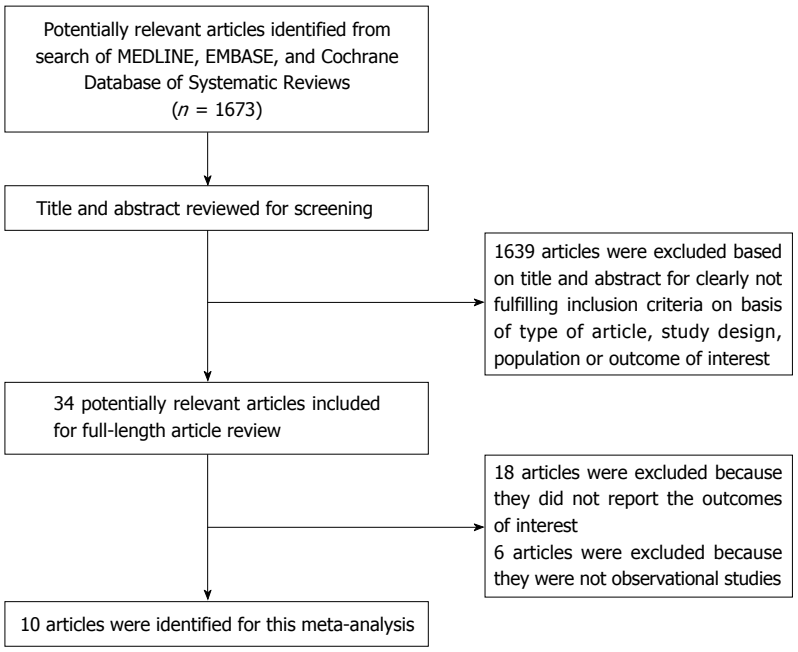

Figure 1 Literature review process.

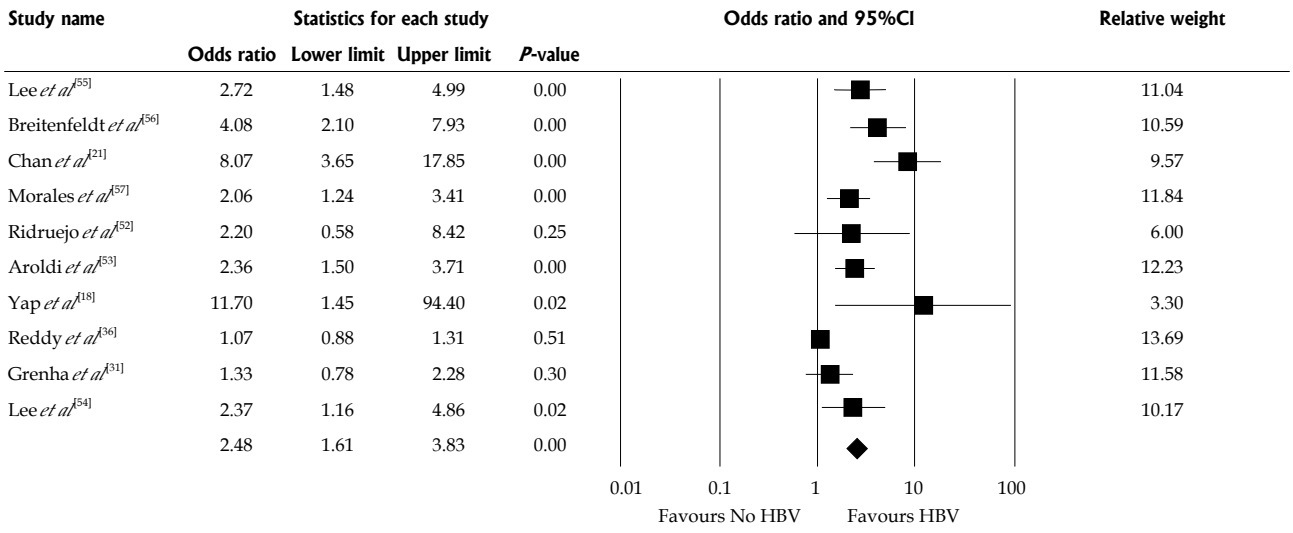

Figure 2 Forest plots of included studies evaluating mortality after kidney transplantation in patients with hepatitis B surface antigen-positive *vs* hepatitis B surface antige -negative.

recipients (> 50%) treated with prophylactic antiviral treatment for HBV, the pooled OR of mortality was 3.85 (95%CI: 0.91-16.23, I^2 = 50%). In a recent study by Lee *et al*[54], which 81% of HBs Ag-positive recipients were treated with prophylactic antiviral treatment, HBsAg-positive status was significantly associated with increased risk of mortality after kidney transplantation with adjusted HR of 2.37 (95%CI: 1.16-4.87).

Yap *et al*[18] demonstrated that recipients treated with nucleoside/nucleotide analogues had significantly better patient survival, when compared to those who were not on treatment (83% *vs* 34% at 20 years, *P* = 0.006). In patients who had lamivudine-resistant HBV, the investigators showed that treatment with adefovir or entecavir was effective with a three-log reduction in HBV DNA by 6 mo. When compared to patients who were treated with lamivudine or adefovir, Lee *et al*[54] demonstrated that those treated with new generation antiviral agent entecavir had better patient survival (log-rank, *P* = 0.050).

Renal allograft failure in patients with HBsAg-positive vs HBsAg-negative

There were 9 studies assessed renal allograft outcomes in HBsAg-positive patients (Table 1). HBsAg-positive status was significantly associated with increased risk of renal allograft loss with pooled OR of 1.46 (95%CI: 1.08-1.96, I^2 = 69, Figure 4). When meta-analysis was

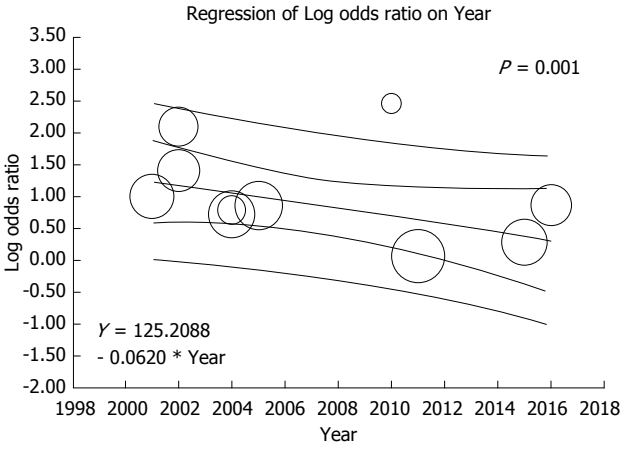

Figure 3 Graphical display of significant negative correlations between mortality risk after kidney transplantation in hepatitis B surface antigen-positive patients and year of study.

limited only to non-HCV patient population, the pooled OR of allograft failure was 1.33 (95%CI: 1.00-1.77, I^2 = 80). When meta-analysis was limited only to studies with adjusted analysis for confounders[36,53,54], the pooled OR of allograft failure was 1.25 (95%CI: 0.90-1.73, I^2 = 54). There was also a significant negative correlation between year of study and risk of allograft failure (slopes = -0.018, P =0.002, Figure 5). Meta-regression showed no significant impact of donor type on the association between HBsAg-positive status and increased risk of renal allograft loss (P =0.52).

Evaluation for publication bias

We found no publication bias as assessed by the funnel plots (Supplementary Figures 1 and 2) and Egger's regression asymmetry test with P = 0.18 and 0.13 for the risks of mortality and allograft failure after kidney transplantation in HBV infected patients, respectively.

DISCUSSION

In this systematic review, we demonstrated that HBsAg-positive status in kidney transplant recipients was significantly associated with poor outcomes after transplantation including a 2.5-fold increased risk of mortality and 1.5-fold increased risk of allograft loss. Theses associations existed in overall analysis as well as in limited cohort of hepatitis C virus-negative patients.

Chronic HBV infection can negatively impact the clinical outcomes of kidney allograft recipients. Compared to the HBsAg-negative recipients, HBsAg-positive recipients carry a higher risk of hepatic complications including chronic hepatitis, liver failure, fibrosing cholestatic hepatitis, and hepatocellular carcinoma[34,36,58]. In addition, some immunosuppressive agents after kidney transplantation may also put patients at higher risks of HBV reactivation[23,40]. HBV genome contains glucocorticoid responsive element that activates transcription of HBV genes[35]. Moreover, cyclosporine may also enhance HBV replication, leading to higher risks of HBV-related complications in kidney transplant recipients[37]. Previously, in 2005, Fabrizi *et al*[27] conducted a meta-analysis of six observational studies and demonstrated a significant association between HBsAg seropositive status and increased mortality after kidney transplantation. Since then, although hepatitis B is still incurable, there have been significant advancements in antiviral agents including the United States Food and Drug Administration approvals of entecavir in 2005 and telbivudine in 2006[59] resulting in reasonably sustained suppression of HBV replication after kidney transplantation[10]. Our meta-analysis with a new era of medicine also demonstrated a 2.7-fold increased risk of mortality in kidney transplant recipients with HBsAg positivity, when compared to HBsAg-negative recipients. In addition, our meta-analysis is the first to demonstrate a significant negative correlation between the mortality risk and year of study, which potentially represents improvements in patient care and management for chronic HBV in kidney transplant patients[60]. Although antiviral treatment has been shown to reduce mortality after kidney transplantation due to decrease in liver complications[18,21,54], in the era of antiviral therapies, Lee *et al*[54] recently showed that deaths from liver complications remained a significant problem accounting for 40% of deaths in HBsAg patients and 22.2% of all mortalities that occurred in recipients treated with antiviral agents[54].

There are several plausible explanations for the increased risk of renal allograft failure in recipients with HBsAg-positivity. Firstly, it is known that chronic HBV infection can result in HBV-related membranous nephropathy, not only in patients with native kidneys but also in kidney transplant recipients[37-43]. Secondly, due to a concern of HBV reactivation, physicians may avoid or limit the use particular immunosuppression or Rituximab in HBsAg-positive patients when it is indicated such as for recurrent glomerulonephritis post-transplantation[61,62]. Lastly, treatment of chronic HBV infection itself such as tenofovir could affect renal function[63]. Thus, the findings from our meta-analysis confirm an increased risk of allograft failure in HBsAg seropositive patients, when compared to HBsAg-negative recipients. Also, we found a significant negative correlation between the risk of allograft failure in HBsAg positive patients and year of study. Recently, data analysis from the Organ Procurement Transplant Network/United Network for Organ Sharing database (OPTN/UNOS) suggested no increased risk for allograft failure or death in HBV-infected kidney transplant patients in a recent era (between 2001 and 2007)[36]. Although follow-up time was limited to only 3 years post-transplant, these data along with the findings from our study suggest potential improvements in patient and graft survivals in HBsAg-positive recipients overtime.

There are several limitations in this meta-analysis that bear mentioning. First, there was low to moderate statistical heterogeneity between studies in meta-

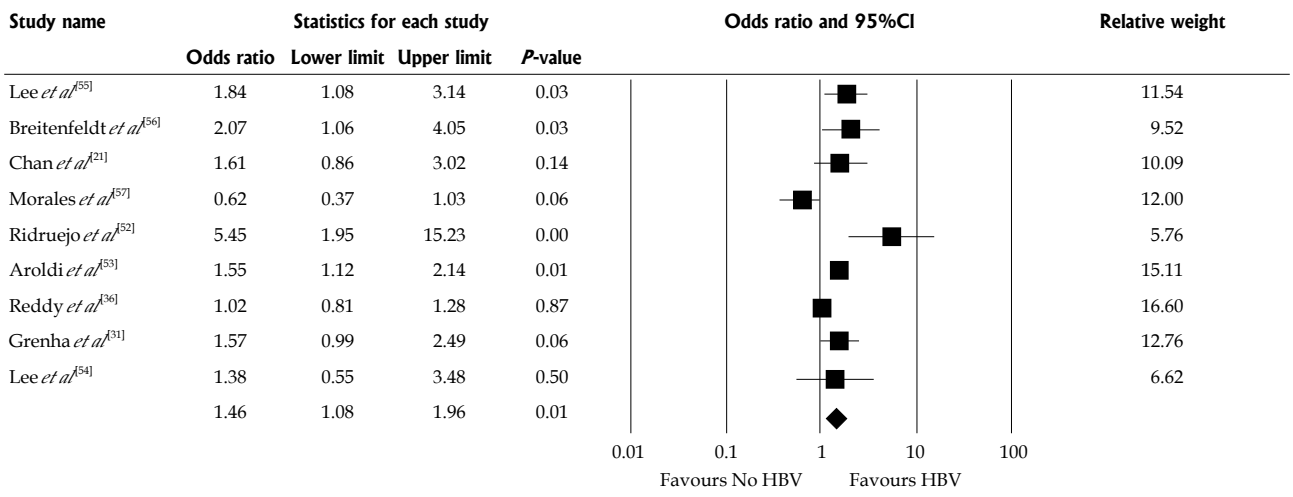

Figure 4 Forest plots of included studies evaluating renal allograft failure in patients with hepatitis B surface antigen-positive *vs* hepatitis B surface antigen-negative.

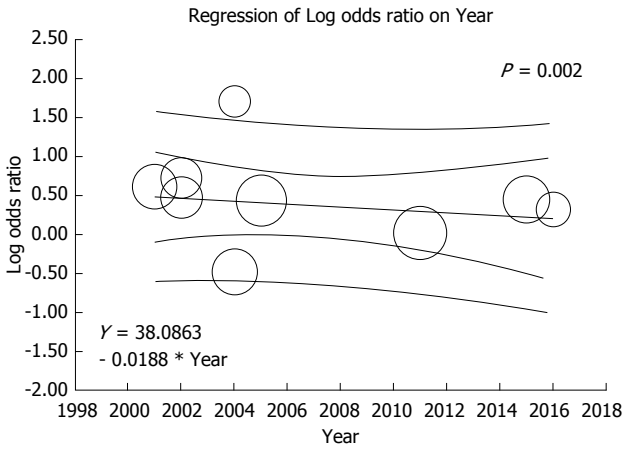

Figure 5 Graphical display of a significant negative correlation between year of study and risk of allograft failure.

analysis assessing the risks of mortality and allograft failure in HBsAg-positive recipients. The possible source of this heterogeneity includes the difference in population, type of donor, number of patients with positive HBeAg, immunosuppression regimens, and difference in confounder adjustments. In addition, the data on the graft quality (*e.g.*, Kidney Donor Profile Index) and surgical technique in HBsAg-positive recipients were limited. Second, despite the associations of HBsAg-positive status with poor kidney transplant outcomes, there is limited evidence whether the treatment with antiviral drugs for chronic HBV helps improve patient and allograft survival. However, with potential improvements in patient and graft survivals overtime demonstrated in our meta-analysis, future studies are required to evaluate if advancement in patient care for chronic HBV plays an important role. Also, additional studies are required to identify optimal antiviral treatment regimens and duration of suppressive therapy for HBV after kidney transplantation, since outcomes after withdrawal of antiviral treatment in kidney transplant recipients with chronic HBV infection remain unknown. While cautious withdrawal of antiviral therapy post kidney transplantation has been described especially in those with stable renal allograft function, low immunological risk for rejection and no evidence for HBV activity[10,23], fatal hepatitis flares in several kidney transplant recipients have been reported after withdrawal of antiviral therapy[64]. Lastly, this is a meta-analysis of observational studies. Thus, it can at best identify only associations of HBsAg-positive status with poor kidney transplant outcomes, but not a causal relationship.

In summary, our study reveals an association between HBsAg-positive status in kidney transplant recipients and higher risks of mortality and allograft failure after kidney transplantation. However, there are also significant negative correlations between the risks of mortality and allograft failure and year of study, representing potential improvements in patient and graft survivals overtime.

ARTICLE HIGHLIGHTS

Research background

Among renal transplant patients with hepatitis B virus (HBV) (HBsAg positive), there have been reported cases of HBV reactivation, massive liver necrosis due to fulminant hepatitis, and severe cholestatic hepatitis after kidney transplantation. In spite of improvement of HBV care, the outcomes of kidney transplantation including patient and allograft outcomes in recipients with HBV infection remain unclear.

Research motivation

Although hepatitis B is still incurable, there have been significant advancements in antiviral agents resulting in reasonably sustained suppression of HBV replication after kidney transplantation. The results of studies on kidney transplant outcomes in patients with renal transplant patients with HBV (HBsAg positive) were inconsistent. To further investigate outcomes of renal transplant patients with HBsAg positivity, the authors conducted this systematic review and meta-analysis reporting the association between HBsAg positivity in kidney transplant recipients and higher risks of mortality and allograft failure after kidney transplantation.

Research objectives
We conducted this meta-analysis to assess the outcomes of kidney transplantation including patient and allograft outcomes in recipients with HBV infection; and the trends of patient's outcomes overtime.

Research methods
A literature search was conducted using databases (MEDLINE, EMBASE and Cochrane Database) from inception through October 2017. Those studies reported odds ratios (OR) of mortality or renal allograft failure after kidney transplantation in HBV patients (defined as HBsAg positive) were included. HBsAg-negative kidney transplant recipients are the comparison group. The effect estimates from the individual study were extracted and combined.

Research results
The authors demonstrated that HBsAg-positive status in kidney transplant recipients was significantly associated with poor outcomes after transplantation. These associations existed in overall analysis as well as in limited cohort of hepatitis C virus-negative patients.

Research conclusions
The authors found significant associations of HBsAg positive status with poor outcomes after transplantation. Significant negative correlations between the risks of mortality and allograft failure and year of study, representing potential improvements in patient and graft survivals overtime were found.

Research perspectives
This study demonstrated significantly increased risks of mortality and allograft failure in HBsAg-positive kidney transplant recipients. This finding suggests that HBsAg positive status may be an independent potential risk factor for poor outcomes after transplantation. However, there are also potential improvements in patient and graft survivals with HBV infection overtime.

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CASE REPORT

Primary hepatic peripheral T-cell lymphoma associated with Epstein-Barr viral infection

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Author contributions: Ramai D, Ofori E and Nigar S designed the report; Ramai D and Reddy M collected the patient's clinical data; Ramai D wrote the paper; Ofori E, Nigar S and Reddy M edited the manuscript for intellectual content.

Informed consent statement: Regarding consent, we contacted our patient's spouse who reported that the patient has since passed.

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Abstract

Primary hepatic peripheral T-cell lymphoma (H-PTCL) is one of the rarest forms of non-Hodgkin lymphoma. We report a patient who presented with worsening jaundice, abdominal pain, and vomiting. Laboratory values were significant for elevated total bilirubin, alkaline phosphatase, and liver aminotransferases. Following a liver biopsy, histopathology revealed several large dense clusters of atypical T-lymphocytes which were CD2+, CD3+, CD5+, CD7-, CD4+, CD8-, CD56-, CD57-, CD30+ by immunohistochemistry. The proliferation index was approximately 70% by labeling for ki67/mib1. The above histological profile was consistent with peripheral T-cell lymphoma of the liver. Epstein-Barr viral serology indicated a remote infection, a likely risk factor for PTCL. Bone marrow biopsy was negative for malignancy, further supporting hepatic origin.

Key words: Primary lymphoma; Liver cancer; Non-Hodgkin's lymphoma; T-cell lymphoma

Core tip: Primary hepatic peripheral T-cell lymphoma (H-PTCL) is one of the rarest forms of non-Hodgkin lymphoma. We report a patient who presented with worsening jaundice, abdominal pain, and vomiting. Laboratory values were significant for elevated total bilirubin, alkaline phosphatase, and liver aminotransferases. Liver biopsy followed by histopathology confirmed the diagnosis of H-PTCL. Furthermore, bone marrow biopsy was negative for malignancy, further supporting hepatic

origin. Our patient's medical history reported a prior Epstein-Barr viral infection, a risk factor for H-PTCL. In the setting of risk factors, H-PTCL should be born in mind when a patient presents with symptoms of malignancy, and an enlarged and infiltrating liver.

Ramai D, Ofori E, Nigar S, Reddy M. Primary hepatic peripheral T-cell lymphoma associated with Epstein-Barr viral infection. *World J Hepatol* 2018; 10(2): 347-351 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/347.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.347

INTRODUCTION

Peripheral T-cell lymphoma (PTCL) is the rarest of all cases of non-Hodgkin lymphoma (NHL). It constitutes approximately 0.0016% of all extranodal lymphomas[1-3]. PTCL not otherwise specified (NOS) is a heterogeneous subset of nodal T-cell lymphomas which does not satisfy the criteria for the other subtypes of PTCLs, namely, angioimmunoblastic T-cell lymphoma, and follicular T-cell lymphoma[4]. The annual incidence rate for PTCL is 1.56 per 100000 persons in non-Hispanic Whites, 1.32 per 100000 in Blacks, 0.89 per 100000 in Asians/Pacific Islanders, 0.63 per 100000 in American Indians/Alaskan natives, and 0.96 per 100000 in Hispanic Whites[5]. The distribution of PTCL NOS among racial groups is reported to be highest amongst non-Hispanic Whites (2689), followed by Blacks (661), Hispanic Whites (418), Asian/Pacific Islanders (322), and lowest in American Indians/Alaskan natives (20)[5]. When the lesion is localized or arises from the liver, it may also be referred as primary hepatic peripheral T-cell lymphoma (H-PTCL). We present a 37-year-old male with worsening jaundice, abdominal pain, and vomiting who was diagnosed with hepatic peripheral T-cell lymphoma with a Ki-67 of 70%.

CASE REPORT

A 37-year-old male with a past medical history of Epstein-Barr Virus (EBV) infection was admitted for jaundice and right upper quadrant abdominal pain. He reported having worsening symptoms for one month duration. The patient was a non-smoker and non-alcohol drinker. Review of systems was positive for decreased appetite and weight loss of 10 lbs. over the past two months. His family history was unknown. Physical examination was significant for mild scleral icterus and abdominal distension. Heart rate was 92/min, blood pressure was 107/67 mm Hg, respiratory rate was 20/min, oxygen saturation was 94% on room air, and temperature was 98.1 °F. Laboratory results were within normal limits with a white blood cell count (WBC) of 11.4/μL, hemoglobin of 12.2 g/dL, hematocrit of 37%, and platelet count of 291 k/cmm. Total bilirubin was 5.7 mg/dL, alkaline phosphatase (ALP) was 1005 U/L, LDH was 830 U/L, albumin was 3.2 g/dL, aspartate aminotransferase (AST) was 257 U/L and alanine aminotransferase (ALT) was 239 U/L. EBV serology was negative for IgM, and positive for IgG and EBV nuclear antigen, consistent with prior infection.

Abdominal magnetic resonance imaging (MRI) showed mild intrahepatic ductal dilatation, peripheral areas of arterial enhancement in liver felt to be related to vascular shunting, periportal edema, a cut off in the course of the biliary tree at the bifurcation, a simple liver cyst, and enlarged left retroperitoneal nodes. Upper endoscopy showed gastropathy in the gastric fundus and body. Endoscopic ultrasound was unremarkable. Following a liver biopsy, histopathology showed several large dense clusters of atypical T-lymphocytes, which appeared to be centered in the portal areas. The atypical lymphocytes were medium to large in size and were CD2+, CD3+, CD4+, CD5+, CD7-, CD8-, CD56-, CD57-, CD30+, by immunohistochemistry (Figure 1).

The proliferation index was approximately 70% by labeling for ki67/mib1. Labeling for CD68 was seen in Kupffer cells, and in a few scattered histiocytes only. There were rare, scattered, unremarkable small B-lymphocytes (CD20+, CD79a+). Stains for CD138, kappa, lambda, were noncontributory. The above histological profile was consistent with hepatic peripheral T-cell lymphoma. The patient was subsequently transferred to a tertiary care center for further management where he had a bone marrow biopsy which was negative for malignancy, further supporting hepatic origin.

DISCUSSION

H-PTCL is mainly diagnosed by the presence of a hepatic mass in the absence of lymphadenopathy, splenomegaly, bone marrow involvement, and associated with normal tumor markers[6]. After six months following diagnosis, other tissues may become involved including the spleen, lymph nodes, peripheral blood, and/or bone marrow[7]. According to literature, H-PTCL commonly occurs around the fifth decade of life[8].

While the etiology of H-PTCL remains unclear, certain risk factors have been described such as Hepatitis C virus (HCV), Hepatitis B virus (HBV), and Epstein-Barr virus (EBV)[9-12]. In patients diagnosed with H-PTCL, HCV was identified in 20%-60% of cases[9]. This finding hints that viruses such as HCV may play a role in the pathogenesis of H-PTCL. Furthermore, H-PTCL has been diagnosed in immunocompromised patients with Human Immunodeficiency Virus (HIV), Human T-Lymphotropic Virus (HTLV), systemic erythematous lupus (SLE), and immunosuppressive therapy[13]. Our patient was negative for HCV and HBV infections, but his medical history indicated a prior EBV infection. While the tumor was CD30+, we did not pursue in-situ hybridization for Epstein-Barr

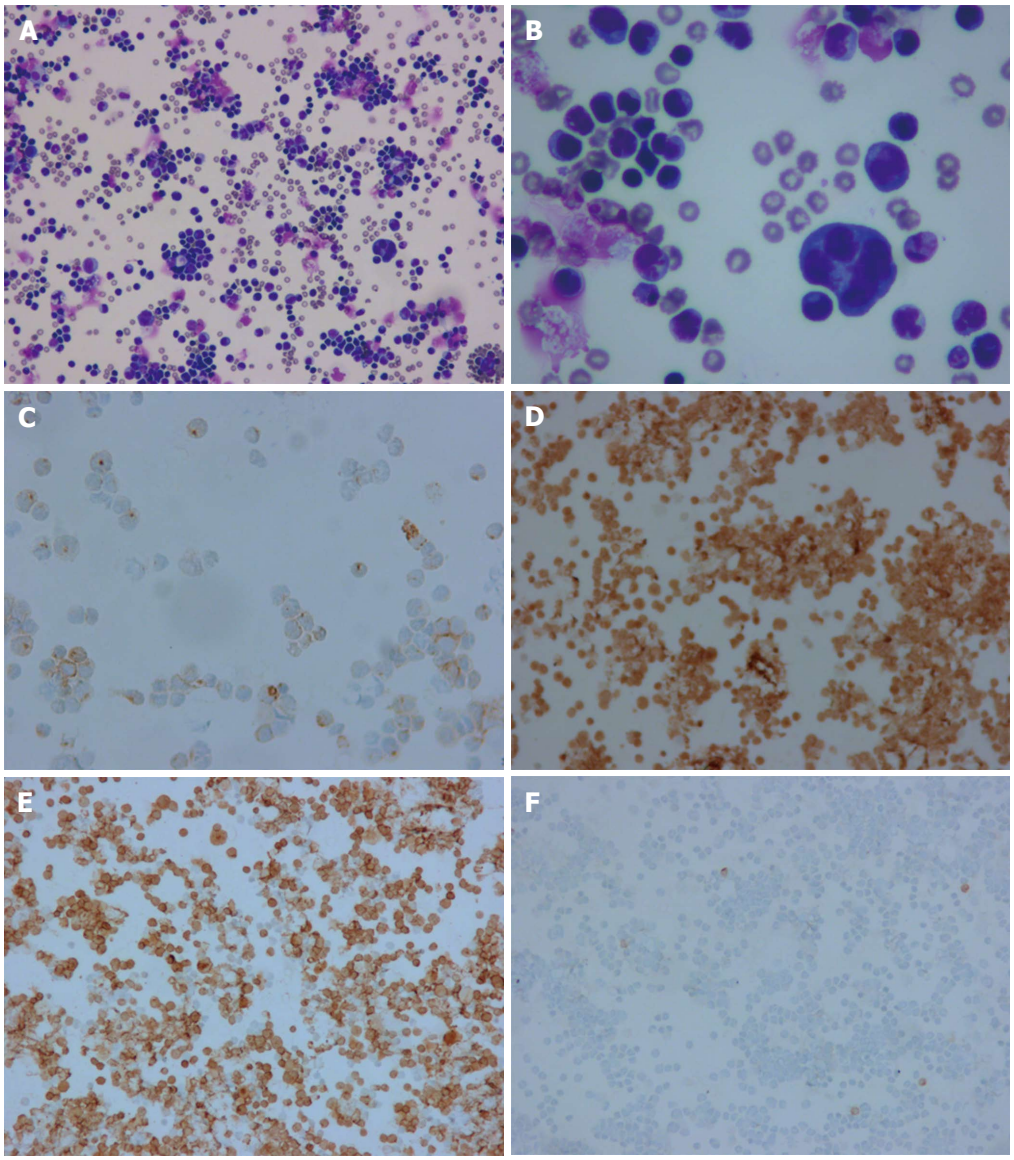

Figure 1 Biopsy results. A: large dense clusters of atypical T-lymphocytes 20 ×; B: atypical T-lymphocytes 40 ×; C: CD2 positive 40 ×; D: CD3 positive 20 ×; E: CD4 positive 20 ×; F: Scantly positive CD79a 20 ×.

virus-encoded RNA in lymphoma cells. Peng *et al*[14] reported the first case of EBV-associated CD30-positive peripheral T-cell lymphoma of cytotoxic phenotype. Our case provides further confirmation of an association of EBV infection and PTCL.

The clinical presentation of H-PTCL is non-specific, with the most reported symptom being abdominal pain in 40%-70% of patients, similar to our patient[3]. About 35% of PTCL patients experience systemic B symptoms including fever, night sweats, and weight loss[15]. Tumor markers alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) are typically normal in these patients[16]. Abnormal laboratory findings include elevated liver function aminotransferases, bilirubin, γ-glutamyl transferase, ALP and LDH. Approximately 70% of cases present with abnormal liver function enzymes, 30 to 80% with elevated LDH, 90% with elevated β2-microglobulin, and 80% with elevated ALP[17-20]. Mitarnun *et al*[20] reported that out of 100 patients with EBV associated H-PTCL, ALP was found elevated in 80%, while LDH was elevated in 65% of cases. Our patient presented with significantly notable ALP, LDH, total bilirubin, and liver function enzymes.

The proliferation index measured by Ki-67 has traditionally been used in assessing patient prognosis and response to therapy. Went *et al*[21] proposed a prognostic model which incorporated age (> 60 years), high lactate dehydrogenase, poor performance status, and Ki-67 greater or equal to 80%. Their model was significantly associated with patient outcome ($P < 0.0001$). Weisenburger *et al*[15] reported a Ki-67 > 25% was an adverse predictor of survival.

Our case was classified as PTCL-NOS according to guidelines outlined by the World Health Organization[4]. The running differential diagnosis included extranodal NK/T-cell lymphoma, nasal type and adult T-cell leukemia/lymphoma. Extranodal NK/T-cell lymphoma, nasal type, was considered due to a prior EBV

infection, however, it was ruled out after being CD56 negative. CD56 is a diagnostic requisite for extranodal NK/T-cell lymphoma, nasal type[22]. Adult T-cell leukemia/lymphoma was ruled out given that the patient's calcium levels and WBC were within normal limits[23].

Treatment for PTCL requires an aggressive course of chemotherapy, typically cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisone (CHOP). A recent study by Kim *et al*[22] reported that patients with whole blood EBV-DNA were more likely to have aggressive clinical characteristics and inferior survival. Overall, H-PTCL has a poor prognosis due to life threatening complications and tumor progression. Clinical studies report that CHOP therapy can provide up to 60% complete remission, and a 30%-50% five-year survival rate[24-26].

More recently, a prospective study of 499 patients showed that patients who received doxorubicin had a significantly longer survival than those who did not (P = 0.03)[27]. Furthermore, in a study involving 775 patients, better survival outcomes were seen in one third of patients who remained in remission 2 years after diagnosis, especially in younger patients less than 60-years of age[28]. However, Abramson *et al*[29] reported that the most dominant prognostic factor was response to initial therapy, with no overall survival difference based on the choice of upfront regimen. These studies further reemphasizes the need for early detection and treatment.

In conclusion, we report a rare case of H-PTCL in a 37-year old male with a medical history of EBV infection who presented with worsening jaundice, abdominal pain, and vomiting. H-PTCL is an aggressive form of NHL which requires early diagnosis and a robust treatment regimen. However, the diagnosis of H-PTCL remains challenging due to the presence of multiple granulomas, histiocytosis, and focal neoplastic infiltrates. In the setting of worsening symptoms and abnormal liver enzymes of unknown etiology, clinicians should consider performing a differential liver biopsy.

ARTICLE HIGHLIGHTS

Case characteristics

A 37-year-old male with a past medical history of Epstein-Barr Virus infection reported having jaundice, right upper quadrant pain, and decreased appetite and weight loss of 10 lbs over the past two months.

Clinical diagnosis

Abdominal magnetic resonance imaging showed mild intrahepatic ductal dilatation, peripheral areas of arterial enhancement in the liver felt to be related to vascular shunting, periportal edema, a cut off in the course of biliary tree at the bifurcation, simple liver cyst, and enlarged left retroperitoneal nodes.

Differential diagnosis

Cirrhosis, hepatocellular carcinoma, cholangiocarcinoma.

Laboratory diagnosis

Laboratory was significant for total bilirubin of 5.7 mg/dL, alkaline phosphatase of 1005 U/L, albumin of 3.2 g/dL, and AST/ALT of 257/239 U/L.

Imaging diagnosis

Upper endoscopy showed gastropathy in the gastric fundus and body. Endoscopic ultrasound was unremarkable.

Pathological diagnosis

A liver biopsy showed several large dense clusters of atypical T-lymphocytes, which appeared to be centered in portal areas. The atypical lymphocytes were medium to large in size and were CD2+, CD3+, CD5+, CD7-, CD4+, CD8-, CD56-, CD57-, CD30+, by immunohistochemistry. The proliferation index was approximately 70% by labeling for ki67/mib1. Labeling for CD68 was seen in Kupffer cells, and in a few scattered histiocytes only. There were rare, scattered, unremarkable small B-lymphocytes (CD20+, CD79a+). Stains for CD138, kappa, lambda, were noncontributory. The above histological profile was consistent with hepatic peripheral T-cell lymphoma (H-PTCL).

Treatment

The patient was transferred to a tertiary center for chemotherapy (CHOP) treatment.

Related reports

H-PTCL has a poor prognosis due to life threatening complications and tumor progression. Clinical studies reports that CHOP therapy can provide up to 60% complete remission, and a 30%-50% five-year survival rate.

Term explanation

H-PTCL is one of the rarest forms of non-Hodgkin lymphoma. It constitutes approximately 0.0016% of all extranodal lymphomas.

Experiences and lessons

In the setting of worsening symptoms and abnormal liver enzymes of unknown etiology, clinicians should consider performing a differential liver biopsy. Clinicians should also be aware of the risk factors for H-PTCL.

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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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REVIEW

Reactivation of hepatitis B after liver transplantation: Current knowledge, molecular mechanisms and implications in management

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Abstract

Chronic hepatitis B (CHB) is a major global health problem affecting an estimated 350 million people with more than 786000 individuals dying annually due to complications, such as cirrhosis, liver failure and hepatocellular carcinoma (HCC). Liver transplantation (LT) is considered gold standard for treatment of hepatitis B virus (HBV)-related liver failure and HCC. However, post-transplant viral reactivation can be detrimental to allograft function, leading to poor survival. Prophylaxis with high-dose hepatitis B immunoglobulin (HBIG) and anti-viral drugs have achieved remarkable progress in LT by suppressing

viral replication and improving long-term survival. The combination of lamivudine (LAM) plus HBIG has been for many years the most widely used. However, life-long HBIG use is both cumbersome and costly, whereas long-term use of LAM results in resistant virus. Recently, in an effort to develop HBIG-free protocols, high potency nucleos(t)ide analogues, such as Entecavir or Tenofovir, have been tried either as monotherapy or in combination with low-dose HBIG with excellent results. Current focus is on novel antiviral targets, especially for covalently closed circular DNA (cccDNA), in an effort to eradicate HBV infection instead of viral suppression. However, there are several other molecular mechanisms through which HBV may reactivate and need equal attention. The purpose of this review is to address post-LT HBV reactivation, its risk factors, underlying molecular mechanisms, and recent advancements and future of anti-viral therapy.

Key words: Hepatitis B virus; Liver transplantation; Reactivation; Hepatitis B immunoglobulin; Recurrence; Prophylaxis; Antivirals

Core tip: Aim of this review is to summarize the current concepts and management of hepatitis B after liver transplantation (LT). There are no clear guidelines regarding hepatitis B therapy after transplantation. Hepatitis B immunoglobulin (HBIG) is expensive and cumbersome to administer and there is no definite time point for discontinuation of HBIG after LT. Here we summarize the indications and duration of hepatitis B immunoglobulin and nucleoside analogs. This review also addresses key molecular mechanisms and the risk factors which are associated with hepatitis B virus reactivation post LT. This review provides up-to-date information not only for the liver transplant specialists but also for the virologists and scientists working in this field.

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INTRODUCTION

Chronic hepatitis B (CHB) caused by hepatitis B virus (HBV) infection remains a major global health problem affecting an estimated 350 million people worldwide with more than 786000 individuals dying annually due to complications of CHB, including cirrhosis and liver cancer. CHB is the leading cause of hepatocellular carcinoma (HCC) accounting for at least 50% of newly diagnosed cases[1]. Furthermore, HCC is the third leading cause of cancer-related mortality in the world[2] with a dismal 5 year survival and the fastest growing rate of cancer death in North America[3]. Liver transplantation (LT) is the most effective treatment in patients with CHB-related liver failure, cirrhosis and HCC. However, HBV reactivation following LT emerges as a major clinical challenge[4].

Over the past decade, a substantial advancement has been made in the treatment of CHB, and to date several potent antiviral medications are available for the treatment of HBV infection, mainly gaining long-term viral suppression[5,6]. However, despite of having strong suppressive antiviral therapy for chronically HBV-infected patients, some patients still develop HCC possibly due to the presence of minimal residual viremia (MRV) and irreversible HBV DNA integration into liver genome. MRV is a consequence of persistent, low-level virus replication in the liver and at the extrahepatic sites, particularly in peripheral blood mononuclear cells (PBMC), coinciding with circulation of virus traces[7-10]. Despite long-term antiviral treatment with suppression of viral DNA, MRV commonly persist[7,11]. One of the major sources of MRV is supercoiled HBV covalently closed circular DNA (cccDNA) and its persistence is mainly responsible for recurrent HBV infection post-LT[12,13]. Prior to introduction of hepatitis B immunoglobulin (HBIG) in 1990s, HBV recurrence in LT was as high as 75% to 89% of patients with 3-year survival rate in 54%[14,15]. The introduction of viral suppression strategy using combination of HBIG and more potent nucleos(t)ide analogs (NAs) has significantly decreased the HBV recurrence in vast majority of these patients improving their long-term survival[16]. However, this strategy does not completely eradicate HBV and, therefore, does not protect against future recurrence of symptomatic HBV infection. It also requires monitoring of LT patients for life, thus significantly increasing the economic burden and manpower engagement.

Evaluating the risk of HBV recurrence is crucial in devising effective strategy against post-LT reactivation. The factors associated with high rates of HBV reactivation are high viral load prior to the transplant, HBV e antigen (HBeAg) reactivity, co-infection with human immunodeficiency virus type 1 (HIV), non-compliance with drug therapy, HCC at the time of LT, and anti-viral drug resistance. On the other hand, low viral load, anti-HBe positivity and anti-HBs presence are factors with lower risk of HBV reactivation[15,17-21].

MOLECULAR MECHANISMS OF HEPATITIS B REACTIVATION IN LIVER TREANSPLANTATION

cccDNA and its role in HBV reactivation

Although HBV is a DNA virus, it replicates by reverse transcription intermediate[22]. Establishment of cccDNA is crucial in the HBV life cycle. This nuclear cccDNA

minichromosomal acts as the powerhouse of HBV transcriptional machinery and constitutes a molecular basis for virus reactivation[12]. HBV cccDNA chronically exists throughout the natural history of HBV infection[23] and it is not yet possible to eradicate this HBV molecule even with current potent anti-viral therapies, such as Entecavir (ETV) or Tenofovir disoproxil fumarate (TDF)[24]. A recent study by Papatheodoritis *et al*[25] showed that despite of the anti-HBV therapy, HCC develops in the context of the cccDNA presence and, thus, MRV and reactivation cannot be ruled out.

When recipients receive transplantation with liver from donors with previous history of HBV infection, but with negative serum HBsAg and HBV DNA, intrahepatic cccDNA could still be detected after LT[4,26]. Notably, detection of anti-HBc alone in the absence of HBsAg and HBV DNA in a donor should be treated as an indicator of occult infection and a low-level virus replication in the liver, which could be reactivated post-LT[18,27,28]. On the other hand, patients with undetectable HBV viremia at LT and no evidence of cccDNA and intrahepatic HBV DNA on repeat examinations -may be safely withdrawn from long-term prophylaxis[29]. However, safe withdrawal also depends on the level of the sensitivity of the assays used for detecting HBV viremia, HBV cccDNA in the liver and the existence of HBV replication at the extrahepatic sites [*e.g.,* peripheral blood mononuclear cells (PBMC)], which in occult cases may be missed even using ultrasensitive tests.

Genotype-specific recurrence of HBV

Ten different HBV genotypes have been identified which are scattered in an ethno-geographically specific manner. Ample of evidence suggested the role of HBV genotypes in disease progression, mode of transmission, disease severity, HCC risk, and response to therapy[30]. Compared to genotype D, HBV genotype A responds well to the interferon therapy[31]. Numerous reports across the globe documented association of HBV genotype B and C with severe liver disease including development of HCC[32], while HBV genotype C has higher risk for mother to child transmission[33]. Since virus evolves within the host, study of HBV genotype is important prior to LT, especially in genotypes, which are associated with the occult HBV infection[34,35]. A study by Devarbhavi *et al*[34] demonstrated that patients with HBV genotype D have the highest risk of HBV recurrence and mortality compared to genotype A. In our recent study, we demonstrated that viral genotypes fluctuate while patient is on the Tenofovir therapy, revealing two important phenomena, first, there is mixture of viral populations present in HBV infected patient and secondly, at a given time, only one of the viral strain is inhibited/exhibits[36]. Although, not with regard to the HBV genotypes, but from the point of HBV quasispecies an elegant study by Buti *et al*[37] identified HBV quasi-species evolution after LT in patients under long-term lamivudine prophylaxis with or without HBIG and there was low transient viremia detected even in the absence of serum HBsAg, showing importance of continuing HBV prophylaxis. In the same context, a recent case study by Mina *et al*[38] showed that HBV genotypes fluctuates after LT, which could possibly be the main reason behind the HBV reactivation in liver transplant settings. Since, in diagnostic assays, the possible source of HBV reactivation is negated, it is an open question, if extrahepatic tissues should be tested to find the origin of such reactivation. Studies focusing on HBV recurrence based on genotype are summarized in Table 1. It would be worthwhile to consider HBV genotyping in both donor and recipient so that each viral strain is tracked in case of the mixed genotype infections, which are emerging as important hidden source for reactivation.

Co-existing hepatitis D virus infection and HBV reactivation

Hepatitis delta virus (HDV) consists of a single-stranded RNA molecule enveloped by hepatitis B surface antigen (HBsAg)[39]. One of the risk factors of HBV recurrence in LT patients is the co-infection with hepatitis delta virus (HDV)[40]. Fulminant hepatitis B reactivation in co-infected patients has been reported[40,41]. HBsAg-positive liver grafts in HBsAg-positive recipients with HDV co-infection has been reported to result in virological recurrence and rapid development of liver cirrhosis, and need for re-transplant[42,43]. HDV is a RNA pathogenic virus that requires presence of HBV for its survival[44]. Studies on post-LT patients suggest that the absence of HBV prophylaxis or lack of proper function of HBIG leads to higher incidence of both HBV and HDV reinfection[43,45,46].

The co-existence or co-infection of HBV and HDV is very commonly observed, obviously due to the dependence of HDV infection on HBV. For instance, 11.9% of HBV-positive patients were also positive for HDV in an Italian liver patient cohort, with a higher incident in patients older than 50 years[47]. It also appears to have a geographical connection, as co-infection HBV-HDV in LT patients was found to be low in Japan[48], possibly due to the differential geographical distribution of HDV genotypes Ⅰ and Ⅱ between other parts of the world and Asian countries, respectively[49]. The helper functions of HBV provide the support to HDV for cell entry, replication, virion assembly and export[50]. The interactions between HBV-HDV occur in two phases, the first phase of active HDV replication occurs with the suppression of HBV, followed by reactivation of HBV and reduction in HDV in the second phase[51]. Due to this nature of HDV and HBV interactions, early recurrence of HDV has been detected in many patients in the absence of HBV recurrence[52]. Studies also imply that HDV could be a cause for many subclinical infections and symptoms develop rapidly upon recurrence of HBV[45]. HBV recurrence has been shown to cause atypical reappearance of HBV infection and HDV relapse in the

Table 1 Recurrence of hepatitis B virus in different genotypes

HBV genotype	No. of patients	Median follow-up (mo)	HBV recurrence number (%)	Mortality number (%)
Girlanda *et al*[175], 2004				
A	15	56	4 (27)	2 (13)
D	13	67	7 (54)	5 (38)
A/D	12	43	4 (33)	2 (17)
A/C	2	66	1 (50)	0
E	2	45	1 (50)	1 (50)
C	1	106	1 (100)	0
Devarbhavi *et al*[34], 2002				
A	10	56	3 (30)	1 (10)
C	6	22.5	3 (50)	1 (10)
D	5	15	3 (60)	1 (10)
E	1	1	0	Lost follow-up
Gaglio *et al*[176], 2008				
A	28	24	3 (10.7)	3 (10.7)
B	8	24	1 (12.5)	1 (12.5)
C	18	24	1 (5.5)	5 (5.5)
D	6	24	0	0
Lo *et al*[177], 2005				
B	43	36	4 (2)	7 (17)
C	74	36	21 (15)	7.5 (11)

HBV: Hepatitis B virus.

allographs[53]. Additionally, the recurrence of HBV-HDV post-LT is the cause of death for many LT patients, prompting need for more research on this subject[45,54]. In a recent study, recurrence rate of HBV after LT was not different from the recurrence rate of HBV-HDV co-infection on long-term low-dose HBIG prophylaxis along with TDF[55].

Genetic variations of host genetic makeup in predicting HBV reactivation

Genetic variations of host genetic makeup may play some role in increased/reduced risk of HBV reactivation after LT. Single-nucleotide polymorphisms (SNP) of two-gene locus cytotoxic T lymphocyte antigen-4 (CTLA-4) +49 and CD86 +1057 were previously reported to influence the outcome of LT with respect to allograft acceptance[56,57]. Homozygosity for CTLA-4 +49 (G/G genotype) was reported to be associated with reduced risk of HBV recurrence in post-LT Chinese patients[56]. CD86 and CTLA-4 are known to stimulate and inhibit T cell activation, respectively.

Role of superinfection in HBV reactivation

Superinfection is defined as the infection with a second virus or a different strain of virus at a later time point, after the establishment of persistent infection of the first virus[51,58].

Superinfection with HDV of an individual chronically infected with HBV may have deleterious consequences[59]. This pattern of infection causes a severe acute hepatitis that may be self-limited but that in most cases (up to 80%) progresses to chronicity[60]. The resultant chronic HDV infection usually exacerbates the preexisting CHB[60]. It is to be noted that HBV replication is usually suppressed by HDV, and this suppression becomes persistent in the case of a chronic HDV infection[61,62]. Due to concern for HDV superinfection in post-LT setting, it is of utmost importance to prevent HBV recurrence after LT. Nonetheless, patients chronically co-infected with HDV are less at risk of HBV recurrence and have a better survival rate than patients infected with HBV alone. Patients co-infected with HDV generally do not require pre-transplant antiviral therapy due to HBV suppression and low viral load. Although potent HBV DNA-polymerase inhibitors can control HBV replication, reappearance of HBsAg and/or the persistence of HBV DNA in serum, liver, or PBMC might have deleterious consequences in the setting of HBV-HDV co-infection as they may provide the biologic substrate to the reactivation of HDV[40]. No effective antiviral drug is available for the treatment of graft infection with HDV, and potentially the best approach is to keep them on long-term potent antiviral therapy along with low dose of HBIG (Figure 1).

As mentioned before, HBV has ten genotypes named A-J, and they influence the disease outcome and treatment to antiviral therapy[63]. Depending on the geographical location, patients may have one or mixed genotypes of HBV in infected patients and consequences of which possibly have the recombinant HBV genotypes[64-66]. The genotype C of HBV was observed in majority of the HBV-infected patients with acute exacerbation[67]. An earlier published review reported that HBV genotypes D and C are associated with a lower rate of favorable response to alfa-interferon and pegylated-interferon alfa-2b therapy than genotypes A and B[68]. The rate of resistance to lamivudine (LAM) was higher in patients with genotype A infection than in patients infected by genotype D, whereas no difference in the risk of LAM resistance is found between patients

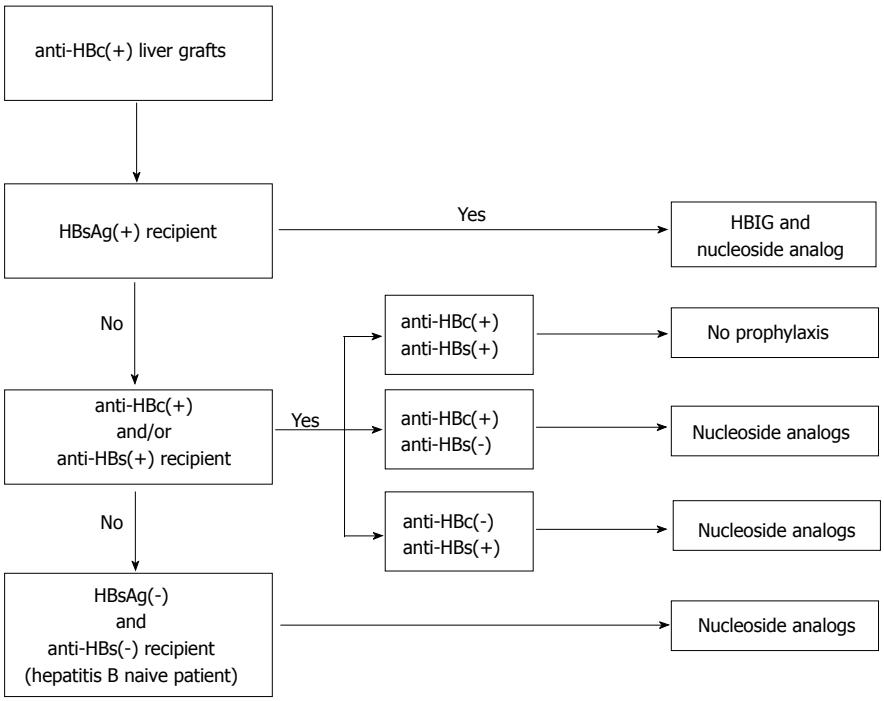

Figure 1 Stepwise approach of anti-hepatitis B core positive grafts allocated to recipients based on their hepatitis B serology. In chronic hepatitis B patients with HBsAg positive and who receive Anti-HBc positive liver grafts should be treated with HBIG and nucleoside analogs. If the recipient is HBsAg negative and Anti-HBc positive and/or anti HBs positive, NA is used for prophylaxis based on anti HBc and anti HBs serologies. No prophylaxis is recommended for anti-HBc positive and anti-HBs positive liver in LT recipient without HBsAg positive serology. These patients should be followed with periodic HBV DNA level guided by ALT to monitor for any relapse. In Hepatitis B naïve patients, NA is recommended for prophylaxis. HBIG: Hepatitis B Immunoglobulin; HBsAg: Hepatitis B surface antigen; Anti-HBs: Hepatitis B surface antibody; Anti-HBc: Hepatitis B core antibody.

with genotype B and patients with genotype C[68]. Later studies using potent nucleotide analogue have shown no genotype specific differences in treatment responses[69]. Another challenge with HBV is the generation of HBV variants through splicing. These variants may get activated with the disease progression post-LT leading to undesirable clinical outcomes as well as the development of drug resistance[70].

Role of HBV integration in HBV reactivation after LT

The role of HBV DNA integration in the genome of host liver cells has been studied from the early 1980s and it had long been postulated to have implications for the antiviral therapy for HBV[71,72]. Recent study demonstrated that HBV can integrate into the host genome immediately after its invasion[73]. HBV DNA integration has been detected in all the stages of HBV infection including occult HBV infection[74]. The potential for oncogenicity has been proven in the woodchuck model with occult WHV infection[75]. In a study from Japan, eighty-two consecutive Japanese patients with cirrhosis, who were negative for serum HBsAg and antibody to hepatitis C virus (anti-HCV) were observed for a median of 5.8 years[76]. The HCC development rates in the patients HBV DNA-positive and HBV DNA-negative were 27.0% and 11.8% at the end of the 5th year, and 100% and 17.6% at the 10th year, respectively.

The clinical significance of occult HBV infection has not been well studied in LT recipients. A recent study investigated the prevalence of occult HBV infection in cirrhotic patients undergoing LT in a Brazilian referral center[77]. Liver samples from 68 adults were analyzed using a nested polymerase chain reaction assay for HBV DNA and occult HBV infection was diagnosed in three (4.4%) patients. Markers of previous HBV infection were available in two patients with occult HBV infection and were negative in both. Clinical impact of occult HBV infection in immunosuppressed individuals has been recently reviewed[78]. These results suggest potential for HBV reactivation post-LT from occult HBV infection. In fact, a recent study with 43 patients with alcoholic cirrhosis, who were negative for serum HBsAg before LT, detectable HBV DNA in the explanted liver was evident in 41.9%[79]. *De novo* HBV infection occurred in 18.6% (8/43) of the recipients at a median of 10 mo after LT.

Extrahepatic replication of HBV and its role in HBV reactivation

Numerous reports demonstrated the presence of HBV DNA, virus genome replicative intermediates and viral proteins in hepatic tissue, and HBV DNA and HBsAg in serum of HBV-infected persons, but the existence of extrahepatic sites of HBV replication are not as well recognized. Nonetheless, the accumulated data indicate that PBMC and different immune cell types can support HBV replication[27,80-83]. Stronger evidence came from the woodchuck model of HBV infection[27,75,84-87]. There are also occasional observations that endothelial cells, epithelial cells, neurons, macrophages and polymorphonuclear leukocytes could be permissive to

HBV infection in humans[88]. HBV replication was also demonstrated in *in vitro* bone marrow cultures and lymphatic tissues of patients with CHB[89-91]. In the woodchuck model of hepatitis B, extrahepatic replication of the woodchuck hepatitis virus and infectivity of the virus derived from lymphoid cells were clearly delineated[75,87]. Interestingly, in some situations, the lymphatic (immune) system might be the only site of virus replication in this model[75,86,87,92].

In one of the xenotransplantation study in patients with baboon liver transplants, Lanford *et al*[93] demonstrated the persistence of HBV DNA in several extrahepatic tissues after HBV replication halted in the liver. In the woodchuck model, the mothers with resident hepadnaviral infection cells transmit the infection to their offspring which is predominantly restricted to their lymphatic system[84]. These observations suggest that the attachment preferences of HBV to cellular receptors on diverse cell types might be responsible for the quasispecies specific compartmentalization of HBV[94]. Studies related to genetic variability, drug resistance and potential immune evasion mechanisms of virus in plasma and PBMC of patients with CHB have also been investigated[95,96]. Because of the diverse nature of the HBV in hepatic and extrahepatic tissues, the response to therapy has been shown to be different in PBMC-restricted HBV compared to hepatic HBV[95]. In these studies, liver, plasma as well as PBMC samples were evaluated using ultrasensitive assays for the quasispecies compatibility in LT patients under long term prophylaxis. The authors inferred that extrahepatic HBV is always detectable in the serum, liver, and PBMC of almost all patients despite prophylaxis, supporting continuation of anti-HBV therapy[95,96]. However, there is not a study yet that demonstrated that reactivation can solely originate from extrahepatic sites.

THE RISK OF HBV REACTIVATION IN LIVER TRANSPLANT PATIENTS UNDERGOING IMMUNOSUPPRESSION THERAPY

Upon HBV entry, the level at which HBV persists depends on the interplay between the viral replication rate and the host immune response. LT patients with prior HBV infection could experience a reactivation of HBV following LT due to immunosuppressive therapy, potentially leading to deleterious consequences, including graft failure and death[97-99].

HBV reactivation in immunosuppressed patients

Immune mechanism: HBV cccDNA and low levels of HBV DNA and RNA remain detectable in host hepatocytes even in patients exposed to HBV who have developed anti-HBs after apparent complete clearance of serum HBsAg and HBV DNA from a recent infection[87,100]. Hence, there seems to be a balance between host HBV-specific T cell and innate immune responses and virus replication that maintains the latency of the viral infection[80,101,102]. Immunosuppressive therapy or cancer chemotherapy may lead to induce imbalance of these mechanisms which causes HBV reactivation[101,103].

Non-immune mechanism: HBV infection can also be flared by steroids[104]. This may include stimulation of a glucocorticoid-responsive element (GRE) in the HBV genome which leads to up regulation of HBV gene expression[105]. In addition, mechanistic target of rapamycin (mTOR) inhibitors, like rapamycin, that are used as immunosuppressive drugs in LT patients and certain cancers, are reported to enhance HBV reactivation in patients[106]. It is also shown that maintaining an immunosuppressive regimen using mTOR-inhibitors post-LT commonly reactivate HBV infection, along with infections with other viruses, such as HCV, cytomegalovirus (CMV), HIV-1, human papilloma virus (HPV), Epstein Barr virus (EBV) and herpes simplex virus (HSV) as well[107].

HCC recurrence after LT

In a Chinese registry study, patients undergoing LT due to HBV-related HCC *vs* HCV-related HCC demonstrated recurrence of HCC at a significantly higher rate in HBV-HCC cohort (26.39%) compared to that in HCV-HCC cohort (9.07%) ($P < 0.001$)[108]. The risk factors for HCC recurrence were: elevated serum alpha fetoprotein, large tumor volume, microvascular invasion, high serum HBV DNA and HBsAg levels, and immunosuppression[109,110].

Younger age has been suggested as a significant risk factor for HBV infection-related HCC recurrence after LT. It has been proposed that this could be due to the vertical transmission of HBV from the occult HBV infection harboring mother and HBV immune tolerant state of the younger patients, triggering HCC recurrence[111,112].

PROPHYLAXIS FOR HBV REACTIVATION AFTER LT

HBsAg-positive patients

Introduction of HBIG in prevention of HBV reactivation following LT was a major milestone. HBIG is pooled polyclonal antibody against HBsAg. Although its mechanism of action remains incompletely understood, it is believed that it prohibits binding of virions to hepatocytes or promotes lysis of infected hepatocytes[113]. In the initial days, prophylaxis for recurrent HBV infection was administered to HBsAg-positive patients using HBIG or LAM monotherapy. This strategy showed significant reduction in re-infection and improvement of graft survival after LT[14,15,114]. Although graft survival was largely improved with either HBIG or LAM monotherapy, the re-infection rates were continued to be 30%-40% of patients[15,19,115]. Furthermore, LAM monotherapy

resulted in development of HBV reverse transcriptase mutations that lead to antiviral drug resistance. When LT patients were on only HBIG prophylactic therapy, their chance of developing HBV escape mutations was significantly higher[116], and this lead to *de novo* HBV infection in some patients after LT[17,117]. First described in 1998, combination therapies of HBIG with NA were successful in controlling HBV infection in most of the patients. None of the 59 patients undergoing LT for HBV-related liver failure who received high dose of HBIG intra- and post-operatively in combination with LAM as prophylaxis, showed detectable HBV DNA after 459 days of treatment[118]. By combining LAM with HBIG, the HBV recurrence rate further dropped to less than 5%. The success of this combination regimen led it to become the most favored antiviral prophylactic regimen in lLT centers worldwide. Despite being effective, HBIG was very expensive and unavailable to a significant percentage of the patient population, and it requires regular parental injections and monitoring. In view of this, lower-dose HBIG in combination with LAM was evaluated and was found to be equally effective[119-121]. However, this combination approach of HBIG with an oral antiviral medication is of historical value only and neither alone was sufficient in preventing HBV reactivation or recurrence. With the availability of newer and more potent oral NA, there has been a shift from HBIG combination therapy to NA alone. A systematic review by Cholongitas *et al*[122] noted a higher recurrence rate with combination of HBIG plus LAM compared to HBIG plus ETV/TDF (6.1% *vs* 1%, $P = 0.004$). A meta-analysis has shown that compared to high dose HBIG-LAM combination, low dose HBIG and potent NAs (TDF or ETV) demonstrated significantly lesser HBV recurrence[123]. Both ETV and TDF have been associated with resistance rate of less than 2% after 5 years in patients with HBV infection[124]. Several earlier studies have demonstrated usefulness of long term HBIG, and more recent studies have demonstrated safe withdrawal of HBIG with continuation of oral antiviral therapies alone by adopting a limited duration of HBIG use in the protocol[119,121,125-138] (Tables 2 and 3).

Hepatitis B core antibody-positive liver donor

LT from hepatitis B core antibody (anti-HBc)-positive donors is being increasingly used due to the shortage of organs. However, due to immunosuppressive therapy, the risk of HBV reactivation is higher after LT in these patients[139]. In a systematic review of 39 studies involving 903 LT patients, Cholongitas *et al*[139] evaluated the risk of HBV recurrence after LT with grafts from anti-HBc-positive donors and effect of anti-HBV prophylaxis. HBV recurrence was found to be 11% in HBsAg-positive LT patients who received anti-HBc-positive grafts compared to anti-HBc-negative grafts, but overall survival was same in both groups. They also noted that *de novo* HBV infection occurred in 19% of HBsAg-negative patients receiving anti-HBc-positive grafts. Without prophylaxis, HBV re-activation was 15% in anti-HBc/anti-HBs-positive recipients and 48% in HBV naïve patients. However, prophylaxis using HBIG, LAM or a combination decreased re-infection rate significantly. Similarly, *de novo* HBV infection rates in HBsAg-negative patients decreased to 19%, 2.6% and 2.8% using HBIG, LAM and combination, respectively.

This study suggests that anti-HBc positive grafts can be donated safely to HBsAg-positive and anti-HBc/anti-HBs-positive sub groups, and antiviral prophylaxis decreases post-LT reactivation significantly. Due to high risk of reactivation in HBV-naïve patients, anti-HBc-positive grafts should only be considered if other two sub-group recipients are not available[140]. Figure 2 shows stepwise approach in allocating anti-HBc-positive grafts based on recipients HBV serology and prophylaxis after LT.

Anti-HBs and Anti- HBc-positive recipients

De novo HBV infection is substantially lower in anti-HBc and/or anti-HBs-positive compared to HBV-naive recipients[141]. The presence of anti-HBs seems to protect from *de novo* HBV infection and both anti-HBc and anti-HBs-positive recipients represent a group that can safely receive anti-HBc-positive liver grafts without any post-transplant HBV prophylaxis (probability of *de novo* HBV infection < 2%)[142-151]. These patients should however be followed with periodic HBV DNA level guided by ALT to monitor for any relapse. Despite this low risk, many centers prefer to continue with NA without HBIG in this subgroup of patients, and future studies will further clarify this concept (author's personal communication). Figure 3 shows stepwise approach in allocating anti-HBc-positive grafts based on recipients HBV serology and prophylaxis after LT.

Duration of HBIG administration

Currently, there is no consensus regarding the duration of use and dose of HBIG as a component of prophylaxis, and many experts believe in an individualized approach to use of HBIG in prophylaxis[152-154]. A recent study has demonstrated that in HBV-infected patients undergoing LT, who have HBV DNA levels less than 100 U/L and an absence of co-infection with HIV or HDV, a very short course of HBIG in combination with long-term antiviral therapy is highly effective in preventing HBV recurrence[130]. Chen *et al*[131] has shown infusion of two high doses of HBIG during surgery in combination with ETV significantly prevented HBV recurrence and improved the 3-year survival after LY. Another, potential cost saving approach could be combination of ETV plus low-dose on-demand HBIG[155]. Additionally, HBIG-free approach has recently been advocated and is discussed in the later part of this review.

HBIG-free prophylaxis and treatment options

Advent of newer and more-potent NAs with high genetic barrier for resistance such as ETV and TDF, have shown great therapeutic potential as prophylactic agents, and achieved a stronger viral suppression,

Table 2 The results of combination therapy of low-dose hepatitis B immunoglobulin and nucleos(t)ide analogues and the effects of withdrawal of hepatitis B immunoglobulin from combination therapy

Ref.	NA	HBIG protocol	Median follow-up (mo)	HBV recurrence
Angus *et al*[119], 2000	32 LAM	400 IU or 800 IU/d for 1 wk from LT followed by 400 IU or 800 IU/ monthly thereafter	18.4	3.1% HBsAg + and 0% HBV DNA+
Gane *et al*[121], 2007	147 LAM	400 IU or 800 IU/d for 1 wk followed by 400 IU or 800 IU/monthly thereafter	62	1% at 1 yr and 4% at 5 yr. Baseline HBV DNA was associated with HBV recurrence
Karademir *et al*[125], 2006	33 LAM, 2 LAM + ADV	All patients received 4000 IU of intramuscular HBIG during surgery, 2000 IU intramuscular daily thereafter, until the HBsAb titer > 200 IU/mL and the HBsAg was seronegative, followed by lifelong 1200 to 2000 IU HBIG on-demand if HBsAb titer fell below 100 IU/mL	16	5.7% (2 of 35 patients) had HBV DNA recurrence. They were LAM resistant
Iacob *et al*[126], 2008	42 LAM	10000 IU within anhepatic phase and daily within the first postoperative week, followed by 2500 IU on demand	21.6	HBV recurrence rate was 4.8% after a median of 1.8 yr
Jiang *et al*[127], 2010	254 LAM	2000 IU in anhepatic phase, followed by 800 IU/d for first day then weekly for the rest of 3 wk in the first post-operative month, then 800 IU monthly	41.2	1-, 3- and 5-yr HBV recurrence rates were 2.3%, 6.2% and 8.2%, respectively 5 cases have YVDD mutations
Nath *et al*[128] , 2006	14 LAM + ADV	1000 IU HBIG in anhepatic phase 1000 IU/daily for week 1, then HBIG withdrawn, replaced with oral ADV	14.1	7.1%
Saab *et al*[129], 2011	18 LAM + HBIG, 16 LAM to LAM + ADV	Randomized trial Patients treated with low dose HBIG + LAM ≥ 1-yr post LT 18 patients continued HBIG 16 patients discontinued HBIG and ADV added	21	0% in HBIG + LMV 6.1% in LMV + ADV Recurrent case: HBsAg + /HBV DNA (-)
Saab *et al*[129] , 2011	19 LAM to LAM + ADV, 41 LAM to LAM + TDF, 1 ETV to ETV + ADV	All patients treated with low dose HBIG + LAM ≥ 1-yr post-LT. All patients discontinued HBIG	15	3.3% recurrent cases: HBsAg (+)/ HBV DNA (-)
Radhakrishnan *et al*[130], 2017	42 (ETV (12%), TDF (83%), or TDF/FTC (5%)	HBIG 5000 IU given in anhepatic phase and daily for 5 d together with nucleos(t)ide analogues after LT and then continued indefinitely.	36	1- and 3-year cumulative incidences of recurrence, defined by positive serum HBsAg of 2.9%
Chen *et al*[131], 2015	50 (ETV before and after LT)	Two doses of HBIG-First dose anhepatic phase (10000 IU) and other dose (10000 IU) during surgery (additional doses as needed to maintain HBIG level > 300 IU/mL from 6 wk to 12 mo)	36	0% recurrence at 3 years defined as reappearance of HBsAg and HBV DNA level
Cholangitas *et al*[132], 2016	34 (LAM = 2, AFV = 1, ETV = 9, TDF = 12)	HBIG 1000-10000 IU bolus during anhepatic phase, followed by daily × 7 d, and then monthly 1000-2000 IU intramuscularly for 6-12 mo post-LT and then discontinued NA were continued indefinitely	28	5.8% recurrence defined as reappearance of serum HDV in LT recipients with detectable serum HBsAg and/or HBV DNA
Wesdorp *et al*[133], 2013	17 (15 of 17 converted from LAM/ADV to TDF/FTC)	All received HBIG ± (10000 IU given during anhepatic phase followed by a 4-7 d course of 10000 IU of IV HBIG daily, and then monthly intramuscularly for > 6 mo and then switched to TDF/FTC	24	No recurrence defined by HBsAg and HBV-DNA positivity. However, 6.7% had isolated HBsAg recurrence
Stravitz *et al*[134], 2012	21 (Patients were initially on LAM = 11, ETV = 4, AFV = 2, LAM + ADV = 2, LAM + ADV = 2. All patients were converted to TDF/FTC)	HBIG ± nucleos(t)ide > 6 mo, then substituted with TDF/FTC	31	0% recurrence of HBV DNA after switching to TDF/FTC
Taperman *et al*[135], 2013	37 patients were randomized to TDF/FTC plus HBIG (*n* = 19) or receive (TDF/FTC) alone (*n* = 18)	HBIG ± nucleos(t)ide for 24 wk, then randomized to TDF/FTC plus HBIG (*n* = 19) or receive TDF/FTC alone (*n* = 18) for an additional 72 wk	72	0% recurrence of HBV DNA in both arms

Gane *et al*[136], 2013	20 patients with initial HBIG for 7 d and then switched to LAM+ ADV	HBIG 800 intramuscularly given immediately after LT and the daily for 7 d and then switched to LAM/ ADV	57	0% recurrence defined as reappearance of HBsAg and HBV DNA
McGonigal *et al*[137,141], 2013	4 (ETV = 2, LAM = 1, TDF = 1)	HBIG + NA for more than one year and switched to TDF/FTC	15	0% recurrence of HBsAg and HBV DNA
Angus *et al*[138], 2008	34 patients randomized after 12 mo of HBIG +LAM to ADV (n = 16) with and without HIBIG (n = 18)	Low dose HBIG × 12 mo along with LAM	4.4 yr for the LAM/ADV and 4.6 yr for the HBIG/LAM group	1 of 15 (6%) in the LAM/ADV and 0 of 15 (0%) in the HBIG/LAM group had HBsAg positive at last follow up

HBIG: Hepatitis B immunoglobulin; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; HDV: Hepatitis delta virus; LAM: Lamivudine; LT: Liver transplantation; ETV: Entecavir; TDF: Tenofovir.

Table 3 Hepatitis B immunoglobulin-free regimens in preventing recurrence of hepatitis B virus infection after liver transplantation

Ref.	No. of patients	Median duration of follow-up (mo)	Therapy	HBsAg loss	Undetectable HBV DNA
Fung *et al*[161], 2017	265	59	ETV	At 1, 3, 5, and 8 yr of follow up, 85%, 88%, 87.0%, and 92% were negative for HBsAg, respectively	At 1, 3, 5 and 8 yr of follow up, 95%, 99%, 100%, and 100% had undetectable HBV DNA, respectively
Fung *et al*[158], 2013	362	53	LAM = 176 (49%), ETV = 142 (39%), and 44 (12%) were on combination therapy (Either LAM or ETV) plus nucleotide analog (either ADV or TDF)	HBsAg seronegativity at 1, 3, 5 and 8 yr was 80%, 82%, 82% and 88%	HBV DNA suppression to undetectable levels at 1, 3, 5 and 8 yr was 94%, 96%, 96%, and 98%. Rate of HBV DNA suppression for LAM, combination therapy, and ETV at 1 yr was 97%, 94%, and 95%, respectively
Fung *et al*[159], 2011	80	26	ETV	The cumulative rate of HBsAg loss was 86% and 91% after 1 and 2 yr, respectively	95% with undetectable HBV DNA and 5% had low level viremia
Wadhawan *et al*[157], 2013	75	21	19 patients received a combination of LAM+ADV, 42 received entecavir, 12 received TDF, and 2 received a combination of ETV + TDF	The cumulative probabilities of clearing HBsAg were 90% and 92% at 1 and 2 yr after transplantation, respectively	Nine patients were HBsAg-positive with undetectable DNA at the last follow-up. The recurrence rate in our series was 8% (6/75)

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; LAM: Lamivudine; ETV: Entecavir; TDF: Tenofovir.

paving the way for a HBIG free regimen for antiviral prophylaxis[13,156]. In a multicenter trial by Gane *et al*[136], no HBV recurrence was detected in 28 HBV patients who received a combination of LAM and ADV after a median follow-up of 22 mo when the pre-transplant HBV-DNA level was below 3 log(10) IU/mL. In a later study of 75 HBV patients who received different oral antiviral treatment after LT (19 received a combination of LAM and ADV, 42 ETV, 12 TDF, and 2 received a combination of ETV and TDF), the HBV recurrence rate was merely 8% at a median follow-up of 21 mo and there was no mortality related to HBV recurrence[157]. There was no significant difference in HBsAg clearance and HBV-DNA suppression between those on LAM, combination treatment, or ETV, but virological relapse rate at 3 years was 17%, 7%, and 0%, respectively (P < 0.001).

Fung *et al*[158] evaluated monotherapy with NAs (LAM, ETV or LAM plus ADV) without HBIG in a large, long-term cohort study involving 362 LT patients with CHB. At the end of 8 years of follow-up, 98% showed undetectable HBV DNA in serum by clinical assay. Overall 8-year survival rate was 83% with no difference between these three treatment groups and, importantly, no mortality was observed due to HBV recurrence in any of the 362 patients. This study showed that at least in low risk patients, HBIG-free regimen with high potency NAs was safe and effective in preventing post-LT HBV reactivation. For patients without preexisting LAM-resistant mutation, the use of ETV as a standalone treatment remains an ideal choice given its lack of nephrotoxicity. In a study of 80 CHB patients undergoing LT where ETV was used alone in a completely HBIG-free regimen with a median follow-up of 26 mo a high HBsAg seroclearance rate of 86 and 91% after 1 and 2 years respectively was observed[159]. Thirteen percent of patients had HBsAg positivity either from reappearance of HBsAg after initial seroclearance or from persistence of HBsAg-positive status after transplantation. It is important to note that there was no incidence of virological rebound or

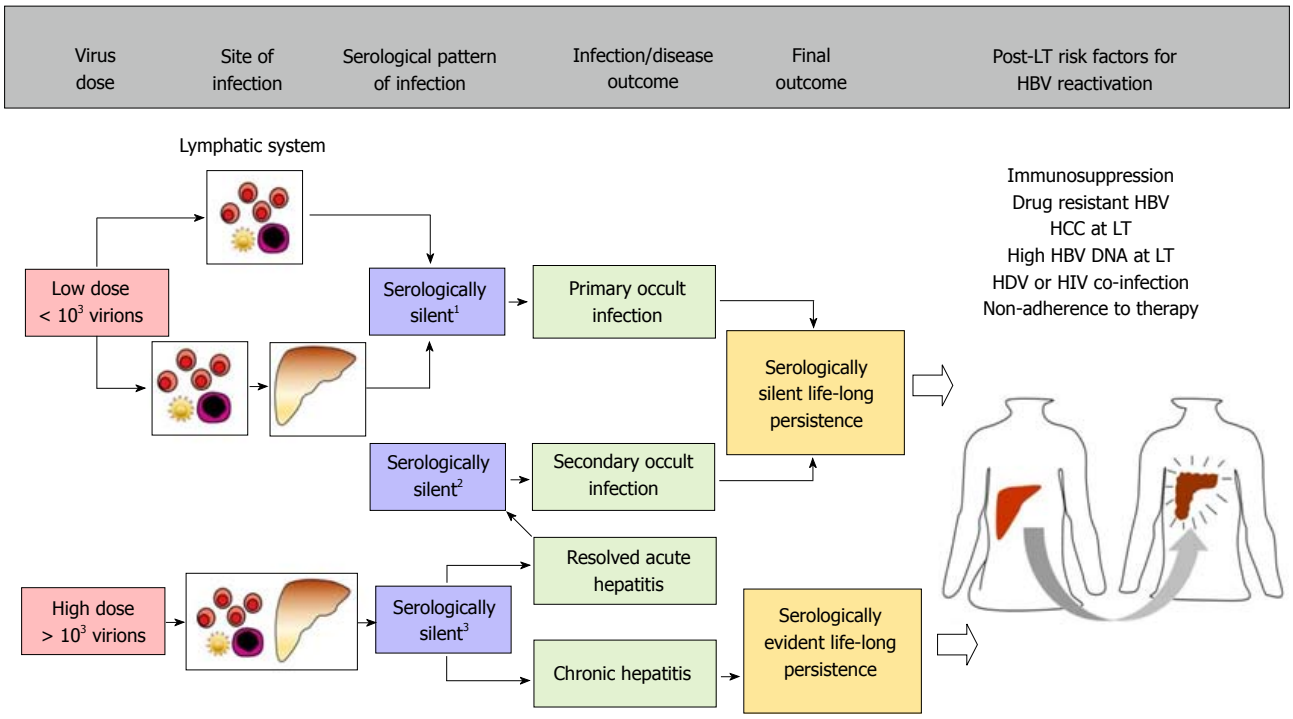

Figure 2 Generalized concept of overt and occult hepatitis B virus infections based on the data from the woodchuck model of hepatitis B, their long-term outcomes, and associated risk factors for hepatitis B virus reactivation following liver transplant. Based on experimental infection in the woodchuck model (Mulrooney-Cousins PM, Michalak TI, 2015[92]). [1]Serologically silent infection: HBsAg, anti-HBc and anti-HBs negative; HBV DNA positive; [2]Serologically silent infection: HBsAg negative, anti-HBc positive, anti-HBs positive or negative; HBV DNA positive; [3]Serologically evident infection: HBsAg and anti-HBc positive, anti-HBs negative. HBV DNA positive. SOI: Secondary occult infection; POI: Primary occult infection; LT: Liver transplant; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HDV: Hepatitis D virus; HBsAg: HBV surface antigen; anti-HBc: Antibodies to HBV core antigen; anti-HBs: Antibodies to HBV surface antigen.

resistance, nor any HBV-related graft hepatitis, graft loss, or mortality. The same group later also followed histological outcomes of CHB patients treated with an HBIG-free regimen, 42 patients were treated with ETV monotherapy who underwent liver biopsies after LT at a median time of 10 mo. Of these, 9 were serum HBsAg-positive at the time of biopsy. All patients were serum HBV DNA-negative- at the time of biopsy. None of these patients had histological evidence of HBV-related graft hepatitis and positive immunohistochemical staining for HBsAg[160]. Fung *et al*[161] also shown the long-term efficacy of using ETV monotherapy in a study involving 165 LT recipients with HBV. The study demonstrated that ETV monotherapy is highly effective at preventing HBV reactivation after LT for CHB, with a durable HBsAg seroclearance rate of 92%, an undetectable HBV DNA rate of 100% at 8 years, and excellent long-term survival of 85% at 9 years.

This approach has been supported by another recent study by Cholngitas *et al*[132]. They have shown that maintenance therapy with NAs prophylaxis after HBIG discontinuation was effective against HBV/HDV recurrence, but it seems that a longer period of HBIG administration might be needed before it is withdrawn after LT. Another large study from Asia has shown long-term ETV monotherapy (without HBIG) is highly effective at preventing HBV reactivation after LT for CHB, with a durable HBsAg seroclearance rate of 92%, an undetectable HBV DNA rate of 100% at 8 years, and excellent long-term survival of 85% at 9 years. The positive outcomes with the use of ETV monotherapy without HBIG has challenged the need for HBIG post-LT[161] A recent network metanalysis has shown that ETV resulted with the highest probability (31%) as the best prophylactic option on reducing the risk of HBV recurrence. ETV is the preferred oral NAs treatment compared to other five different prophylactic regimens (LAM, TDF, ADV, LAM plus ADV, LAM plus TDF) in the prevention of HBV recurrence after LT[162]. With currently preferred antivirals, namely, those with high barrier to resistance, more patients are likely to have low or undetectable viral load at the time of transplantation and an HBIG-free regimen will more likely be acceptable in the vast majority (Figures 2 and 4). On the other hand, HBIG is still an integral part of prophylaxis in high-risk patients with high pre-transplant HBV DNA level, presence of HCC at LT, co-infection with HIV and HDV, presence of drug-resistance and non-compliance with therapy[152]. However, duration of HBIG in such patients can be guided by testing of serial serum HBV DNA level, and HBsAg status (Figures 2 and 4). A recent study however has challenged this notion, and noted that oral antiviral therapy alone without HBIG is highly effective in preventing reactivation of HBV infection and graft loss from recurrent hepatitis B after LT in patients with preexisting HBV LAM resistance[163]. The cumulative rate of HBsAg seroclearance at 1, 5, and 10 years was 82%, 88%, and 91%, respectively. At the time

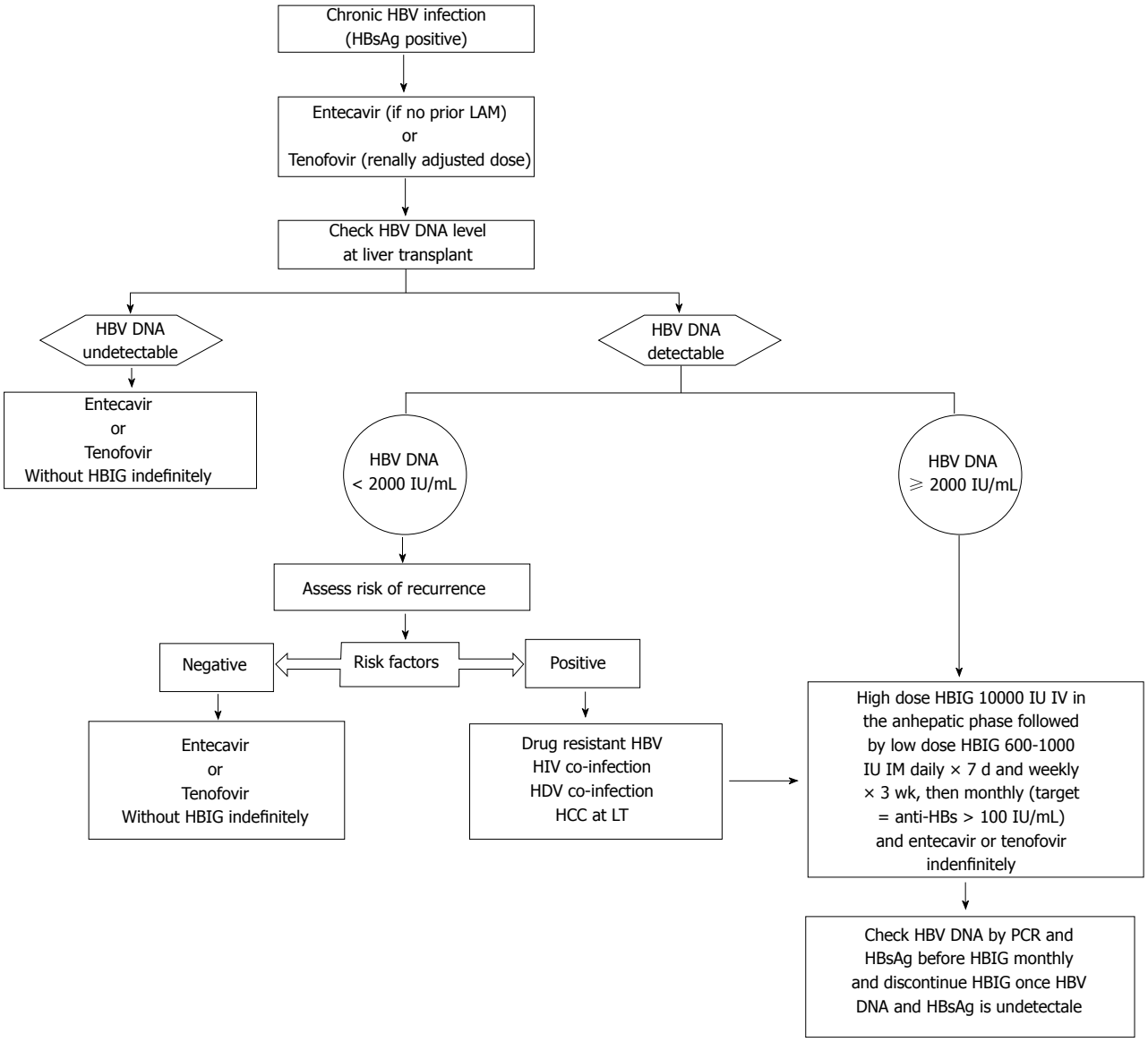

Figure 3 Proposed algorithm for hepatitis B prophylaxis in liver transplant patients. In chronic hepatitis B patients Entecavir (if no prior Lamivudine therapy) or Tenofovir (adjusted to renal function) is recommended as the first line therapy. Based on HBV DNA level at the time of transplant and risk factors, HIBG should be initiated, if associated risk factors for HBV recurrence post LT. High risk patients include drug resistant HBV, HIV co-infection, HDV co-infection, HCC. This group of patients receive high dose IV HBIG 10000 IU given during the anhepatic phase followed by low dose HBIG to achieve target anti HBs > 100 IU/mL along with NAs. HBIG is discontinued once HBV DNA is undetectable and loss of HBsAg is achieved. HBIG: Hepatitis B immunoglobulin; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; HDV: Hepatitis delta virus; LAM: Lamivudine; LT: Liver transplantation.

of transplantation, 39 (72%) patients had detectable HBV DNA, with a median of 4.5 log copies/mL. The cumulative rate of HBV undetectability was 91% at 1 year, increasing to 100% by 5 years. After 1 year of LT, over 90% of the patients had undetectable HBV DNA, and from 8 years onward, 100% had undetectable HBV DNA in serum. The long-term outcome was excellent, with survival of 87% at 12 years after transplantation, without any mortality related to HBV reactivation. However, HBIG does provide additional benefits beyond preventing HBV recurrence in LT recipients such as its association with reduced rates of rejection[164,165], and modifying risk of developing HCC post-LT[166]. Another important consideration is the potential for preventing graft reinfection such that subsequent discontinuation of all immunoprophylaxis can be considered[167]. The proposed algorithm for HBV prophylaxis for CHB patients undergoing LT is summarized in Figures 1 and 3.

Complete discontinuation of all prophylaxis

Based on the previous data and clinical studies, lifelong prophylaxis is currently advocated to LT patients to prevent HBV recurrence. Lenci *et al*[167] investigated the safety of withdrawal of prophylactic measures in selected LT patients using a stepwise protocol. The LT patients underwent liver biopsies after receiving a HBIG-LAM combination therapy. It was shown that careful withdrawal of HBIG was safe in patients with undetectable HBV viremia at transplantation and no evidence of total and intrahepatic cccDNA.

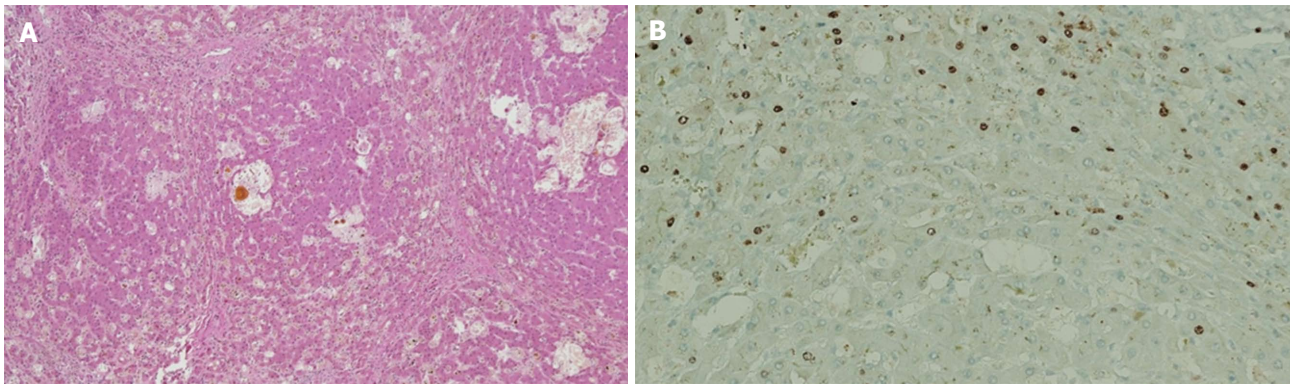

Figure 4 Immunostaining. A: Recurrent hepatitis B virus infection leading to cirrhosis in a post-liver transplantation patient. Figure shows cirrhotic nodules with cholestasis but no appreciable inflammation; B: Immunostaining for hepatitis B core antigen shows strong nuclear accumulation of antigen in a small proportion of hepatocytes indicating active virus propagation.

More recent study showed that complete prophylaxis withdrawal is safe in patients transplanted for HBV-related disease at low risk of recurrence and is often followed by spontaneous anti-HBs seroconversion[168]. However, based on previous studies many centers continue prophylaxis indefinitely as low level HBV viremia is known to persist even after many years of therapy[167,169], and complete discontinuation of all preventative therapy cannot be recommended at this time and should only be performed in the setting of a clinical trial[170].

HBV vaccination and active immunity

Although there is no effective clearance, to ensure a maximum suppression of HBV in LT patients and avoidance of escape mutations caused by long-term administration of HBIG or NAs, it is crucial to develop a strong and long lasting immune response against HBV. Several trials have noted an increase in anti-HBs titer in up to 65% of patients who received HBV vaccination after LT following HBIG withdrawal[141]. More recent study looking at active immunization in *de novo* HBV infection after LT with a HBV core antigen-positive graft have shown that active immunization is effective in preventing *de novo* infection if the post-transplant anti-HBs level is maintained above 100 IU/L with vaccination and antiviral prophylaxis. Prophylaxis can be safely discontinued in this group of patients who obtain this immunity[171].

Emerging therapies and the future of HBV treatment

Current HBV prophylaxis and treatment modalities can only suppress but do not eradicate HBV infection completely; therefore there is a lifelong need for the therapy. Recently, there is a renewed interest to target various stages HBV replication cycle and its interaction with the host.

DAAs and host-targeting agents (HTA) are the two major categories that are being developed and are at various phases of clinical trials[2,13]. Among these, DAAs act by inhibiting viral enzymatic activities or protein function, and generally have excellent safely profile, therefore present an attractive option for drug manufacturers. Major HBV target-specific classes of DAAs that are being developed are inhibitors of cccDNA (*e.g.,* CRISPR/Cas9, sirt1/2, MC2792), hepatocyte entry receptor inhibitors (*via* NTCP; *i.e.,* mycludex, ezetimibe), HBV DNA polymerase inhibitors (HB pol; *e.g.,* GS-7340, besifovir), siRNA target (ARC-520/521), core allosteric modulators (CpAM; *e.g.,* NVR 3-778), immune modulators (*e.g.,* GS9620, nivolumab, pidilizumab), and therapeutic vaccines (*e.g.,* TG-1050)[2,172-177]. These drugs are at various stages of clinical trials and they indicate a promising future for HBV prophylaxis and treatment.

CONCLUSION

With the advent of LT is currently regarded as the ultimate option for treatment for liver cirrhosis, liver failure and HCC associated with chronic HBV infection. Phenomenal success in allograft survival has been achieved by use of HBIG and oral antiviral medications. Prophylaxis with low dose HBIG and oral anti-HBV nucleotides is universally accepted as an effective option to reduce post-transplant viral reactivation. Availability of newer oral anti-HBV nucleos(t)ide analogs (NA), such as ETV and TDF, with higher barriers to resistance and better knowledge of risk factors associated with post-LT HBV reactivation have allowed incorporating these newer NA as part of the antiviral regimen after LT for CHB patients. The use of combination HBIG and lamivudine remains only of historical interest at this time as neither alone was sufficient to prevent HBV recurrence. ETV with its excellent safety profile, low nephrotoxicity, remains the agent of choice for patients without prior lamivudine resistance. For those with prior resistance, the addition of TDF is likely the best treatment option. LT with anti-HBc-positive donors is now possible due to better understanding of the balance between recurrence risk and availability of individualized prophylaxis strategies, and has expanded the pool of donor in an era with high demand for cadaveric donor with scarce supply. Current treatment regimen for

HBV can only control HBV replication, but cannot fully eradicate. As such, efficacy of HBV prophylaxis should be measured by its ability to prevent graft hepatitis and loss secondary to HBV infection, and not in terms of achieving a cure. With currently available potent NA we can achieve substantial suppression of HBV replication, but we are far from achieving viral eradication, although newer antiviral treatments approaches are in development. Hence, a positive HBsAg in post-LT period does not necessarily means HBV recurrence, as the patient has never achieved a virological cure. It is for the same reason we can argue that continuation of HBIG to achieve seroclearance of HBsAg does not achieve any clinical utility as long as viral suppression is achieved with NA. By administering HBIG to keep the antibody titers above a certain arbitrary level, serum HBsAg logically becomes undetectable because of the formation of immune complexes, which evades detection. However, this does not equate to complete eradication, nor the reappearance of serum HBsAg upon stopping HBIG signifies reactivation. In fact, hepatitis B core antigen remains detectable in the liver throughout HBIG administration despite serum HBsAg negativity. As such, long-term prophylaxis with HBIG does not serve any clinical utility and early discontinuation of this practice should be considered as long as complete viral suppression is achieved.

Emerging therapies are now focusing on newer targets of HBV replication and virus-host interaction with an ambitious goal of eradicating HBV infection in the near future rather than mere viral suppression.

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MINIREVIEWS

Metabolomics: From liver chiromancy to personalized precision medicine in advanced chronic liver disease

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Abstract

Currently there is a lack of accurate biomarkers for diagnosis and prognosis in advanced liver diseases. Either the occurrence of first decompensation, or diagnosis of acute on chronic liver failure, severe alcoholic hepatitis, or hepatocellular carcinoma (HCC), none of the available biomarkers are satisfactory. Metabolomics is the newest of omics, being much closer than the others to the actual phenotype and pathologic changes that characterizes a certain condition. It deals with a much wider spectrum of low molecular weight bio-compounds providing a powerful platform for discovering novel biomarkers and biochemical pathways to improve diagnostic, prognostication and therapy. Until now metabolomics was applied in a wide spectrum of liver conditions, but the findings were contradictory. This review proposes a synthesis of the existing evidences of metabolomics use in advanced chronic liver diseases, decompensated liver cirrhosis, severe alcoholic hepatitis and HCC.

Key words: Metabolomics; Biomarker; Prediction; Advanced chronic liver disease; Decompensation; Alcoholic hepatitis; Hepatocellular carcinoma

Core tip: Currently there is a lack of accurate biomarkers for diagnosis and prognosis in advanced liver diseases. Either the occurrence of first decompensation, or diagnosis of acute on chronic liver failure, severe alcoholic hepatitis, or hepatocellular carcinoma (HCC), none of the available biomarkers are satisfactory. This review proposes a synthesis of the existing evidences

of metabolomics use in advanced chronic liver diseases, decompensated liver cirrhosis, severe alcoholic hepatitis and HCC.

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INTRODUCTION

Hepatic fibrosis is a dynamic process that may progress to liver cirrhosis in the context of an active etiological factor. In compensated stages, physical exam by itself cannot distinguish between severe fibrosis and constituted liver cirrhosis. This is why in recent years the term compensated advanced chronic liver disease (cACLD) was introduced[1]. In this stage it is essential to establish the risk of decompensation and the best method to do it is by measuring hepatic venous pressure gradient (HVPG)[2]. However, HVPG measurement is not widely available and it is considered invasive[3]. Therefore, in the last years huge efforts were done to find new biomarkers or non-invasive methods to assess prognosis.

The occurrence of decompensation, with its various manifestations (ascites and variceal bleeding most often) is in direct relation with the increase in portal pressure, namely clinically significant portal hypertension, which represent an HVPG > 10 mmHg[4]. The life expectancy of these patients without liver transplantation is significantly lower than in compensated stages[5].

Acute decompensation associated with organ failures and increased short-term mortality is defined by the concept of acute on chronic liver failure (ACLF) syndrome, which was recently defined[6]. The most frequent precipitating factors for ACLF are bacterial infections, acute flares in viral B advanced liver disease and alcohol consumption but the clinical features are identical regardless the precipitating factor[7]. There are some clinical situations where making a therapeutic decision based on the available non-invasive diagnostic tools proves to be difficult. Thus, without liver biopsy it is impossible to differentiate between severe alcoholic hepatitis and decompensated cirrhosis, which is essential for the indication of cortisone treatment[8]. Moreover, around one third of decompensated patients are infected at presentation[9,10] and without routine cultures the clinical suspicion and diagnosis of bacterial infections is very difficult. To reduce in hospital-morbidity and mortality, early initiation of empiric antibiotherapy can be crucial, but bacterial cultures last long, and infection markers represented by CRP, leucocyte count, procalcitonin are of limited value in cirrhosis[11]. Therefore, in these specific clinical scenarios new biomarkers for diagnosis and prognosis are also needed.

Apart from acute decompensation, the prognosis of patients with cACLD is deeply influenced by hepatocellular carcinoma (HCC) occurrence[12]. The high mortality rate of HCC is owed partly to the absence of adequate monitoring in high-risk populations, and partly to insufficient diagnostic resources - especially for early tumor identification, which could still allow curative interventions. Serum alpha-fetoprotein (AFP) - which has been widely and commonly used as biomarker, either as a screening tool for early HCC detection or as a prognostic tool for tumor recurrence and patient survival, has a poor sensitivity since up to 40% of HCC and cirrhosis patients have normal AFP levels and only 10%-20% of patients with early-stage HCC have elevated AFP levels[13]. Therefore, more sensitive markers of disease are needed, particularly for the early detection of HCC disease and for HCC recurrence after curative treatment.

Given the reserved prognosis and the difficulty of management, this review proposes a synthesis of the existing evidences of metabolomics use in cACLD, decompensated liver cirrhosis, severe alcoholic hepatitis and HCC.

METABOLOMICS - NEW OPPORTUNITY FOR BIOMARKERS DISCOVERY

Although its recognition as a distinct scientific area is much more recent than the other "omics" such as genomics, transcriptomics, or proteomics, metabolomics provides a powerful platform for discovering novel biomarkers and biochemical pathways to improve diagnostic, prognostication, and therapy (Figure 1)[14,15]. It has the advantage of being much closer to the actual phenotype than the other omics, but the number of possible compounds is much higher. In contrast to genomics, transcriptomics, and proteomics, which address macromolecules with similar chemical properties, such as DNA, RNA, and proteins, metabolomics deals with diverse properties of low molecular weight bio-compounds[16].

Metabolomics allows small metabolites, usually with a molecular weight under 1 kDa, and metabolic processes to be studied using nuclear magnetic resonance (NMR) spectroscopy, gas chromatography mass spectrometry (GC-MS), and liquid chromatography mass spectrometry (LC-MS)[17]. Given the nonvolatile character of biological materials (serum, urine, tissue or faeces) the most commonly used technique in clinical trials is LC-MS. The common pattern recognition methods of metabolomics include unsupervised and supervised ones: Principal component analysis (PCA) for the former and partial least squares-discriminant analysis (PLS-DA) for the latter group.

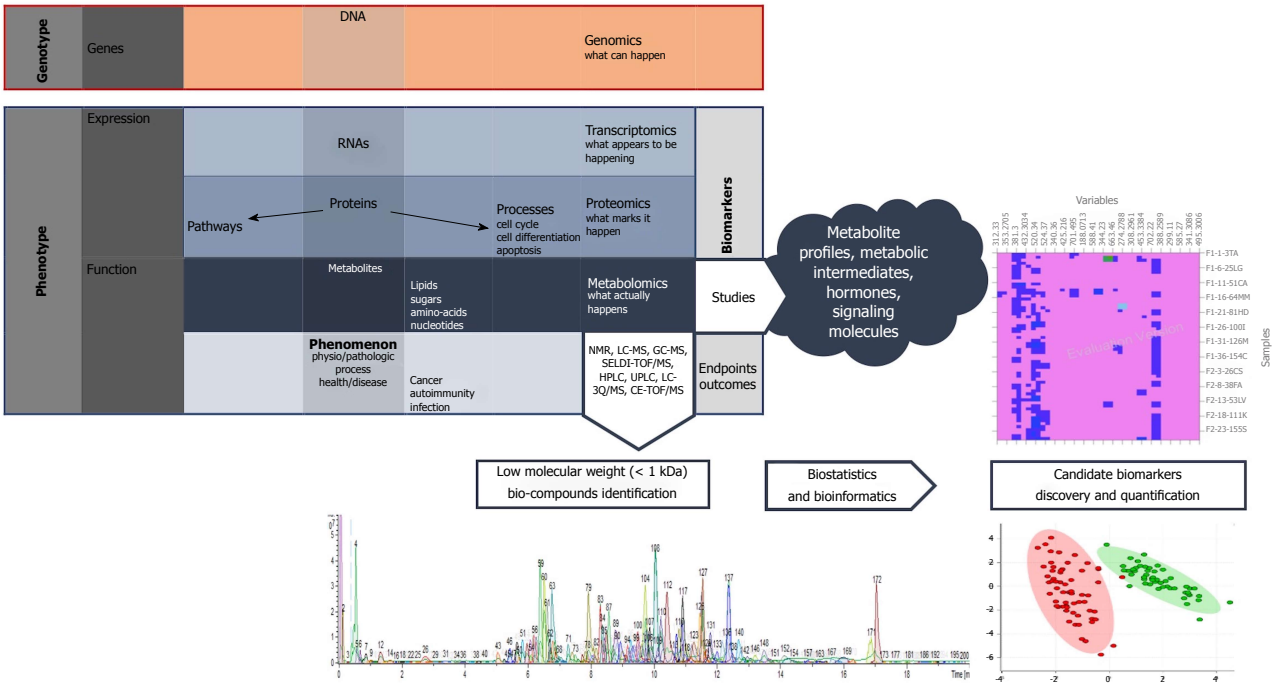

Figure 1 Integration of metabolites into the "omics" pathway and basic principles of metabolomics.

METABOLOMICS AND ADVANCED CHRONIC LIVER DISEASE

Once the cACLD patients develop decompensation the progression of the disease is very clear. The annual transition rate from compensated to decompensated stage is the highest in HBV cirrhosis, around 10% per year, being lower in HCV and alcoholic etiologies[18,19]. However, without HVPG measurement, the most difficult task is to identify the patients at risk of decompensation or to accurately identify the precipitating factor.

It seems that with the progression of chronic liver diseases the core metabolic phenotype is characterized by a decrease in phosphatidylcholines (PC) and increase in serum biliary acids[20]. This core metabolic phenotype appears early in the natural history of chronic liver diseases, regardless the etiology, and remains stable in the evolution, including different stages of cirrhosis or hepatic tumors, either cholangio or HCC.

When comparing the metabolic profile of patients infected with HCV without fibrosis with HCV cirrhosis, along with this core metabolic phenotype, there are other several disorders involving lipid, carbohydrate, protein, and energetic metabolism[21]. The HDL cholesterol and choline levels were lower in patients with cirrhosis compared to those without fibrosis. The perturbations of glucose metabolism are caused, firstly, by impaired tricarboxylic acid cycle activity due to mitochondrial dysfunction, and possibly on the second hand by insulin resistance that characterizes HCV infection, leading to increased serum glucose and citrate levels in the cirrhotic group. As a response to the relative carbohydrate deficiency, ketone bodies (hydroxybutyrate and acetoacetate) are used as preferential energy sources in the mitochondria, explaining their lower serum levels with the evolution of hepatic disease.

Regarding protein metabolism, there is an imbalance in the ratio of aromatic amino acids and branched chain amino acids in cirrhosis. In fact, only phenylalanine was founded elevated in serum of patients with cirrhosis, probably because disturbances of the gut microbiota in this situation[22].

Jimenez *et al*[23] has attempted to identify by NMR spectroscopy the metabolic profile of cirrhotic patients with minimal hepatic encephalopathy (MHE) *vs* cirrhotic patients without encephalopathy. MHE patients displayed increased serum concentrations of glucose, lactate, methionine and glycerol, as well as decreased levels of choline, branched chain amino acids, alanine, glycine, acetoacetate, and lipid moieties.

When serum metabolic profile by NMR spectroscopy of patients in different stages of chronic liver failure (CLF) according to the MELD score was analyzed, there is an evolutionary trend involving the representatives of the metabolism of lipids, carbohydrates and proteins[24]. Thus, there is a decrease in HDL cholesterol, choline and phosphatidylcholine, which are the more expressed in higher MELD patients. The glucose, lactic acid, butyrate, pyruvate and citrate levels increase in severe CLF and the protein metabolism is modified because increased skeletal muscle catabolism, expressed by increased levels of free amino acids (leucine, isoleucine, glutamine, methionine and valine) in parallel with the severity of liver disease.

Recently, it was proved that phosphatidylcholine and lysophosphatidylcholine may have also prognosis

Table 1 Principal metabolic changes in advanced liver diseases

Condition	Lipids	Bile acids	Carbo-hydrates	Energy and oxidative stress	Proteins and Aminoacids
ACLD	↓ HDL cholesterol ↓ Choline ↓ Phosphatidylcholine ↓ Lipid moieties		↑ Glucose ↑ Glycerol	↓ OH-butyrate ↓ Aceto-acetate ↑ Lactate ↑ Pyruvate ↑ Citrate	↑ Phe ↓ Gli, Ala ↓ Branched AA ↑ Leu, Iso-Leu, Val, Glu ↑ Methionine
ACLF	↓ HDL cholesterol			↑ Lactate ↑ Pyruvate	↑ Aromatic AA
ALD	↓ Lyso-phosphatidilcholine ↑ Eico/doco -sapentaenoate ↑ Tetra/hexa/octa -decanedioate	↑ Sulphated bile acids	↑ Fumarate, succinate, malate, citrate ↑ Xylonate	↑ Indole 3-acetic acid (u) ↑ Betaine ↑ Citruline	↓ Val, Iso-Leu
HCC	↓ Lysophosphatidilcholine ↑ Oleamide ↑ Stearoyl-coa desaturase ↓ 3-Hydroxybutyrate ↓ Choline	↓ (Lito)cholic, (cheno)deoxy-cholic acids ↓ GCA, GDCA, GCDCA, TCA, TCDCA	↑ Glucose, glycerol	↓ OH-butyrate ↓ Xantine ↑ Canavanino succinate	↓ BCAAs: Leu, Iso-Leu and Val ↑ AAAs: Phe, Trp, Tyr, His ↑ Methionine, hydroxy-methyldeoxyuridine, dimethyl-guanosine, uric acid ↑ Methylhistidine

ACLF: Acute on chronic liver failure; ACLD: Advanced chronic liver disease; ALD: Alcoholic liver disease; HCC: Hepatocellular carcinoma; AA: Aminoacids; AAA: Aromatic AA; BCAA: Branched chain AA; Ala: Alanine; Arg: Arginine; Gli: Glicine; Glu: Glutamate; His: Histidine; Phe: Phenylalanine; Leu: Leucine; Val: Valine; Trp: Triptofan; Tyr: Tyrosine; CA: Cholic acid; GCA: Glyco CA; GDCA: Glycodeoxy CA; GCDCA: Glycochenodeoxy CA; TCA: Tauro CA; TCDA: Tauro cheno deoxi CA; u: Urinary.

relevance in decompensated liver cirrhosis, serum levels of these compounds being negatively correlated with survival[25].

Therefore, there is no single biomarker for the different stages of advanced chronic liver disease, but a complex of biomarkers, a so-called metabolic fingerprint. This implies, regardless of the etiology of liver disease, progressive changes of the same classes of compounds in parallel with disease evolution.

METABOLOMICS AND ACUTE-ON-CHRONIC LIVER FAILURE

ACLF is a distinct syndrome that can occur in approximately one third of patients with decompensated liver cirrhosis[6]. The most common causes are bacterial infections, alcohol consumption and digestive bleeding, although in a large percentage of cases a precipitating factor cannot be identified[7].

Amathieu *et al*[26] compared the metabolic profile of patients with ACLF with the one of patients with decompensated liver cirrhosis who do not meet the criteria for ACLF. The patients with ACLF had decreased HDL cholesterol, increased lactic acid, pyruvate, and aromatic amino acids but these changes are rather the expression of the severity of liver disease.

Because indirect infection markers have limited value in cirrhotic patients and bacteriological studies last long, identifying bacterial infections in a patient with decompensated liver cirrhosis or ACLF could be difficult. Although there is a strong need for new biomarkers in infection, there are no publications regarding the metabolic profile of the infected cirrhotic patients.

In severe sepsis and septic shock in non-cirrhotic patients it was identified a urinary metabolic profile with prognostic value, characterized by higher levels of ethanol, glucose, hippurate, but lower levels of methionine, glutamine, arginine and phenylalanine in patients with lower survival[27]. A retrospective multicenter study in Greece and Germany, which enrolled a large number of patients proposed as a primary endpoint to differentiate the metabolic profile of patients with SIRS from patients with sepsis and as a secondary endpoint, to identify specific biomarkers for the different types of infections[28]. A regression model combining the sphingolipid SM C22:3 and the glycerophospholipid lysoPCaC24:0 was created for sepsis diagnosis with a sensitivity of 84.1% and specificity of 85.7%. The glycerophospholipid lysoPCaC26:1 was characteristic for patients with community-acquired pneumonia complicated with severe sepsis or septic shock. For the other types of infection, no biomarker or significant metabolic profile was found.

For diagnosis of sepsis in emergency department, one study identified a panel of 6 metabolites, represented by myristic acid, citric acid, isoleucine, norleucine, pyruvic acid and a phosphocholine like derivative, to have very good sensitivity (95%) and specificity (90%)[29].

It is to be demonstrated if all these metabolic markers of infection may be applied in the context of decompensated cirrhosis or ACLF.

METABOLOMICS AND ALCOHOLIC LIVER DISEASE

Alcohol liver disease encompasses a spectrum of injury ranging from simple steatosis to frank cirrhosis and alcohol consumption may represent a precipitating factor for decompensation or ACLF[8]. Because most cases of alcoholic hepatitis occur in patients with established cirrhosis, most of the times it is impossible to differentiate between severe alcoholic hepatitis and decompensated cirrhosis without liver biopsy[8]. Accordingly, new biomarkers capable to differentiate between severe alcoholic hepatitis and decompensated cirrhosis as well as markers capable to predict early the response to corticosteroid therapy, would be of great help in clinical practice.

Urinary indole-3-acetic acid has been identified as a potential biomarker for early alcoholic liver disease on animal model, by two studies performed by LC-MS[30,31]. Our group, in a pilot study, proved that lysophosphatidylcholine (LPC) 16:1 and 20:4 decease progressively with the severity of alcoholic liver disease and this is correlated with survival and the occurrence of liver related events[32]. However, if these metabolic changes are rather general in ACLD or specific to alcoholic liver disease remains to be proved.

There are only few small studies in the literature regarding the metabolic profile of the patient with severe alcoholic hepatitis. Enhanced adipose tissue lipolysis with increased fatty acid supply to the liver is a phenomenon observed in severe alcoholic hepatitis[33]. In severe alcoholic hepatitis, there is an impaired long-chain fatty acid beta-oxidation in the liver, first because of the oversaturation of hepatic metabolic capacity due to excessive fatty acid supply and second because of impaired mitochondrial function. Eicosapentaenoate (EPA; 20:5n3) and docosapentaenoate (DPA;22:5n6), 2 long chain essential fatty acids, have been identified as potential biomarkers for severe alcoholic hepatitis by Rachakonda *et al*[33,34], capable to differentiate severe alcoholic hepatitis from compensated alcoholic liver cirrhosis.

As a consequence of faulty beta-oxidation, the same study demonstrated a relative transition to lipid omega-oxidation in severe alcoholic hepatitis, with the increase of dicarboxylic acids such as tetradecanedioate, hexadecanedioate, octadecanedioate, which are endogenous ligands of the peroxisome proliferator activated receptor (PPAR) alpha, mechanism at least partially responsible for the developing of severe steatosis in alcoholic hepatitis[34]. Severe alcoholic hepatitis is characterized by intrahepatic cholestasis. An increase in sulphated bile acids has been demonstrated in the serum of patients with severe alcoholic hepatitis, with a decrease in bile acids from intestinal bacterial origin, reflecting probably gut microbiota disturbances[34]. Carbohydrate metabolism is also impaired and implicates mainly the dysfunction of the Krebs cycle activity, with an increase in serum concentrations of the intermediates of the cycle, like fumarate, succinate, malate, citrate. Glucose is poorly used in severe alcoholic hepatitis, and there is a shunting from glycolysis to the pentose phosphor pathway, the end product of this, xylonate, being an important biomarker for severe alcoholic hepatitis.

Branched chain amino acids originating in skeletal muscles appear to be an important energy substitute in severe alcoholic hepatitis, as their serum levels (valine and isoleucine) are reduced in parallel with the increase of their metabolites[34].

Ascha *et al*[35] identified two compounds, betaine and citrulline, as important biomarkers for the differentiation of severe alcoholic hepatitis from decompensated liver cirrhosis. Betaine is a methylating agent involved in preserving the integrity of the hepatic cell, while citrulline has intestinal origin and appears to be elevated alongside NO, secondary to an excessive nitric oxyde synthaseactivity in the context of significant portal hypertension in alcoholic hepatitis[35].

METABOLOMICS AND HCC

LPC is an important signaling molecule, involved in regulating cellular proliferation, cancer cell invasion, and inflammation[36] and it has been reported to be significantly decreased in the sera of HCC patients[37,38]. In a recent study, lower levels of LPC and PC, such as LPC (16:0), LPC (18:0), PC (16:0), and PC (18:0) were observed in HCC and liver cirrhosis samples compared with healthy controls[37]. Low levels of LPCs imply an anti-inflammatory status in HCC patients, and markedly low levels of LPCs represent a severe immune suppression status in cirrhotic patients. Similar LPC trends have also been found in other malignant diseases, such as renal cell carcinoma[39].

Other serum lipid compounds found to be discriminative between HCC and healthy controls are Free Fatty Acids (FFA). Amongst them, oleamide (cis-9, 10-octadecenoamide), the amide of FFA C18:1 (oleic acid), may represent a specific marker for HCC[36,40]. Gao *et al*[41] reported a gradual up-regulation of the ratio of FFA C16:1 to C16:0 and FFA C18:1 to C18:0 during hepatocarcinogenesis as a result of significantly increased level of stearoyl-CoA desaturase 1(SCD1), due to the increased demand for lipid synthesis in HCC.

Bile acids are synthesized in the liver and aid in fatty acid absorption and digestion and constitutes one of the most frequently reported compound classes suggested as discriminating between HCC patients and a control group. Cholic acid, chenodeoxycholic acid, lithocholic acid and deoxycholic acid had lower levels in HCC patients compared with cirrhosis[40,42]. Also, glycochenodeoxycholic acid 3-sulfate (3-sulfo- GCDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), taurocholic acid (TCA), and taurochenodeoxycholate

(TCDCA) are down regulated HCC *vs* cirrhosis[37,42]. Bile acid downregulation in HCC may also reflect a metabolic shift away from β-oxidation and the reduced de novo bile acid production caused by the obliteration of healthy hepatocytes during chronic liver disease[43].

As the liver is the major organ of protein metabolism it is not surprising that a dysregulation of amino acids was found in several studies specifically a decrease in branched chain amino acids (BCAAs: leucine, isoleucine, and valine) and an increase in aromatic amino acids (AAAs: phenylalanine, tryptophan, tyrosine, and histidine) in HCC patients *vs* healthy controls, indicating enhanced BCAA catabolism and reduced AAA breakdown in the failing liver[44,45]. BCAAs have been reported to have a crucial role in cancer by regulating the anabolic process involving protein synthesis and degradation. Alteration of these metabolic pathways was observed after RFA intervention, indicating that application of electrical current during RFA treatment causes burns in the liver and produces coagulative necrosis which results in parenchymal and tumor cell death, enhancement of consumption of BCAA, such as isoleucine which may characterize the inflammatory response in liver[46].

Baniasadi *et al*[47] used a targeted approach based on liquid chromatography resolved tandem mass spectrometry (LC-MS/MS) on 73 metabolites out of which 16 were statistically different between the serum of HCC *vs* cirrhotic HCV patients. Among them, 4 metabolites (methionine, 5-hydroxymethyl-2′-deoxyuridine, N2,N2-dimethylguanosine and uric acid) showed an excellent separation between the two group patients with a sensitivity of 97% and specificity of 95% and an AUROC of 0.98.

Prognostication for HCC after curative treatment is difficult, in part due to the lack of useful biomarkers that would allow for the selection of patients at higher risk of tumor recurrence or enable accurate assessment of treatment response.

Goossens *et al*[46] evaluated through 1H-NMR analysis, preoperatively and at various time points post-RFA, the metabolic profile of serum samples from HCC patients in order to identify factors associated with treatment response and recurrence in viral and non-viral HCC patients. The analysis was able to discriminate in the serum of viral HCC between t0 (pre-ablation) and t2 (at 1 to 4 mo post ablation), the t2 being mainly characterized by an increase of glucose, glycerol, methylhistidine , and a decrease of lipids, 3-hydroxybutyrate, and choline but it was not able to predict HCC recurrence.

Zhou *et al*[48] evaluated early postoperative recurrence metabolic disturbances in HCC patients and demonstrated that bile acids, steroids and fatty acids showed significant variation in the early recurrent HCC group compared to the late recurrence group. Moreover, with the combination of methionine, GCDCA and cholesterol sulfate, 80% of the early recurrent HCCs can be predicted correctly with the corresponding AUROC equal to 0.91.

As previously shown there are no specific metabolic changes during the carcinogenetic process and, therefore, by now there is no specific marker to be proposed for diagnostic and prognostic of patients with HCC. Although metabolomics is a powerful strategy for identifying a large panel of metabolites that exhibit promise in accurately diagnosing HCC, integrating two or more "omics" approaches can unveil the complex genomic-proteomic-metabolic network galvanizing cancer development. With this regard, Beyoğlu *et al*[49] performed a combined transcriptomics and metabolomics study and was able to evaluate the metabolic profile of G1 to G6 transcriptomics groups of HCC.

CONCLUSION

The main limitation to the generalization of the results of existing publication is that the methodology used by different groups is not uniform and, thus, the results are sometimes contradictory. Other possible explanation for this variability is the fact that the majority of the studies use non-targeted metabolic analysis and identification of different metabolites is based on molecular mass, which can be similar for different compounds. Despite the important progress that has been made in technology and in understanding the pathological processes, when talking about specific biomarkers for advanced chronic liver diseases we are still in an era of uncertainty and chiromancy.

However, what appears to be a fact, is that during the progression of liver diseases, regardless the etiology, there is a core represented by decrease in serum lysophosphatidylcholine and an increased in bile acids[20] (Table 1). These changes augment with the progression of the disease and that's explains the prognostic relevance of these changes. Besides that, several candidate metabolomic biomarkers have been identified in these clinical scenarios. They reflect the changes that occur mainly in lipids, amino-acids and energetic metabolism. Nevertheless, none of them was widely and independently validated, or have been translated into clinical practice.

It is our strong belief that the diversity and quality of emerging data would allow the selection of the best method for metabolomics and further studies would validate new biomarkers for those scenarios where clinical needs are still unmet. Probably, the solution would be to interdisciplinary analyze the data through system's biology, allowing the integration of clinical, biochemical, imaging and "omics" findings, so that we'll be moving towards the era of personalized precision medicine in advanced chronic liver diseases.

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ORIGINAL ARTICLE

Observational Study

Management of restless legs syndrome in chronic liver disease: A challenge for the correct diagnosis and therapy

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Abstract

AIM

To investigate the association between restless legs syndrome (RLS) and well-defined chronic liver disease, and the possible therapeutic options.

METHODS

Two hundred and eleven patients with chronic liver disease, complaining of sleep disturbances, painful leg sensation and daily sleepiness, were included. Patients with persistent alcohol intake, recent worsening of clinical conditions, or hepatitis C virus were excluded. Diagnosis of RLS was suggested by the Johns Hopkins questionnaire and verified by fulfilling the diagnostic criteria by Allen. All patients were tested, both at baseline and during follow-up, with the Hamilton rating scale for depression, sleep quality assessment (PSQI), Epworth sleepiness scale (ESS), International Restless Legs Syndrome Study Group evaluation, and international RLS severity (IRLS) scoring system. Iron-free level, ferritin, folate, vitamin B12 and D-OH25 were detected. Neurological examinations and blood test

occurred at the beginning of the therapy, after 2 wk, and at the 28th, 75th, 105th, 135th, 165th and 205th day. Regarding therapy, pramipexole or gabapentin were used.

RESULTS

Patients were moderately depressed, with evident nocturnal sleep problems and concomitant daily sleepiness. Sleep problems and involuntary leg movements had been underestimated, and RLS syndrome had not been considered before the neurological visit. All (211/211) patients fulfilled the RLS diagnostic criteria. Twenty-two patients considered their symptoms as mild, according to IRSL, but 189 found them moderate to very severe. No correlation was found between ammonium level and ESS or PSQI. Augmentation was rather precocious in our patients (135th day), and more frequent (35%) than previous data (8.3%-9.1%). The dosage of dopamine agonists was found to be associated with augmentation and appears in range with the literature. Previous intake of alcohol and lower levels of vitamins have been related to the phenomenon in our study.

CONCLUSION

RLS is a common disorder, requiring rapid diagnosis and treatment. Further research is therefore fundamental.

Key words: Restless legs syndrome; Chronic liver disease; Dopamine agonist treatment; Augmentation

Core tip: The diagnosis of restless legs syndrome (RLS) relies on the presence of unpleasant sensation in the legs associated with the urge to move. Symptoms mostly begin during periods of rest or inactivity and worsen in the evening or night. Partial or total relief is related to movement. Chronic hepatic failure was recently described in association with RLS, but there are very limited studies, with no mention to treatment. We describe RLS syndrome associated with well-defined chronic liver disease along with therapeutic options, discussing risks, benefits and potential side effects, with a particular look at the augmentation phenomenon in hepatic failure.

Moretti R, Caruso P, Tecchiolli M, Gazzin S, Tiribelli C. Management of restless legs syndrome in chronic liver disease: A challenge for the correct diagnosis and therapy. *World J Hepatol* 2018; 10(3): 379-387 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i3/379.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i3.379

INTRODUCTION

Restless legs syndrome (RLS) is defined as a very sickening, bilateral (even if also unilateral) sensation, almost described as affecting a very limited zone, between the knees and ankles, sometimes involving thighs and feet and resulting in feelings of scrambles, creeps or crawls. The discomfort is experienced only during the rest phase and it is relieved by active movement of the legs. Patients describe the symptoms of RSL as unbearable, when they are strained to maintain the sit-down position, such as during long flights or social events. But, usually, sleep is the worst moment of the day and RLS can disturb their sleep for hours. The American patients' organization Restless Legs Syndrome Foundation reminds us that RLS is "the most common disorder you have never heard of" (http://www.rls.org).

RLS remains a clinical diagnosis based on confirming the four essential diagnostic features of RLS: (1) An urge to move the legs, usually but not always accompanied by or felt to be caused by uncomfortable and unpleasant sensations in the legs; (2) the urge to move, as any accompanying unpleasant sensation begins or worsens during periods of rest or inactivity, such as lying down or sitting; (3) the urge to move, as any accompanying unpleasant sensation is partially or totally relieved by movement, such as walking or stretching; and (4) the urge to move, as any accompanying unpleasant sensation during rest or inactivity only occurs or is worse in the evening or night compared to during the day[1,2]. Moreover, supportive criteria should be found in family history, response to dopaminergic therapy and the presence of involuntary, rhythmic muscular jerks in the lower limbs, including dorsiflexion or fanning of toes, flexion of ankles, knees and hips, the so-called periodic limb movements during sleep (PLMS)[1,3].

Helpful tools to make an accurate RLS diagnosis include the Johns Hopkins telephone diagnostic interview, medical history (evaluating for four essential diagnostic features of RLS and iron deficiency), and evaluating and ruling out mimics[4]. RLS frequently occurs in patients with kidney disease.

The prevalence of RLS, which is high in dialysis patients and which has been associated with increased risk for cardiovascular disease in the general population, could also play a role in the pathogenesis of hypertension during sleep in renal patients. It should be noted that intravenous iron treatment reduces the RLS symptoms in patients with end-stage renal disease[2]. RLS is common in rheumatologic disorders, such as rheumatoid arthritis or Sjögren's syndrome[1,2], but not in isolated peripheral neuropathy, as in hereditary neuropathic patients[2]. Some data seem to indicate that there is a considerably higher risk for developing RLS in migraneous patients, especially in those who experienced the dopaminergic anticipatory symptoms, such as nausea, somnolence and yawning[2]. RLS is also common during pregnancy, especially during the last trimester, and iron deficiency may be a major cause; the symptoms of RLS usually disappear soon after childbirth. An increased

prevalence of RLS has been described in patients with liver cirrhosis in the United States[5] and Japan[6]. Very recently, Goel *et al*[7] described in India RLS in a series of chronic hepatic failure patients.

MATERIALS AND METHODS

This study included 267 adult patients with chronic liver disease, referred to our Neurological Unit by the Liver Unit of the University of Trieste between June 1, 2008 and December 1, 2015. The patients had been referred to the neurologist for the three complaints of sleep disturbances, painful leg sensation, daily sleepiness not correlated to hepatic encephalopathy. Patients with chronic liver disease included primary liver tumor and liver cirrhosis. We excluded 13 patients with chronic and persistent significant alcohol intake (> 30 g/d in men and > 20 g/d in women; to avoid acute alcohol polyneuropathy, which might mimic some symptoms of RLS and low compliance), 25 patients with recent worsening of clinical condition (jaundice, ascites or encephalopathy, gastrointestinal bleeding, or hospitalization), and 12 patients with hepatitis C virus (HCV; to exclude HCV-related peripheral complications).

All the other 211 patients were followed up by a neurologist at least for 24 mo (Table 1). According to neurological exams, the diagnosis of RLS was suggested by the Johns Hopkins questionnaire[4] and verified by fulfilling the diagnostic criteria by Allen *et al*[1]. Only 3 patients mentioned a possible familiar history of RLS. Iron-free level, ferritin, folate, vitamin B12 and vitamin D-OH25 was measured in all patients (Table 2).

At baseline, patients were tested with the Hamilton rating scale for depression[9], sleep quality assessment (PSQI)[10], Epworth sleepiness scale (ESS)[11], International Restless Legs Syndrome Study Group (IRLSSG) evaluation[12], and international RLS severity (IRLS) scoring system[13].

The Pittsburgh sleep quality index (PSQI) is an effective instrument, employed to measure the quality and pattern of sleep in adults. It differentiates "poor" from "good" sleep quality by measuring the following seven areas (components): Subjective sleep quality; sleep latency; sleep duration; habitual sleep efficiency; sleep disturbances; use of sleeping medications; and, daytime dysfunction over the last month. A total score of 5 or greater is indicative of poor sleep quality[10].

The ESS questionnaire asks the subject to rate the probability of falling asleep on a scale of increasing probability from 0 to 3 for eight different situations that most people engage in during their daily lives, though not necessarily every day. The scores for the eight questions are added together to obtain a single number. A number in the 0-9 range is considered to be normal, while a number in the 10-24 range indicates that expert medical advice should be sought. For instance, scores of 11-15 are shown to indicate the possibility of mild to moderate sleep apnea, where a score of 16 and above indicates the possibility of severe sleep apnea or narcolepsy[10].

Table 1 Baseline general conditions of patients recruited

Characteristic	Hepatic failure, *n* = 211
Male/female	107/104
Age in year, mean and standard deviation	59 ± 4.7
(median range)	(36-74)
BMI, kg/m^2	25.43 ± 4.1
Cause of liver disease, *n*	211
Previous alcohol abuse	139
Hepatic venous outflow tract obstruction	14
Cryptogenic	12
Liver primary tumor	46
Child-Pugh class; number	211
A	132
B	54
C	25

BMI: Body mass index.

The IRLSSG[1] evaluation is based on the assessment of the following five questions, with the necessary fulfillment of three or more: (1) An urge to move the legs, usually accompanied by uncomfortable and unpleasant sensations in the legs; (2) which begins or worsens during periods of rest or inactivity; (3) which occurs only or is worse in the evening or night than during the day; (4) which is partially or totally relieved by repeated leg movements; and (5) for which the occurrence of the above features is not solely accounted for by another medical or behavioral condition.

The IRLS score[11,12] consists of a set of 10 self-administered questions, each of which is scored on a scale extending from 0 to 4. The scores of individual questions are aggregated to yield a total score ranging from 0 to 40. Based on the IRLS score, RLS is graded as mild (0-10), moderate (11-20), severe (21-30), or very severe (31-40).

The drugs used to treat RLS belong to many different pharmacological classes, including the dopaminergic agents, opioids, benzodiazepines and antiepileptic drugs. We decided to avoid the employment of opioids and benzodiazepine due to the hepatic conditions. Therefore, the first-choice drug was pramipexole, a dopamine agonist[14] .

Neurological examinations and laboratory tests were performed at the beginning, after 2 wk, at the 28th, 75th, 105th, 135th and 165th day, and at the final day of the follow-up, the 205th day. If major side effects induced by the neurological therapy occurred, such as nausea, vomiting, somnolence, hypotension or optical illusions, the pramipexole was stopped.

The second-choice drug was gabapentin, due to its beneficial properties and to the limited hepatic side effects[15-18].

Another aim of this study was to define the augmentation phenomenon in the liver patients.

Table 2 Baseline metabolic parameters of 211 patients recruited

Labs parameter (normal values)	Average of 211 patients (range)
Hemoglobin (14-16 g/dL)	11.1 (7.5-12.3)
Platelets counts (150-400 × 1000/μL)	97 (65-423)
Serum protein (g/dL)	7.6 (3.4-10.1)
Serum bilirubin (0.1-1.3 mg/dL)	1.7 (0.9-12)
Alanine aminotransferase (8-55 IU/L)	77 (24-452)
Aspartate aminotransferase (8-48 IU/L)	71 (34-715)
International normalized ratio (INR)	1.8 (1.0-4.9)
Serum creatinine (0.6-1.2 mg/dL)	1.0 (0.6-2.1)
Serum albumin (3.7-5.0 g/dL)	3.5 (1.5-5.1)
Ammonium (40-80 μg/dL)	97 (45-134)
Folate (3.89-26.0 ng/mL)	2.3 (1.9-12.3)
Iron free level (40-150 μg/dL)	26.5 (12-89)
Ferritine (20-200 ng/mL)	235 (126-456)
Vitamin B12 (205-870 pg/mL)	189 (121-245)
Vitamin D-OH25 (30-100 ng/mL)	41 (12-130)

Augmentation is a characteristic phenomenon, well known in RLS patients, even if its mechanisms are not fully understood and most importantly, the possible inducing factors have not been identified[11,13,18]. It seems to be a pejorative condition of the earliest symptoms of RLS, or an expansion to other body parts, such as the trunk or upper limbs, compared with the initial benefits of the therapy[19]. It has been related to long-term duration of dopaminergic therapy, to higher dosage, and to the dopamine stimulation (up to 14.2%-73% with L-DOPA, and from 8.3 up to 70% with dopamine agonists)[19-22]. Opioid analgesics, such as tramadol, methadone and oxycodone, may be considered for RLS treatment; although, trials reviewing long-term efficacy are lacking. The potential for abuse and adverse effects including dizziness, nausea and constipation limit the usefulness of these medications. In addition, tramadol has been rarely associated with RLS symptom augmentation[23].

As far as we know, no study has ever been conducted in hepatic patients to consider this phenomenon.

Titration, side effects, augmentation phenomenon and whichever alterations in laboratory test findings were checked and are reported here.

All procedures complied with ethical standards for human investigations and the principles of the Declaration of Helsinki. All the patients gave written informed consent for participation at the first visit.

RESULTS

Baseline characteristics of patients are reported in Tables 1 and 2. A synopsis of the various test scores are reported in Table 3.

Patients were moderately depressed, with evident nocturnal sleep problems and a concomitant daily sleepiness. The most relevant aspect is that the sleep problem had been underestimated, and RLS syndrome had not been considered before the neurological visit, since 211/211 patients fulfilled the IRLSSG criteria for RLS.

Table 3 Synopsis of the tests at baseline

Test (range)	Results
Hamilton rating scale (0-66)	18.5 ± 4.5
PSQI (0-5)	3.4 ± 0.5
ESS (0-24)	11 ± 2.1
IRLSSG	Fulfillment of criteria: 211/211
IRSL (0-40)	0-10 (mild) = 22 11-20 (moderate) = 76 21-30 (severe) = 109 31-40 (very severe) = 4

ESS: Excessive diurnal sleepiness; IRLS: International RLS severity scoring system; IRLSSG: International Restless Legs Syndrome Study Group evaluation; PSQI: Depression, sleep quality assessment.

All the patients pointed out that their involuntary leg movements had not been considered previously, or had been interpreted as neuropathic pain and therefore treated with nonsteroidal antiinflammatory drug. Of the 211 RLS patients, 22 considered their symptoms as mild, according to IRSL, but 189 found them moderate to very severe (Table 3).

Patients were moderately depressed according to an objective test, such as the Hamilton scale. Symptoms included depressed mood, insomnia, work and activities production, retardation as slowness of thought and speech, anxiety and somatic symptoms, insight and diurnal variation, and not in the more psychiatric-related scores, such as feelings of guilt, suicide thoughts, agitation, genital symptoms, hypochondriasis, loss of weight, depersonalization and derealization, paranoid symptoms, obsession and compulsive symptoms.

A Spearman's rank correlation analysis showed the following: (1) A positive correlation between IRLSSG fulfillment criteria and poor nocturnal sleep quality (PSQI: $r = 0.89$, $P < 0.01$); (2) a positive correlation between IRLSSG fulfillment criteria and excessive diurnal sleepiness (ESS: $r = 0.92$, $P < 0.01$); (3) a positive correlation between IRLSSG fulfillment criteria and Hamilton's score ($r = 0.76$, $P < 0.05$); and, (4) a positive correlation between the four levels of IRSL and PSQI (IRSL 0-10 *vs* PSQI: $r = 0.71$, $P < 0.05$; IRSL 11-20 *vs* PSQI: $r = 0.78$, $P < 0.05$; IRSL 21-30 *vs* PSQI: $r = 0.83$, $P < 0.01$; IRSL 31-40 *vs* PSQI: $r = 0.89$, $P < 0.01$). There was no correlation found between ammonium level and ESS or PSQI.

At the beginning, all patients were prescribed pramipexole at an average dosage of 0.18 mg, to be taken in the evening for the first 2 week. We then duplicated the dosage for 2 more weeks, up to 0.36 mg, once a day; this dosage was maintained till the 75th day. At the 75th day, we prescribed 0.7 mg daily, which was then increased to 0.88 mg daily at the 105th day. Forty-one patients reported side effects at the 135th day, such as persistent nausea, optical illusions and visual hallucinations, and decided to stop the pramipexole therapy (see below). The remaining

Table 4 Synopsis of pramipexole titration

Patients	Baseline	75th day	105th d	135th day	165th day	205th day
211	0.18 mg					
211		0.7 mg				
211			0.88 mg			
170				1.4 mg		
134					1.4 mg	
36					0.88 mg	
110						1.4 mg
60						0.7 mg

Table 5 Synopsis of gabapentin titration

Patients	45th day	75th day	105th day	135th day	165th day	205th day
41	100 mg					
41		300 mg				
35			300 mg			
6			400 mg			
30				300 mg		
11				500 mg		
27					300 mg	
14					600 mg	
16						300 mg
25						600 mg

Table 6 Results for pramipexole therapy during follow-up of 170 patients

Test (range)	135th day	165th day	205th day
Hamilton rating scale (0-66)	9.2 ± 0.1 (-9.3 ± 3.0; < 0.01)	8.7 ± 1.3 (-9.8 ± 1.7; < 0.01)	9.0 ± 1.1 (-9.5 ± 0.2; < 0.01)
PSQI (0-5)	2.2 ± 0.7 (-1.2 ± 0.2; < 0.05)	1.9 ± 0.7 (-1.32 ± 0.2; < 0.05)	2.3 ± 0.7 (-1.1 ± 0.2; < 0.05)
ESS (0-24)	8. 3 ± 0.7 (-7.1 ± 0.4; < 0.01)	8. 5 ± 0.4 (-7.3 ± 0.7; < 0.01)	8 .7 ± 1.1 (-7.7 ± 0.2; < 0.01)
IRSL (0-40)	0-10 (mild) = 51	134	110
	11-20 (moderate) = 100	12	45
	21-30 (severe) = 19	14	15
	31-40 (very severe) = 0	0	0

Within-group analysis was done by comparing results at each day's visit *vs* baseline. ESS: Excessive diurnal sleepiness; IRLS: International RLS severity scoring system; PSQI: Depression, sleep quality assessment.

170 patients were prescribed 1.4 mg daily, which seemed quite appropriate in respect of their average body mass index. At the following scheduled visit, on the 165th d, we reported that 134 patients (65%) felt well with the 1.4 mg/daily dose (the maximum allowed dosage being 2.1 mg daily). On the contrary, 36 patients (25%) reported the reappearance of unpleasant sensations in their legs and feet, with the urgency to rise up and move, during night and early morning (augmentation phenomenon). These patients were treated with 0.88 mg daily. At the 205th day, 110 patients (52%) continued to feel good with the 1.4 mg daily dosage; on the other hand, 60 patients (48%) reported the augmentation symptoms

Table 7 Results for gabapentin therapy during follow-up of 41 patients

Test (range)	135th day	165th day	205th day
Hamilton rating scalec(0-66)	9.7 ± 0.4 (-9.8 ± 0.2; < 0.01)	9.7 ± 0.5 (-9.8 ± 0.3; < 0.01)	10.0 ± 0.7 (-9.9 ± 1.2; < 0.01)
PSQI (0-5)	2.7 ± 0.7 (+ 0.7 ± 0.2; NS)	2.9 ± 0.3 (+0.5 ± 0.2; NS)	3.0 ± 0.5 (+0.6 ± 0.3; NS)
ESS (0-24)	9 9 ± 0.7 (-0.7 ± 1.0; NS)	9. 5 ± 0.4 (-0.5 ± 0.1; NS)	12 .7 ± 1.1 (-3.3 ± 0.1; < 0.05)
IRSL (0-40)	0-10 (mild) = 21	18	17
	11-20 (moderate) = 14	19	22
	21-30 (severe) = 6	4	2
	31-40 (very severe) = 0	0	0

Within-group analysis was done by comparing results at each day's visit *vs* the 45th day results. ESS: Excessive diurnal sleepiness; IRLS: International RLS severity scoring system; NS: Nonsignificant; PSQI: Depression, sleep quality assessment.

and were titrated to 0.7 mg daily (Table 4).

The 41 patients who abandoned pramipexole, after 2 wash-out weeks, were administered gabapentin at 100 mg daily for 10 d, then 200 mg daily for 20 d, and then 300 mg for 40 d. At the 105th day, 6 patients (14%) required 400 mg daily. At the 135th day, 11 patients (27%) needed 500 mg gabapentin daily. At the 165th day, 14 patients (34%) needed gabapentin up to 600 mg daily, and at the 205th day, 25 patients (61%) needed 600 mg gabapentin (Table 5).

Considering the 170 patients who completed the 205 d of follow-up with pramipexole, the results were rather satisfactory (Table 6), with an evident and constant amelioration of depression, of sleep quality and of daily sleepiness, as far as Wilcoxon signed rank test within-group comparison (*vs* baseline) showed. At the final visit, their subjective feeling of the intensity of RLS disturbances was perceived as mild to moderate in 155 patients and severe in 15 of them. Nobody declared a very severe score (Table 6).

The 41 patients who abandoned pramipexole, due to side effects, were treated with gabapentin (Table 5). According to a Wilcoxon signed rank test, there was a slight worsening of nocturnal sleep quality, significantly evident at the 205th day (Table 7) according to reporting of an increase in daily sleepiness. The quality of RLS disturbances was perceived at final visit as mild to moderate in 29 patients and severe in 2 of them. All the 41 patients who took gabapentin reported abdominal weight gain (5.2 ± 1.1 kg, range: 2.4-7.6) at the final visit.

We have determined the onset of augmentation symptoms in 170 patients who carried on with pramipexole. Logistic regression analysis to identify factors associated with the augmentation was performed with independent variables, including age, body mass index, IRLS alcohol abuse, iron-free levels, folate, vitamin B_{12} and D-OH25, alanine and

Table 8 Analysis of factors for association with the presence of augmentation

Factor	*n*	Univariate		Multivariate	
		OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age	170	1.07 (0.7-1.2)	0.24	1.1 (0.9-1.3)	0.20
BMI	170	1.3 (0.9-1.5)	0.45	1.6 (1.0-1.9)	0.40
IRLS	170	1.5 (1.1-2.2)	0.36	1.7 (1.2-2.2)	0.57
Alcohol abuse	139	2.3 (0.9-4.1)	< 0.001	3.75 (2.7-6.2)	< 0.001
Daily pramipexole	170	3.6 (2.1-6.8)	< 0.001	7.2 (4.1-15.2)	< 0.001
Treatment duration, > 75 d/< 75 d	170	2.3 (1.3-4.2)	0.036	6.6 (3.1-11.2)	0.01
ALT	170	1.3 (0.9-1.6)	0.21	3.1 (1.7-3.9)	0.54
AST	170	1.6 (0.8-1.7)	0.50	2.7 (0.7-4.2)	0.76
Iron-free level	170	2.9 (0.9-4.1)	0.01	5.05 (1.1-12.2)	0.06
Vit. B12	170	4.25 (1.3-9.7)	0.01	6.9 (4.7-7.6)	0.01
Folate	170	4.1 (3.1-13.6)	0.01	5.7 (4.2-8.2)	0.01
Vit. D-OH25	170	4.8 (3.4-12.9)	0.01	5.67 (2.4-8.9)	0.01

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CI: Confidence interval; IRLS: International restless leg syndrome severity; OR: Odds ratio; Vit.: Vitamin.

aspartate aminotransferases, treatment duration of pramipexole, and daily pramipexole doses. Univariate and multivariate logistic regression analyses were performed, and the Wald test was used to assess the significance of each variable, as reported in Table 8. Daily pramipexole dose, duration of treatment, previous alcohol abuse, iron-free levels as well as lower levels of B_{12}, D-OH25 and folate were significantly associated with augmentation in univariate analysis (Table 8). On the other hand, abuse of alcohol, dose of pramipexole and its duration, level of vitamin B12 and D-OH25 and of folate, in the multivariate regression analysis, seemed to be significantly associated with augmentation (Table 8).

DISCUSSION

In this study, we found that patients with chronic liver disease, such as liver cirrhosis or primitive tumors, who were referred for sleep disorders might have RLS as well. The presence of RLS was not associated with sex, and cause or severity of the liver disease was in line to what has been demonstrated by Goel *et al*[7]. As previously reported[3], many causative factors can induce RLS in hepatic chronic disease patients, such as low iron levels, high ferritin levels and associated low folate and vitamin B12 levels. It has also been described that the increased prevalence of RLS in chronic medical conditions (such as renal failure and, limited to few studies, hepatic failure) might be related to altered electrolyte levels, such as diuretic-induced hypokalemia, dilutional and diuretic-induced hyponatremia, hypocalcemia, or hypomagnesemia. Furthermore, it is possible that vitamin D deficiency, reduced physical activity, reduced muscular tone and increased serum levels of endotoxins and inflammatory cytokines (due to porta-systemic shunting resulting in low-grade inflammation) account for this phenomenon.

In particular, iron deficiency (present in all our patients) has been associated with dopamine pathology in RLS[24]. More specifically, it has been hypothesized that brain iron deficiency produces a dopaminergic pathology, resulting in the RLS symptoms[2]. Cerebral spinal fluid (CSF), autopsy and brain imaging studies clearly showed the expected brain iron deficiency, particularly affecting the dopamine-producing cells in the substantia nigra and their terminal fields in the striatum. A low content of iron in the brain is a well-established finding of RLS[24,25]. The dopamine pathology was, however, elusive and only recently has it been more clearly identified.

Animal and cellular iron deficiency studies have shown an increased activity of tyrosine hydroxylase in the substantia nigra[26] and decreased D2 receptors in the striatum[26]. These variations were associated with a decreased function of the cell membrane dopamine transporter[28] with increased concentration of the extracellular dopamine, with a 4-times increase in the amplitude of the circadian variation of extracellular dopamine (night-day difference)[29]. These same findings have been confirmed in RLS patients[2]. The CSF of these patients has significantly more 3-O-methyldopa, that correlates with the CSF homovanillic acid and RLS severity, indicating that increased dopamine production is proportional to the severity of RLS symptoms[30]. Moreover, the CSF tetrahydrobiopterin is significantly increased in the morning compared to night[30], and this finding is consistent with the larger circadian extracellular dopamine pattern observed in the iron-deprived rat.

As pointed out by Salas *et al*[2], RLS, unlike Parkinson's disease, is a hyper-dopaminergic condition with an apparent postsynaptic desensitization that overcompensates during the circadian low point of dopaminergic activity in the evening and night. This overcompensation leads to the RLS symptoms that can be easily corrected by adding dopamine stimulation at that time. The primary finding from multiple studies indicates that the iron deficiency affects dopaminergic function, by increasing tyrosine hydroxylase, which then increases extracellular dopamine[2,32-34]. Our study confirms an effective and rapid benefit by the use of dopamine agonist (as well-recognized and reported in the literature[3,32,33]).

On the other hand, RLS is a hyperdopaminergic condition, with an apparent postsynaptic desensitization that overcompensates during the circadian low point of dopaminergic activity in the evening and night. This overcompensation leads to the RLS symptoms that in turn often lead to increasing postsynaptic desensitization and augmentation of the RLS[2,32,34-36]. In fact, patients with RLS with mood and stress states may be at greater risk of developing compulsive behaviors while receiving standard dosage treatment of dopamine agonists (but

also of other drugs, such as tramadol[22]).

It appears evident that RLS remains a still confounding, not immediately recognized condition requiring rapid diagnosis and treatment. Augmentation seems rather precocious in our patients (135th day) and more frequent (35%) than previously described by Ferini-Strambi (8.3%)[20] and by Takahashi *et al*[22] (9.1%). The dosage of dopamine agonists found to be associated with augmentation in this study appears in range with the literature[14,19-22]. Previous intake of alcohol and lower levels of vitamins have been related to the phenomenon in our study.

RLS is a major cause of insomnia, and the structure of sleep of sufferers may be severely impaired. Sleep disruption has, in consequence, a great impact on health and daytime functioning of RLS patients. Despite the fact that RLS is a very common disorder, it is frequently undiagnosed in primary care, and therefore inadequate therapy may be prescribed.

Additional neurophysiologic studies and more extended clinical trials are strenuously needed to explain some crucial pathologic mechanisms of the syndrome, in order to better employ common therapeutic agents and to find novel ones.

ARTICLE HIGHLIGHTS

Research background

Restless legs syndrome (RLS) remains a clinical diagnosis based on confirming the four essential diagnostic features of RLS, including: (1) An urge to move the legs, usually but not always accompanied by or felt to be caused by uncomfortable and unpleasant sensations in the legs; (2) the urge to move, as any accompanying unpleasant sensation begins or worsens during periods of rest or inactivity, such as lying down or sitting; (3) the urge to move, as any accompanying unpleasant sensation is partially or totally relieved by movement, such as walking or stretching; and (4) the urge to move, as any accompanying unpleasant sensation during rest or inactivity only occurs or is worse in the evening or night compared to during the day. Chronic medical situations (dialysis, end-stage renal disease and rheumatologic disorders) have a higher prevalence of RLS.

Research motivation

An increased prevalence of RLS has been described in patients with liver cirrhosis in the United States and Japan. Very recently, RLS has been described in India in a series of chronic hepatic failure patients. Data in hepatic patients are limited.

Research objectives

According to neurological exams, the diagnosis of RLS was suggested by the Johns Hopkins questionnaire and verified by fulfillment of the diagnostic criteria by Allen. Iron-free level, ferritin, folate and vitamin B12 and vitamin D-OH25 were measured in all patients. Drugs used to treat RLS belong to many different pharmacological classes, such as the dopaminergic agents, opioids, benzodiazepines and antiepileptic drugs. We decided to avoid the employment of opioids and benzodiazepine due to the hepatic conditions. Therefore, the first-choice drug was pramipexole, a dopamine agonist. The second-choice drug was gabapentin, due to its beneficial properties and to the limited hepatic side effects. Neurological examinations and laboratory tests were performed at the beginning, after 2 wk, at the 28th, 75th, 105th, 135th and 165th day, and at the final day of the follow-up, the 205th day. Another aim of this study was to define the augmentation phenomenon in the liver patients.

Research methods

The study included 267 adult patients with chronic liver disease, referred to our Neurological Unit by the Liver Unit of the University of Trieste, for three complaints, including sleep disturbances, painful leg sensation, and daily sleepiness not correlated to hepatic encephalopathy. Patients with chronic liver disease included primary liver tumor and liver cirrhosis cases. We excluded 13 patients with chronic and persistent significant alcohol intake, 25 patients with recent worsening of clinical condition, and 12 patients with hepatitis C virus infection. All the other 211 patients were followed up by a neurologist for at least 24 mo. At baseline, patients were tested with the Hamilton rating scale for depression, sleep quality assessment (PSQI), Epworth sleepiness scale (ESS), International Restless Legs Syndrome Study Group (IRLSSG) evaluation, and international RLS severity (IRLS) scoring system. Alterations in titration, side effects, augmentation phenomenon and laboratory test findings were checked and reported. The first-choice drug was pramipexole, a dopamine agonist. If major side effects induced by the neurological therapy occurred, such as nausea, vomiting, somnolence, hypotension or optical illusions, the pramipexole was stopped. The second-choice drug was gabapentin, due to its beneficial properties and to the limited hepatic side effects. Another aim of this study was to define the augmentation phenomenon in the liver patients.

Research results

Patients included in the study fulfilled the IRLSSG criteria for RLS; they were moderately depressed, with evident nocturnal sleep problems and a concomitant daily sleepiness. Of the 211 RLS patients, 22 considered their symptoms as mild, according to IRSL, but 189 found them moderate to very severe. A Spearman's rank correlation analysis showed the following: (1) a positive correlation between IRLSSG fulfillment criteria and poor nocturnal sleep quality (PSQI); (2) a positive correlation between IRLSSG fulfillment criteria and excessive diurnal sleepiness (ESS); (3) a positive correlation between IRLSSG fulfillment criteria and Hamilton's score; (4) a positive correlation between the four levels of IRSL and PSQI; and (5) no correlation between ammonium level and ESS or PSQI. At the beginning, all patients were prescribed pramipexole at an average dosage of 0.18 mg, to be taken in the evening for the first 2 week. Titration was standard; we duplicated the dosage for 2 more weeks, up to 0.36 mg, till the 75th day. At the 75th day, we prescribed 0.7 mg daily, which was then increased to 0.88 mg daily at the 105th day. Forty-one patients reported heavy side effects at the 135th day and decided to stop the pramipexole therapy. The remaining 170 patients were prescribed 1.4 mg daily, which seemed quite appropriate in respect of their average body mass index. At the 205th day, 110 patients (52%) continued to feel good with the 1.4 mg daily dosage; on the other hand, 60 patients (48%) reported the augmentation symptoms and were titrated at 0.7 mg daily. The 41 patients who abandoned pramipexole, after 2 wash-out weeks, were administered gabapentin, at increasing dosages. Considering the 170 patients who completed the 205 d of follow-up with pramipexole, the results were rather satisfactory, with an evident and constant amelioration of depression, of sleep quality and of daily sleepiness, as far as Wilcoxon signed rank test within-group comparison (*vs* baseline) showed. At the final visit, their subjective feeling of the intensity of RLS disturbances was perceived as mild to moderate in 155 patients and severe in 15 of them. Nobody declared a very severe score. The 41 patients who abandoned pramipexole, due to side effects, were treated with gabapentin, reporting a slight worsening of nocturnal sleep quality and an increase of daily sleepiness. All the 41 patients who took gabapentin reported abdominal weight gain at the final visit. As far as the augmentation phenomenon was concerned, a logistic regression analysis to identify factors associated with the augmentation were performed with independent variables, including age, body mass index, IRLS alcohol abuse, iron-free levels, folate, vitamin B12 and D-OH25 levels, alanine and aspartate aminotransferase, treatment duration of pramipexole, and daily pramipexole doses. Univariate and multivariate logistic regression analyses were performed and the Wald test was used to assess the significance of each variable. Daily pramipexole dose, the duration of the treatment, previous alcohol abuse, iron-free levels as well as lower levels of B12, D-OH25 and folate were significantly associated with augmentation in univariate analysis. On the other hand, abuse of alcohol, dose of pramipexole and its duration, level of vitamin B12 and D-OH25 and of folate, in the multivariate regression analysis, seemed to be significantly associated with augmentation.

Research conclusions

In this study, we found that patients with chronic liver disease, such as liver cirrhosis or primitive tumors, who were referred for sleep disorders might have

RLS as well. The presence of RLS was not associated with sex, and cause or severity of the liver disease was in line with what has been demonstrated by the few other studies. As previously reported, in our study, many causative factors induce RLS in hepatic chronic disease patients, such as low iron levels, high ferritin levels, and associated low folate and vitamin B12 levels. Our study confirms an effective and rapid benefit for the use of dopamine agonist (as is well-recognized and reported in the literature). On the other hand, RLS is a hyperdopaminergic condition with an apparent postsynaptic desensitization that overcompensates during the circadian low point of dopaminergic activity in the evening and night. This overcompensation leads to the RLS symptoms that in turn often lead to increasing postsynaptic desensitization and augmentation of the RLS. In fact, patients with RLS with mood and stress states may be at greater risk of developing compulsive behaviors while receiving standard dosage treatment of dopamine agonists (but also of other drugs, such as tramadol). Augmentation seems rather precocious in our patients (135th day), and more frequent (35%) than previously described by the most important study on the topic (8.3%-9.1%). The dosage of dopamine agonists reported in our study to be associated with augmentation appears to be in range with the literature. Previous intake of alcohol and lower levels of vitamins were related to the phenomenon in our study.

Research perspectives

It appears evident that RLS remains a still confounding, not immediately recognized condition requiring rapid diagnosis and treatment. Despite the fact that RLS is a very common disorder, it is frequently undiagnosed in primary care, and therefore inadequate therapy may be prescribed. Additional neurophysiologic studies and more extended clinical trials are strenuously needed to explain some crucial pathologic mechanisms of the syndrome, in order to better employ common therapeutic agents and to find novel ones.

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CASE REPORT

Clostridium paraputrificum septicemia and liver abscess

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Author contributions: Kwon YK and Cheema FA wrote the initial manuscript; Maneckshana BT, Rochon C and Sheiner PA performed a literature search and analyzed the data; all authors contributed equally to editing and revising the final manuscript.

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Abstract

We report the first case of a healthy 23-year-old female who underwent an interventional radiology-guided embolization of a hepatic adenoma, which resulted in a gas forming hepatic liver abscess and septicemia by *Clostridium paraputrificum*. A retrospective review of Clostridial liver abscesses was performed using a PubMed literature search, and we found 57 clostridial hepatic abscess cases. The two most commonly reported clostridial species are *C. perfringens* and *C. septicum* (64.9% and 17.5% respectively). *C. perfringens* cases carried a mortality of 67.6% with median survival of 11 h, and 70.2% of the *C. perfringens* cases experienced hemolysis. All *C. septicum* cases were found to have underlying liver malignancy at the time of the presentation with a mortality of only 30%. The remaining cases were caused by various *Clostridium* species, and this cohort's clinical course was significantly milder when compared to the above *C. perfringens* and *C. septicum* cohorts.

Key words: *Clostridium*; Hemolysis; Liver cell adenoma; Morbidity; Mortality; Pyogenic liver abscess

Core tip: To our best knowledge, this is the first case where a liver abscess grew *C. paraputrificum*. Although pyogenic liver abscesses caused by *Clostridium* species are extremely rare, early and accurate diagnosis of clostridial hepatic abscess and timely interventions are paramount, as it carries an extremely high morbidity and mortality. However, depending on the exact causative *Clostridium* species, the clinical course can vary unexpectedly.

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INTRODUCTION

Pyogenic liver abscesses caused by *Clostridium* species are extremely rare[1], and only 57 cases have been reported in the English medical literature (Table 1). *C. perfringens* was responsible for more than a half of these reported cases. This species carries an extremely high mortality rate, especially when associated with hemolysis[2-4]. The previously reported 20 *C. perfringens* cases showed a median age of 65 years at the time of presentation[5]. Advanced age, underlying malignancy, liver cirrhosis, and immunocompromised conditions including dialysis, transplant and diabetes mellitus were identified as risk factors[2,5-8]. Here we present a very unusual case of a healthy 23-year-old female who underwent interventional radiology (IR) embolization for a hepatic adenoma and presented within 24 h with a gas forming hepatic liver abscess and septicemia. Due to the extremely rapid clinical presentation where the embolized tumor was completely replaced by a gas forming abscess within a day, *C. perfringens* was suspected as the causative organism. Unlike many other fatal *C. perfringens* hepatic abscess cases, our patient did not have any signs of hemolysis nor experienced any end-organ failure. Future speciation work-up revealed *C. paraputrificum*. There have been five case reports of septicemia caused by *C. paraputrificum*[9-13]. However, this is the first case of a gas forming hepatic abscess.

CASE REPORT

A 23-year-old healthy female with obesity (body mass index of 37 kg/m^2) and Polycystic Ovarian Syndrome on oral contraceptive pills was evaluated for intermittent, right upper quadrant abdominal pain. She was found to have a hepatic adenoma measuring 5.2 cm × 3.3 cm × 6.6 cm abutting the liver capsule in segment 7 (Figure 1) on imaging. The patient's oral contraceptive pill was discontinued for the more than three months, since the adenoma was diagnosed. A repeat computerized tomography (CT) scan did not show regression of the mass (Figure 2). Due to ongoing intractable abdominal right upper quadrant pain and risk of potential rupture, a surgical resection was presented as an option *vs* IR-guided embolization as an alternative option given her body habitus and fatty liver on magnetic resonance imaging study. The patient elected to proceed with IR embolization.

Angiogram showed conventional hepatic artery anatomy, and the adenoma was exclusively fed by a single branch coming off of the posterior right hepatic artery (Figure 3). The tumor was completely embolized with 100-300 μm trisacryl gelatin microspheres (Embosphere®, Merit Medical Systems, Inc., South Jordan, United States). The patient was discharged home the same day.

The next day, the patient began to experience a rapid onset of right upper abdominal pain, nausea, vomiting and fever of 101.5 °F. In the emergency room, the patient was tachycardic with a heart rate in the 120 s. She experienced right upper abdominal tenderness on physical exam. Blood tests showed a white blood cell (WBC) count of 16.4 Thou/μL, a lactic acid of 2.4 nmol/L, a serum aspartate transaminase (AST) of 671 U/L, a serum alanine transaminase (ALT) of 310 U/L, and a total bilirubin (T. bili) of 1.4 mg/dL. A CT scan showed the embolized tumor in segment 7 completely replaced with multiple gas pockets (Figure 4). A set of blood cultures was sent, and the patient was started on vancomycin, levofloxacin and metronidazole (patient has a penicillin allergy). The next day, the set of blood cultures grew gram positive rods. The patient's serum WBC was elevated to 25 Thou/μL. Later that day, the preliminary blood culture revealed *clostridium* species. With ongoing fever and the newly diagnosed *clostridium* species infection, a repeat CT scan was performed to rule out potential life threatening gas gangrene. The repeat CT scan showed no changes.

The patient remained persistently febrile, despite antibiotic therapy and subsequent blood cultures showing no growth. The culture speciation showed *Clostridium paraputrificum* and no other organisms were isolated. Despite improving leukocytosis, an IR-guided drain was placed on hospital day 10 due to the persistent fevers. One hundred and twenty cc of dark turbid sterile fluid was aspirated, and the gram stain showed many neutrophils. No bacteria were isolated. Aspirin was started because the patient's platelet count rose above 500 Thou/μL. Over the next a few days since the drain placement, the fluid character became less turbid. However, the color became frankly bilious. The daily drain output persistently remained less than 200 cc, indicating a low output bile leak. Thus an ERCP was not performed. On Hospital day 16, the patient was afebrile for the first time. The patient was discharged home on hospital day 17 since the patient was afebrile for 48 hours. At the time of discharge, the drain output was less than 100 cc per day and the patient was discharged on oral metronidazole only.

The patient presented two weeks after discharge with a follow-up CT, which revealed a significantly reduced gas filled abscess cavity (Figure 5). The IR drain was taken out as the daily output remained minimum, less than 5 cc per day. Oral metronidazole was continued for two more weeks post drain removal. Upon completion of the antibiotic course, blood tests showed a WBC of 9.5 Thou/μL, a platelet count of 379 Thou/μL, an AST of 27 U/L, an ALT of 30 U/L, and a T. bili of 0.6 mg/dL.

Table 1 Fifty-seven reported clostridial hepatic abscess cases in the English medical literature

Case	Author	Year	Age	Sex	Species	Underlying disease	HML	SSE	TTD	PLM	PMI
1	Fiese[35]	1950	67	M	*C. perfringens*	Cholecystitis	No	Yes	-	No	Yes
2	Kivel *et al*[36]	1958	68	F	*C. perfringens*	DM	Yes	No	5 d	No	No
3	Kahn *et al*[37]	1972	44	F	*C. septicum*	Colon cancer	No	Yes	-	Yes	Yes
4	D'Orsi *et al*[38]	1979	52	F	*C. septicum*	Colon cancer	No	Yes	-	Yes	No
5	D'Orsi *et al*[38]	1979	51	F	*C. ramosum*	Melanoma	Yes	No	2 d	Yes	No
6	D'Orsi *et al*[38]	1979	29	M	*C. ramosum*, *C. sporogenes*	Peri-ampullaryCa	No	Yes	-	Yes	Yes
7	Mera *et al*[39]	1984	6	F	*C. perfringens*	Fanconi's anemia	Yes	No	14 h	No	No
8	Nachman *et al*[40]	1989	6	M	*C. bifermentans*	Blunt trauma	No	Yes	-	No	No
9	Yood *et al*[41]	1989	64	F	*C. perfringens*	Systemic vasculitis	No	Yes	-	No	No
10	Batge *et al*[42]	1992	61	M	*C. perfringens*	Pancreatic cancer, DM	Yes	Yes	-	No	No
11	Rogstad *et al*[43]	1993	61	M	*C. perfringens*	None	Yes	No	3 h	No	No
12	Thel *et al*[32]	1994	39	F	*C. septicum*	Breast Ca, Bone M. txp	No	Yes	-	Yes	No
13	Gutierrez *et al*[44]	1995	74	M	*C. perfringens*	None	Yes	No	6 h	No	No
14	Jones *et al*[45]	1996	66	F	*C. perfringens*	OLT, DM	Yes	No	10 h	No	No
15	Lee *et al*[34]	1999	33	F	*C. septicum*	Uterine cancer	No	Yes	-	Yes	No
16	Eckel *et al*[46]	2000	65	F	*C. perfringens*	Cholangiocarcinoma	Yes	Yes	-	Yes	Yes
17	Urban *et al*[47]	2000	68	M	*C. septicum*	Colon cancer	No	Yes	-	Yes	No
18	Sakurai *et al*[48]	2001	75	F	*C. difficile*	Hepatic cyst	No	Yes	-	Yes	No
19	Kreidl *et al*[8]	2002	80	M	*C. perfringens*	DM, dialysis	Yes	No	11 h	No	No
20	Sarmiento *et al*[49]	2002	57	M	*C. septicum*	Colon cancer	No	Yes	-	Yes	No
21	Hsieh *et al*[50]	2003	23	M	*Unusual C. spp.*	Blunt trauma	No	Yes	-	No	No
22	Quigley *et al*[51]	2003	73	M	*C. perfringens*	Hepatic cyst	-	No	0 h	Yes	Yes
23	Elsayed *et al*[52]	2004	27	M	*C. hathewayi*	Cholecystitis	No	Yes	-	No	No
24	Fondran *et al*[53]	2005	63	M	*C. perfringens*	Pancreatic cancer	No	Yes	-	Yes	Yes
25	Au *et al*[7]	2005	65	M	*C. perfringens*	DM, dialysis	Yes	No	3 h	No	No
26	Kurtz *et al*[54]	2005	50	F	*C. septicum*	Colon cancer	No	Yes	-	Yes	No
27	Ohtani *et al*[55]	2006	78	M	*C. perfringens*	DM	Yes	No	3 h	No	No
28	Daly *et al*[56]	2006	80	M	*C. perfringens*	DM	Yes	No	3 h	No	No
29	Loran *et al*[57]	2006	69	F	*C. perfringens*	None	Yes	No	6 h	No	No
30	Chiang *et al*[58]	2007	46	F	*C. perfringens*	Cholecystitis	No	No	7 d	No	No
31	Abdel-Haq *et al*[59]	2007	11	M	*C. novyi type B*	Blunt trauma	No	Yes	-	No	No
32	Umgelter *et al*[60]	2007	87	F	*C. perfringens*	Colon cancer	No	Yes	-	Yes	No
33	Tabarelli *et al*[61]	2009	65	F	*C. perfringens*	Pancr. Ca s/p whipple	No	No	27 d	No	Yes
34	Merino *et al*[62]	2009	83	F	*C. perfringens*	None	Yes	No	3 d	No	No
35	Saleh *et al*[63]	2009	53	M	*C. septicum*	Colon cancer	No	Yes	-	Yes	No
36	Meyns *et al*[64]	2009	64	M	*C. perfringens*	DM	Yes	No	2 d	No	No
37	Ng *et al*[4]	2010	61	F	*C. perfringens*	DM	Yes	Yes	-	No	Yes
38	Rajendran *et al*[65]	2010	58	M	*C. perfringens*	None	Yes	Yes	-	No	No
39	Bradly *et al*[66]	2010	52	M	*C. perfringens*	OLT	Yes	No	6 h	No	No
40	Ogah *et al*[67]	2012	6	F	*C. clostridioforme*	None	No	Yes	-	No	No
41	Qandeel *et al*[68]	2012	59	M	*C. perfringens*	DM, s/p elective chole	Yes	Yes	-	No	No
42	Kim *et al*[69]	2012	80	F	*C. perfringens*	Hilar cholangiocarcinoma	No	No	3 d	No	Yes
43	Huang *et al*[70]	2012	54	M	*C. baratii*	Cholecystitis	No	Yes	-	No	No
44	Sucandy *et al*[71]	2012	65	M	*C. septicum*	Colon cancer	No	No	2 d	Yes	No
45	Law *et al*[5]	2012	50	F	*C. perfringens*	Rectal cancer	Yes	No	7 d	Yes	No
46	Raghavendra *et al*[72]	2013	63	M	*C. septicum*	Colon cancer	No	Yes	-	Yes	No
47	Kitterer *et al*[73]	2014	71	M	*C. perfringens*	OLT, Gastroenteritis	Yes	No	13 h	No	No
48	Imai *et al*[74]	2014	76	M	*C. perfringens*	None	Yes	No	6.5 h	No	No
49	Kurasawa *et al*[2]	2014	65	M	*C. perfringens*	DM	Yes	No	6 h	No	No
50	Eltawansy *et al*[75]	2015	81	F	*C. perfringens*	DM, Gastroenteritis	No	No	N/A[1]	No	Yes
51	Li *et al*[76]	2015	71	M	*C. perfringens*	HCC, Hepatitis B	Yes	Yes	-	Yes	No
52	Rives *et al*[77]	2015	63	M	*C. perfringens*	Colon cancer	No	Yes	-	Yes	No
53	Lim *et al*[6]	2016	58	M	*C. perfringens*	None	Yes	No	7.5 h	No	No
54	Hashiba *et al*[78]	2016	82	M	*C. perfringens*	DM	Yes	No	2 h	No	No
55	Kyang *et al*[79]	2016	84	M	*C. perfringens*	Gastric adenoCA	No	Yes	-	Yes	Yes
56	Ulger *et al*[80]	2016	80	F	*C. difficile*	DM	No	No	18 d	No	No
57	García *et al*[81]	2016	65	M	*C. perfringens*	DM	Yes	Yes	-	No	Yes

[1]Exact time of TTD was not discussed, but terminal vent weaning was initiated and subsequently expired. HML: Hemolysis; SSE: Survival of septic episode; TTD: Time to death; PLM: Presence of liver mass; PMI: Polymicrobial infection.

DISCUSSION

Pyogenic liver abscess (PLA) is an uncommon disease. Various incidences have been reported throughout the world: 1.1 in Denmark[14], 2.3 in Canada[15] and 17.6 per 100000 population in Taiwan[16]. In the United States, the incidence is 3.6 per 100000 population with a reported in-hospital mortality rate of 5.6%[17].

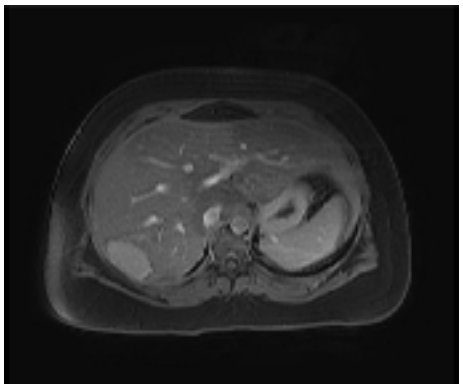

Figure 1 Magnetic resonance imaging of the segment 7 hepatic adenoma measuring 5.2 cm × 3.3 cm × 6.6 cm.

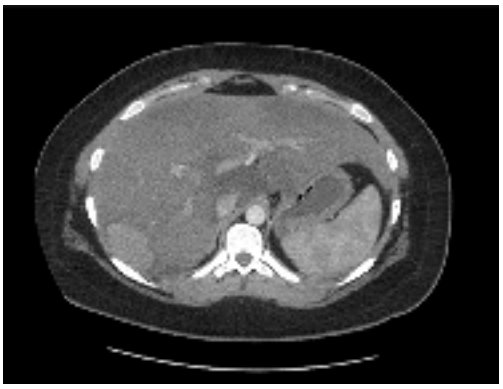

Figure 2 Computed tomography after stopping oral contraceptive pills for 3 mo. No change in size.

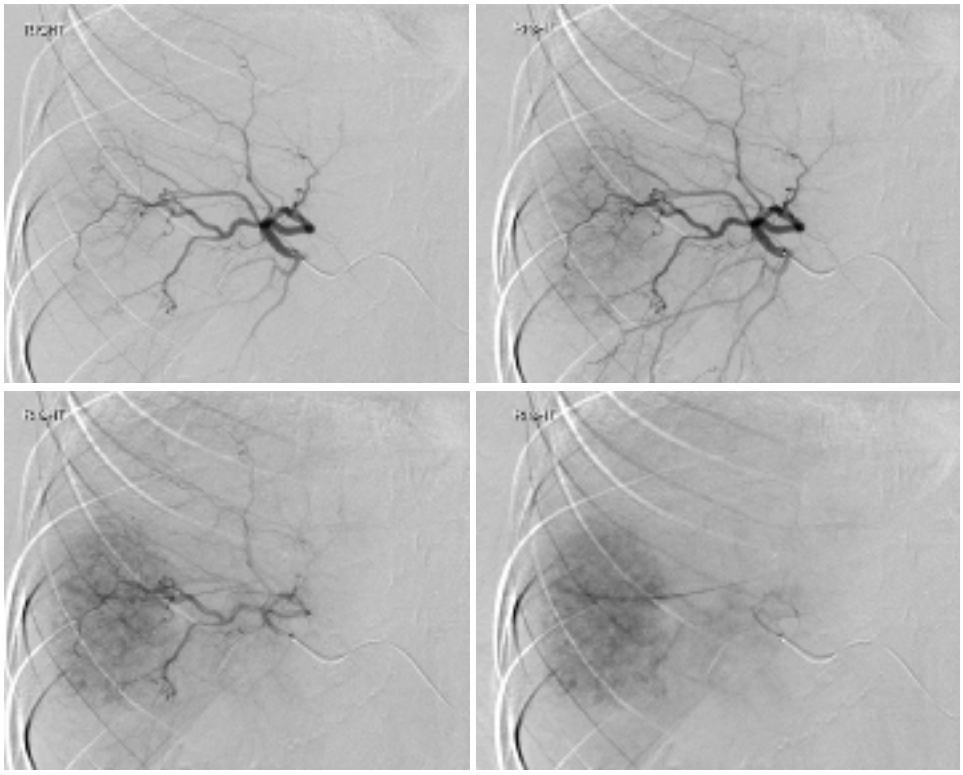

Figure 3 Interventional radiology angiogram of the hepatic adenoma.

The incidences of gas forming pyogenic liver abscess (GFPLA), also known as emphysematous liver abscess, are even rarer, contributing 6.6% to 32% of PLA[16,18-21]. It carries a significantly higher mortality rate, 27.7% to 37.1%[22-25]. For those who presented with GFPLA, their incidence of septic shock was higher (32.5% *vs* 11.7%) and they presented with a shorter duration of symptoms (5.2 d *vs* 7.6 d) when compared to those who presented with non-gas forming pyogenic liver abscess (NGFPLA)[22].

The single strongest risk factor for GFPLA appears to be the presence of diabetes and poorly controlled blood glucose[15,18,22]. According to a case report series done in Taiwan which compared 83 patients with GFPLA against 341 NGFPLA patients, 85.5% of those with GFPLA had diabetes mellitus with an initial glucose level of 383.0 ± 167.7 (mg/dL) *vs* 33.1% with an initial glucose level of 262.6 ± 158.0 (mg/dL)[22]. Similar findings were reported from another single center series from South Korea, where 76% (19 out of 25) were found to have diabetes when comparing 25 patients with GFPLA against 354 NGFPLA patients[18]. The most common causative organism for GFPLA was *Klebsiella pneumoniae* contributing 77% to 88%[18,22,25]. *Escherichia*, *Streptococcus*, *Enterococcus*, *Pseudomonas*, *Morganella*, *Enterobacter*, *Serratia*, *Bacteroides* and *Clostridium* species were responsible for the remaining[22].

An extremely small portion of GFPLA is caused by clostridial species. The two most commonly reported

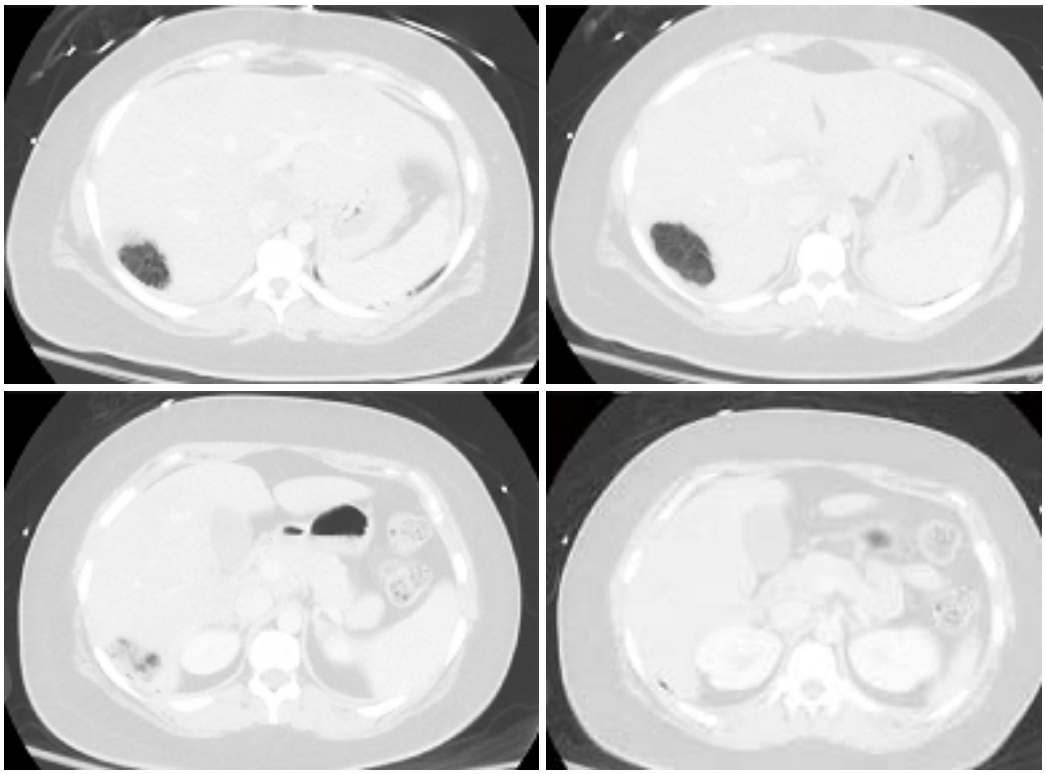

Figure 4 The tumor completely replaced by gas pockets.

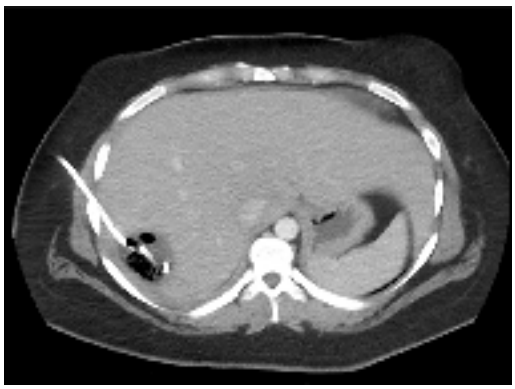

Figure 5 Follow-up computed tomography. The gas pocket reduced.

clostridium species are *C. perfringens and C. septicum*. We performed a PubMed literature search and identified 57 *clostridium* hepatic abscess cases reported in the English medical literature (Table 1). Our search showed that *C. perfringens* was responsible for 37 cases (64.9%) and *C. septicum* was responsible for 10 cases (17.5%). Nine cases were caused by *C. difficile, C. ramosum, C. sporogenes, C. baratii, C. bifermentans, C. clostridioforme, C. hathewayi,* and *C. novyi type B*. In one case, the exact speciation was not provided due to the institution's microbiology limitation for identifying rare clostridial species.

C. perfringens septicemia has been reported to carry a mortality rate ranging from 70%-100%[4]. Massive intravascular hemolysis is a well-known complication, occurring in 7%-15% of *C. perfringens* bacteremia cases[26-28]. *C. perfringens*'s alpha-toxin has been shown be the key virulent factor for this clinical course, by inducing gas gangrene and causing massive hemolysis by destroying red cell membrane integrity[3]. In our 37 cases of *C. perfringens* hepatic abscess, the mortality rate was 67.6% (25/37). 70.2% (26/37) experienced hemolysis (Table 1). Among the 25 patients who died, one patient died prior to arriving to the hospital. The mean time of survival for these 24 patients was 11 h. Among the 25 patients who died, only 4 patients (16%) were found to have poly-microbial infection, whereas among those who survived, 6 patients (50%) were found to have poly-microbial infection. The most common underlying disease was diabetes (11/37) followed by underlying malignancy (10/37). Interestingly, 7 patients were found to have no clear underlying medical disease.

Among the 10 cases of *C. septicum* species (Table 1), the patient survival was greater, 70% (7/10). Furthermore, no hemolysis was reported in contrast to the *C. perfringens* cases. Of note, *C. septicum also* produces alpha toxin, but it was shown to be unrelated to the alpha toxin of *C. perfringens*[29]. *C. septicum* infection has been well known to be associated with underlying occult malignancy[30-33]. It has been hypothesized that a rapidly growing tumor with anaerobic glycolysis provides a relatively hypoxic and acidic environment for germination of the clostridial spores[34]. In fact, all of the ten patients had infected liver tumors at the time of the presentation, and only one patient (10%) was found to have a poly-microbial infection.

The remaining 10 cases where the infection was

caused by various clostridial species, including the one with no provided speciation, appeared to have a milder clinical course when compared to the above *C. perfringens* and *C. septicum* cohorts (Table 1). The mortality rate was lower, only 20%, and median age at the time of presentation was significantly younger, 27 years. Interestingly, trauma was the underlying disease for the three cases.

Here, we report a young, healthy 23-year-old female who was diagnosed with a hepatic abscess caused by *Clostridium paraputrificum*. Due to the extremely rapid clinical presentation and from the initial imaging study where the mass was completely replaced with multiple gas pockets, a *C. perfringens* infection was highly suspected. Unlike many typical *C. perfringens* hepatic abscess cases, our patient did not experience hemolysis nor had any end organ failure requiring ICU care. In addition, our patient did not have the typical risk factors for *C. perfringens* nor *C. septicum* infections, except for having a tumor in the liver. At the end, the causative organism was identified as *Clostridium paraputrificum*, which has not been reported before in the literature. A *Clostridium* hepatic abscess is an extremely rare case and *C. perfringens* is the most common causative organism. Early accurate diagnosis and timely interventions are paramount, as it carries an extremely high mortality. However, depending on the exact causative clostridial species, the clinical course can vary significantly.

ARTICLE HIGHLIGHTS

Case characteristics

A healthy 23-year-old female developed a *Clostridium paraputrificum* gas forming liver abscess within 24 h after interventional radiology hepatic adenoma embolization.

Clinical diagnosis

The patient's source of sepsis was unequivocally identified once an imaging study showed a gas forming liver abscess.

Differential diagnosis

Klebsiella pneumonia was suspected to be the causative organism initially as it is known to contributing 77% to 88% of all gas forming pyogenic liver abscesses.

Laboratory diagnosis

In addition to severe leukocytosis and lactic acidosis, elevated lactate dehydrogenase, deceased haptoglobin and elevated bilirubin, signs of massive hemolysis, can be also seen in certain patients.

Imaging diagnosis

A gas forming liver abscess can be diagnosed with an abdominal X-ray or ultrasound, but typically a computed tomography scan is commonly used for the diagnosis.

Pathological diagnosis

A needle aspiration of the hepatic abscess and/or blood culture often will yield the causative organism.

Treatment

An early recognition and treatment with antibiotics is paramount as *Clostridium* hepatic abscess infections are often extremely aggressive and lethal.

Related reports

There have been five case reports of septicemia caused by *C. paraputrificum*, however, none of them caused hepatic abscess.

Term explanation

Pyogenic liver abscess (PLA) is an uncommon disease. The incidences of gas forming pyogenic liver abscess (GFPLA) also known as emphysematous liver abscess, are even rarer, contributing 6.6% to 32% of PLA.

Experiences and lessons

A *Clostridium* hepatic abscess requires early accurate diagnosis and timely interventions, as it carries an extremely high mortality. However, depending on the exact causative clostridial species, the clinical course can vary significantly.

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CASE REPORT

Liver failure caused by prolonged state of malnutrition following bariatric surgery

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Abstract

Bariatric surgery is an effective tool in the treatment of patients with morbid obesity. In these case reports we describe 2 patients who developed liver failure after currently-practiced types of bariatric surgery, caused by a prolonged state of malnutrition provoked by psychiatric problems. Despite intensive guidance of a psychologist and dieticians after surgery, our patients deteriorated psychologically, resulting in a prolonged state of severe malnutrition and anorexia. Finally, a state of starvation was reached, passing a critical level of the liver capacity. Patients who present with signs of severe protein malnutrition after bariatric surgery should be closely monitored and checked for nutritional status. Specific attention should be given to patients who develop psychiatric problems post-bariatric surgery. If refeeding does not result in clinical improvement, reversal surgery should be considered in a timely manner.

Key words: Protein deficiency; Hyperbilirubinemia; Hyperammonemia; Liver failure; Urea cycle

Core tip: Monitoring of patients after bariatric surgery is important. When psychiatric problems appear, you should be alert and treat your patients proactively. Unfortunately, these case reports show that psychiatric deterioration can lead to severe malnutrition and anorexia, although rarely resulting in liver insufficiency and failure.

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INTRODUCTION

Morbid obesity is an increasing healthcare problem in the Western world, with development of important complications, such as diabetes, cardiovascular disease and fatty liver disease. Among morbidly obese individuals, nonalcoholic fatty liver disease is highly prevalent and a substantial number of patients may develop advanced liver fibrosis or cirrhosis over time[1,2]. Ultimately, these conditions will lead to liver failure and death.

Bariatric surgery provides an effective tool in the treatment of patients with morbid obesity and its comorbidity[3]. Short-term effects, such as significant weight loss and remission of diabetes, have been extensively documented[4]. Several clinical studies have shown that bariatric surgery has an important positive impact on the liver, with improvements of liver enzymes and liver histology[5,6].

The development of liver failure after bariatric surgery has previously been described after jejunoileal bypass and biliopancreatic diversion (Scopinaro) surgery[7], but is rare in modern bariatric surgery. A common idea is that nonuse of the bypassed intestine can lead to changes in the mucosa and bacterial flora. As a result of bacterial overgrowth hepatotoxic macromolecules are produced, passing the damaged mucosa and reaching the liver through the portal venous system and resulting in damage of hepatocytes.

In these case reports we describe 2 patients who developed liver failure after currently-practiced types of bariatric surgery, caused by a prolonged state of malnutrition provoked by psychiatric problems.

CASE REPORT

Case 1

A 43-year-old female underwent endoscopic gastric bypass surgery because of morbid obesity [body mass index (BMI) 59 kg/m^2]. After 1 year, she underwent banded gastric bypass surgery because of insufficient weight loss [BMI: 47 kg/m^2, %excess weight loss (EWL): 34.9%, total body weight loss: 20%]. After surgery, she suffered from episodes of abdominal pain and dysphagia. Therefore, 1 year later the gastric band was removed with revision of the gastric bypass to a distal bypass (alimentary limb 735 cm, biliopancreatic limb 60 cm, common channel 100 cm). In the following period, additional weight loss was recorded (BMI: 32 kg/m^2, %EWL: 79.4%, total body weight loss: 46%) with a relative good quality of life. Another year later, she became pregnant. Unfortunately, after 22 wk she gave birth prematurely, resulting in fetal death. In the following 6 mo, she was hospitalized four times with malnutrition, hypoalbuminemia (serum albumin 12 g/L), generalized edema and depression. During this period, she refused any involvement of psychiatrists.

At her final admission to the hospital, she had abstained from food for more than a week, with suspicion of anorexia. Common causes of hypoalbuminemia, such as protein-losing enteropathy and nephrotic syndrome were excluded. Enteral tube feeding was started with protein plus multi-fiber (protein: 95 g/L). However, on day 8 of admission, she developed a somnolent state caused by a hyperammonemic encephalopathy (serum ammonia: 224 μmol/L) and hypoglycemia, for which she was admitted to the intensive care unit (ICU). No urea cycle disorders were found. Liver test results are presented in Table 1. She was treated for hepatic encephalopathy with lactulose and rifaximin, and enteral feeding was changed to a low-protein diet. Additional imaging studies of the liver did not show parenchyma abnormalities or portal flow disturbance. Common causes of liver disease were excluded. No liver biopsy was performed duo to coagulopathy. Unfortunately, she developed progressive liver failure in the following days, followed by aspiration pneumonia. Liver transplantation was deemed not feasible. On day 15, she died of multiorgan failure.

Case 2

A 34-year-old female underwent gastric sleeve resection because of morbid obesity (BMI: 42 kg/m^2), which was complicated by anastomotic leakage, abdominal sepsis and recurrent esophageal stenosis with stenting. Subsequently, after 5 mo, a gastric bypass (alimentary limb 150 cm, biliopancreatic limb 60 cm) was performed (BMI: 31 kg/m^2, %EWL: 62.5%, total body weight loss: 25%). Unfortunately, she suffered from episodes of nausea and vomiting due to persistent gastrojejunal ulcerations distal of the esophageal stent. With regard to these complications, an esophageal-jejunostomy was performed 3 mo later. In the following 28 mo, she was admitted to the hospital 4 times for recurrent problems of malnutrition due to psychosocial problems and depression as a result of the aforementioned complications. During her hospitalization she refused psychiatric treatment.

Finally, she was hospitalized in the ICU in a malnourished (BMI 16 kg/m^2, %EWL: 153%, total body weight

Table 1 Results of liver test at presentation of hyperammonemic encephalopathy

	Case 1	Case 2	Normal values
Albumin	12	10	> 35 g/L
Total bilirubin	53	9	< 17 μmol/L
Alkaline phosphatase	103	149	< 120 U/L
AST	25	43	< 31 U/L
ALT	21	54	< 31 U/L
γ-GT	76	55	< 35 U/L
Antithrombin Ⅲ	10	20	> 80%
Thrombocytes	105	196	150-400 $^{1}10^{9}$/L
PT-INR	> 7[1]	> 7[1]	
Vitamin B12	1068	273	130-700 pmol/L
Vitamin B1	74	106	75-225 nmol/L
Vitamin B6	37	142	50-180 nmol/L
Vitamin D	17.4	< 10	> 50 nmol/L

[1]Under anticoagulant therapy. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; γ-GT: Gamma-glutamyl transpeptidase; PT-INR: Prothrombin time-international normalized ratio.

loss: 62%) and somnolent state. She did not eat the days before hospitalization, likely due to psychiatric deterioration and suicidal ideation. She was diagnosed with a hyperammonemic encephalopathy (serum ammonia 86 μmol/L) due to liver failure. Liver test results are presented in Table 1. The hepatic encephalopathy was treated with lactulose and rifaximin, and enteral feeding was started with Nutrison Protein plus Multifibre (Nutricia Medical, Dublin, Ireland). Despite these treatments, the patient's condition declined and 2 d after admission she died due to progressive liver failure.

DISCUSSION

In this case series, we present 2 patients who developed severe protein malnutrition after bariatric surgery, followed by hyperammonemic encephalopathy and liver failure provoked by psychiatric deterioration.

Both patients were hospitalized in a period of 1-3 years after bariatric surgery in a malnourished state with dehydration, severe protein deficiency and anasarca. Importantly, common causes of protein loss, such as nephrotic syndrome or protein-losing enteropathy, were excluded, and no clues of decreased synthesis capacity of the liver were observed as cause of hypoalbuminemia. Most likely, hypoalbuminemia was caused by post-bariatric malabsorption and/or self-induced food restriction.

In malabsorptive procedures, such as distal gastric bypass, malnutrition has been described and bariatric surgeons should be aware of this complication[8,9]. Macronutrient deficiencies after restrictive procedures, such as modern gastric bypass surgery, are very rare[10]. In the cases presented herein, hypoalbuminemia was enhanced by very poor intake due to psychosocial problems postoperatively, probably resulting in anorexia, despite successful psychiatric screening as part of the work-up prior to bariatric surgery. During repeated hospital admissions, intensive guidance of psychologists and dieticians was provided. Despite these efforts, both patients remained critically malnourished, finally resulting in liver failure and death. From a clinical perspective it is of utmost importance to recognize patients at risk of psychiatric deterioration after bariatric surgery. Our cases underlined that even close monitoring by a psychiatrist does not guarantee a stable clinical course.

Liver insufficiency in our patients became manifest during hospitalization. Both patients developed somnolence caused by hyperammonemic encephalopathy. In our patients, urea cycle disorders as cause of hyperammonemia were unlikely and excluded. Liver insufficiency was present, as reflected by the laboratory results (Table 1). Common causes of liver disease, such as alcohol abuse, viral infection and autoimmunity, were excluded. Therefore, we consider it likely that our patients developed liver insufficiency due to a prolonged state of severe malnutrition and anorexia, which was not well recognized.

Liver insufficiency has been described after malabsorptive bariatric procedures, such as the Scopinaro procedures. Bacterial overgrowth with the production of hepatotoxic macromolecules was considered the main cause. Malnutrition as cause of liver insufficiency is rare and has been described in non-bariatric patients with anorexia nervosa. The following hypotheses have been proposed in the literature: Liver insufficiency may be caused by acute liver cell necrosis, the result of autophagy[11] or dehydration and hypovolemia with poor blood circulation through the liver[12]. We hypothesize that our patients developed anorexia following bariatric surgery, reaching a state of starvation and a critical level of the liver reserve capacity, finally resulting in a state of liver insufficiency and death.

In conclusion, liver failure due to severe malnutrition is a very rare but critical complication after bariatric surgery. Patients who present with signs of severe protein malnutrition after bariatric surgery should be closely monitored and checked for nutritional status. Specific attention should be given to patients who develop psychiatric problems post-bariatric surgery. If refeeding does not result in clinical improvement, reversal surgery should be considered in a timely manner.

ARTICLE HIGHLIGHTS

Case characteristics

Patients who underwent bariatric surgery in the past developed unconsciousness and liver failure after self-induced food restriction.

Clinical diagnosis

Development of hepatic encephalopathy and hepatic failure.

Differential diagnosis

Hypoglycemia or neurological disorders were excluded as the cause of unconsciousness. No viral, autoimmune or toxic agents were found to have caused the liver failure.

Laboratory diagnosis
Signs of severe hypoalbuminemia, liver failure and hyperammonemia.

Treatment
Lactulose and rifaximin to treat hepatic encephalopathy.

Term explanation
Hyperammonemia refers to high blood level of ammonia.

Experiences and lessons
Specific attention should be given to patients who develop psychiatric problems post-bariatric surgery. If refeeding does not result in clinical improvement, reversal surgery should be considered in a timely manner.

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LETTERS TO THE EDITOR

Do Ayurveda drugs induce liver injury?

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Abstract

Drugs fulfilling the criterion of a standard drug will always become panacea provided, if they are used properly. On the other hand, a poorly manufactured drug however used skillfully, will prove to be a poison. Texts of Ayurveda, do mention hazards of drugs, which are not properly manufactured or administered. Art of drug administration is unique in this ancient medical science that cautions towards concentrating on dose, indications, contra-indications, suitable vehicle, specific diet, certain restrictions *etc*., while administering medicines in suitable individuals. Though a huge amount of information is available and evidences are being generated on the usefulness of traditional practices in global healthcare; there is a need of generating awareness on Promoting rational use of traditional medicines in particular to Ayurvedic drugs. Conventional researchers wish to work on traditional formulations have to understand traditional principles and involve traditional physicians in their researches in the benefit of mankind.

Key words: Ayurveda; Traditional medicines; Safety; Posology; Punarnava Mandura; Guggulu

Core tip: Ayurveda principles of treatment and concepts of drug administration are entirely different than the conventional approach. Besides other basic requirements; understanding digestive ability, metabolic capability, tolerability of the patient to a specific dose of the drug, psycho-somatic constitution, *etc*., of the patient is essential before starting treatment. In absence of which, adverse manifestations are likely. It is also unwise using traditional formulations by procuring over-the-counter for a longer period without any supervision of qualified physician. Considering holistic approaches of traditional remedies, joining hands together respecting fundamental principles of each other will be beneficial in global healthcare.

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TO THE EDITOR

We read with great interest the case report entitled "Ayurvedic drug induced liver injury" that was published in *World J Hepatol* 2017 November 8; 9(31): 1205-1209[1]. Though the Case Report highlights a newer aspect of some Ayurveda formulations; there are few aspects that need to be addressed.

Both the formulations (Punarnava Mandura and Kanchanara guggulu) are well known in Indian parlance and are being used in therapeutics since centuries. No hepatic injury was noticed or reported till date with such usage and no such scientific data that can convincingly prove harmful nature of these formulations is available till date.

The Case Report mentions that the patient used three different herbal and homoeopathic formulations. Though identity of two herbal formulations is revealed; the nature of the third one is unclear. It also not known these drugs was procured and how they have been used for how much duration from where. A drug can be panacea or poison. Drugs fulfilling the criterion of a standard drug will always become panacea provided, if they are used properly. On the other hand, a poorly prepared or manufactured drug however used skilfully, will always prove to be a poison. Classics of Ayurveda do mention the hazards of drugs, which are not properly manufactured and not used judiciously. There is no sufficient evidence in the article to confirm the posological considerations of the formulations used. Ayurvedic formulations are not used in similar way as that of conventional medicines. Besides other basic requirements; understanding of digestive ability, metabolic capability, tolerability of a patient to a specific dose of the drug, psycho-somatic constitution, *etc*., of the patient is essential before starting treatment. After meticulous examination; suitable preparations are to be administered orally in specified quantities with great caution along with requisite vehicles like ghee, milk, honey, *etc*. In absence of a vehicle, adverse reactions are likely[2].

The Case Report also didn't focus on how the drugs have been procured. These medicines are not OTC products and should be used under the supervision of any authorized Ayurveda/Homoeopathy physician, who are the registered authorities, have been trained in that specific field as per the syllabus provided by Ministry of AYUSH, Govt. of India. We are not sure about the identity of the healer referred in the current study. In addition; the nature of the third drug is also not known, in such case why to blame only Ayurvedic formulations for the manifested pathology. There is a possibility of drug-drug interaction too that was not considered in the current work.

As referred in the Case Report; Punarnava Mandura is not an extract of Boerhavia diffusa. Authors need to verify the validity of information being cited from the article. Besides this, editors also should be vigilant and prefer to restrict the authors from citing such articles from predatory journals. Similarly, Kanchanara Guggulu is not an extract of Bauhinia variegata. These two drugs are poly herbal combinations prepared by following standard guidelines explained in the classical text books of Ayurveda.

Punarnava Mandura is made-up of twenty ingredients which is familiar hematinic drug. Its efficacy has been well established in geriatric and gestational anaemias[3,4]. Kanchanara Guggulu is also a poly herbal formulation, whose efficacy has been well established[5]. Traditional medicines, which usually have multi components are helpful in counteracting multi factors of any pathology[6]. Different components of a traditional formulation act synergistically exerting various activities like metabolic enhancers, immuno-modulators, antioxidants, rejuvenators, increases bio-availability and help in countering toxic nature of other ingredients. All these activities indicate towards multi-variant nature of compound formulations that are actually need of the time.

Based upon a single and incomplete observation; inferring Ayurvedic drugs with liver injury is unwise. Authors have to understand that Ayurveda always advocate using drug as a whole and never prefer using extracts in a formulation except aqueous or hydro-alcoholic extracts.

This article indirectly clears the need of paying attention towards generating awareness on use of traditional medicines. The impact of such reports in a leading scientific journal like WJH is a serious matter as it may unnecessarily cause disrepute to herbal remedies and ultimately to the system of Ayurveda.

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CASE REPORT

ABOUT COVER

Editorial Board Member of *World Journal of Hepatology*, Mohammad K Parvez, PhD, Associate Professor, Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 22451, Saudi Arabia

AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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CASE REPORT

Review of the literature laparoscopic surgery for metastatic hepatic leiomyosarcoma associated with smooth muscle tumor of uncertain malignant potential: Case report

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Author contributions: Takase N and Takamatsu M operated on the patient and designed the report; Miyake T, Hisano K, Maeda E, Nishimura T, Abe K, Kozuki A, Tanaka T and Harada N drafted the paper; Kaneda K critically revised the paper with an important conceptual and editorial input.

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Abstract

Metastatic hepatic leiomyosarcoma is a rare malignant smooth muscle tumor. We report a case of metastatic hepatic leiomyosarcoma associated with smooth muscle tumor of uncertain malignant potential (STUMP). A 68-year-old female presented with a liver mass (60 mm × 40 mm, Segment 4). She underwent left salpingo-oophorectomy for an ovary tumor with STUMP in a broad ligament 6 years ago. Though FDG-PET showed obvious metabolically active foci, abnormal metabolically active foci other than the lesion were not detected. A malignant liver tumor was strongly suspected and laparoscopic partial liver resection was performed with vessel-sealing devices using the crush clamping method and Pringle maneuver. Immunohistochemical findings revealed metastatic liver leiomyosarcoma associated with STUMP in a broad ligament. This case is an extremely rare case of malignant transformation from primary STUMP to metastatic hepatic leiomyosarcoma.

It provides important evidence regarding the treatment for metastatic hepatic leiomyosarcoma associated with STUMP.

Key words: Leiomyosarcoma; Smooth muscle tumor; Hepatic neoplasm; Neoplasm metastasis; Laparoscopic surgery; Liver resection

Core tip: Metastatic hepatic leiomyosarcoma is a rare malignant smooth muscle tumor. This case report presents a case of smooth muscle tumor of uncertain malignant potential (STUMP) occurring in a broad ligament that developed into metastatic hepatic leiomyosarcoma over a period of 6 years, and an ultrasound-guided pure laparoscopic partial liver resection with vessel-sealing devices using the crush clamping method and Pringle maneuver was performed safely. This case is an extremely rare case of malignant transformation from primary STUMP to metastatic hepatic leiomyosarcoma. We share this case to provide important evidence regarding the treatment for metastatic hepatic leiomyosarcoma associated with STUMP.

Fukui K, Takase N, Miyake T, Hisano K, Maeda E, Nishimura T, Abe K, Kozuki A, Tanaka T, Harada N, Takamatsu M, Kaneda K. Review of the literature laparoscopic surgery for metastatic hepatic leiomyosarcoma associated with smooth muscle tumor of uncertain malignant potential: Case report. *World J Hepatol* 2018; 10(4): 402-408 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i4/402.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i4.402

INTRODUCTION

Metastatic hepatic malignancies are encountered more frequently (approximately 18-40 times) than primary liver cancers[1]. Among such malignancies, metastatic hepatic leiomyosarcoma is a rare malignant smooth muscle tumor (SMT). The hepatic metastasis associated with primary female genital system arises not only in leiomyosarcoma but also in other SMTs including leiomyoma and smooth uterine muscle of uncertain malignant potential (STUMP)[2,3]. However, STUMP usually does not metastasize because it is a mesenchymal uterine tumor lying between benign leiomyomas and leiomyosarcomas[4]. We herein report a case of metastatic hepatic leiomyosarcoma associated with STUMP in a broad ligament. A pure laparoscopic partial liver resection was performed successfully.

CASE REPORT

A 68-year-old female who had been admitted to another facility presented with a liver mass, which was palpable from the body surface. She had non-alcoholic steatohepatitis (NASH) and cholelithiasis in her past medical history. She underwent hysterectomy for uterine leiomyoma 21 years earlier and left salpingo-oophorectomy for an ovary tumor with STUMP in a broad ligament 6 years earlier.

Her laboratory tests showed a mild dysfunction of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) which would be consistent with suspected NASH. She had no hepatitis virus infection, and tumor markers including alpha-fetoprotein and protein induced by vitamin K absence-2 (PIVKA-2) were negative. Ultrasonography of the liver showed a hypoechoic smooth mass (60 mm × 40 mm) with a heterogeneous hyperechoic region in the median section of the left lobe of the liver (Segment 4) (Figure 1A). Contrast-enhanced computed tomography (CE-CT) and ethoxybenzyl-magnetic resonance imaging (EOB-MRI) showed a heterogeneous mass enhancement (Figure 1B and C). Though fluorodeoxyglucose-positron emission tomography (FDG-PET) also showed obvious metabolically active foci (Figure 1D), no other abnormal metabolically active foci were detected in addition to the lesion.

Though the clinical findings did not lead to a preoperative diagnosis because of the non-specific characteristics, malignant liver tumor was strongly suspected and a pure laparoscopic partial liver resection was performed. Briefly, 5 ports were placed with the patient in the supine position. Three ports were placed in the epigastric region. The other 2 ports were placed in the umbilicus and left lateral abdominal region (Figure 2A). As for the liver resection, we performed ultrasound-guided pure laparoscopic partial liver resection with vessel-sealing devices using the crush clamping method and Pringle maneuver (Figure 2B). The resected partial liver was retrieved from the umbilical port with auxiliary incision. The surgical specimen showed a white colored solid mass with smooth surface (Figure 2C and D).

Hematoxylin and eosin (H-E) stain showed the proliferation of spindle-shaped cells with enlarged nuclei and eosinophilic cytoplasm, and the cells exhibited diffuse moderate-to-severe atypia and multiple mitoses. The mitotic count activity showed the presence of more than 10 mitotic figures (MFs)/10 high power fields (HPFs) (Figure 3A). Immunohistochemical findings were strongly positive for smooth muscle actin (SMA) and desmin, and negative for c-kit (Figure 3B-D). In addition, the expressions of cluster of differentiation 34 (CD34) and s-100 were negative (data not shown). Moreover, more than 10% ki-67 positive cells were seen in the lesion (Figure 3E).

Next, we histologically reconfirmed the past resected specimens including uterus and broad ligament specimens to explore the primary lesion. In the uterus, MFs were absent, and the ki-67 proliferation-index (MIB-1) was zero (Figure 4A-1 and 4B-1). The pathological findings of MFs and the MIB-1 index showed malignant transformation from STUMP to leiomyosarcoma (Figure 4A-2 and A-3) (Figure 4B-2 and B-3). The final diagnosis was STUMP

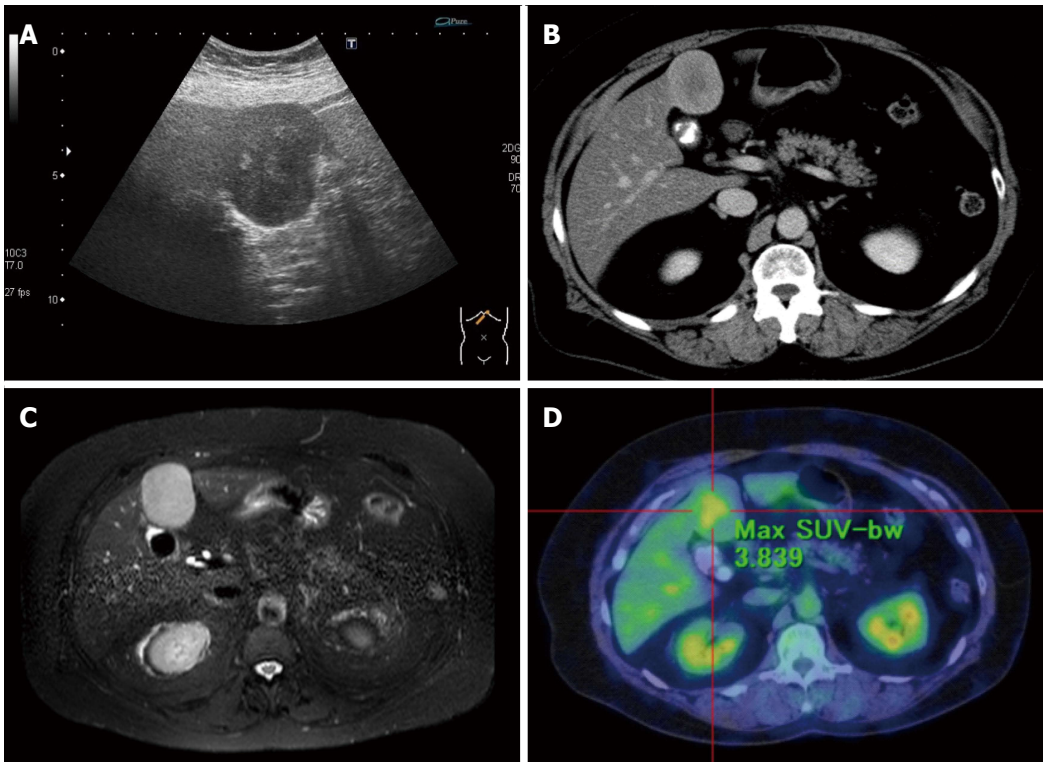

Figure 1 Evaluation of clinical findings. A: Ultrasonography of the liver showed a very-low-echoic smooth mass (60 mm × 40 mm) with a heterogeneous high-echoic region in the median section of the left lobe of the liver (Segment 4); B: Axial delayed phase CE-CT showed the lesion to be gradually enhanced heterogeneously; C: The lesion showed a hyperintense heterogeneous region on axial T2-weighted EOB-MRI; D: The lesion showed obvious metabolically active foci by 18-fluorodeoxyglucose-PET-CT evaluation, while other lesions were not detected.

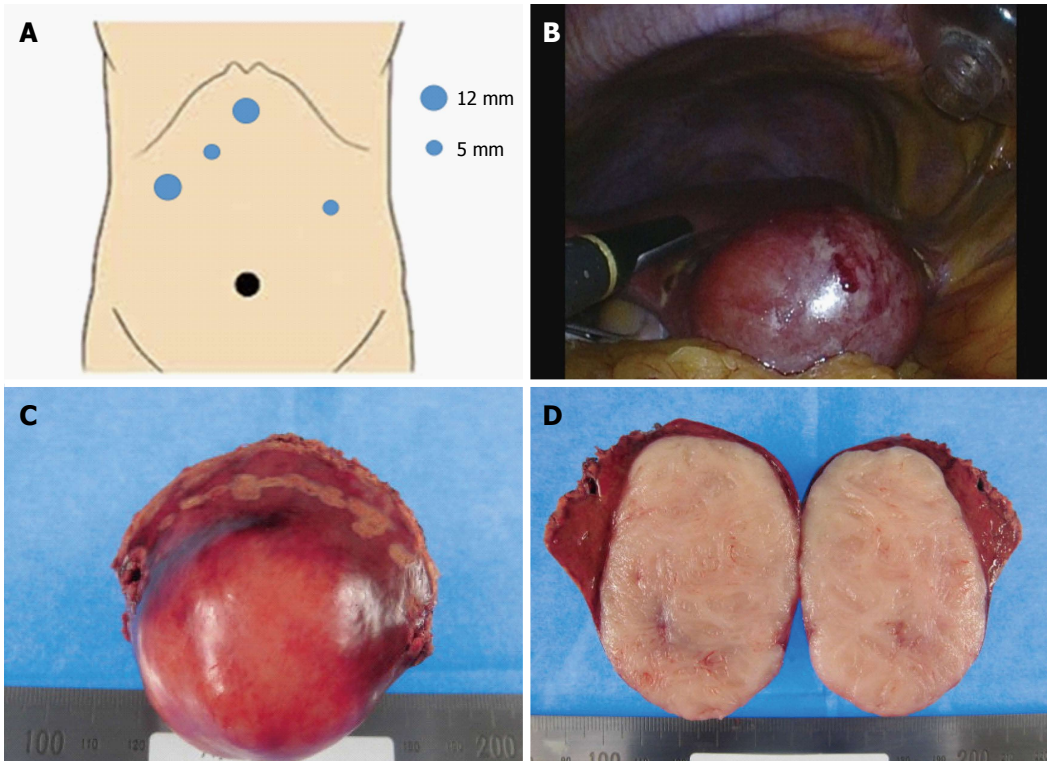

Figure 2 Ultrasound-guided pure laparoscopic partial liver resection (Segment 4) and surgical specimen. A: Five ports were placed for liver partial resection. Three ports were placed around the epigastric region as working ports (blue circles). An umbilical port was used as the camera port (black circle). The resected partial liver was retrieved from the umbilical port with auxiliary incision. A Pringle maneuver was used for the left lateral port; B: We performed ultrasound-guided pure laparoscopic partial liver resection with vessel-sealing devices (LigaSure™ Maryland Jaw 37 cm Laparoscopic Sealer/Divider, Medtronic, Dublin, Ireland) using the crush clamping method and Pringle maneuver; C: Macroscopic image of the resected specimen showed a smooth surface mass; D: Cross-section of the resected specimen showed a milky-white-colored solid mass without necrotic lesion.

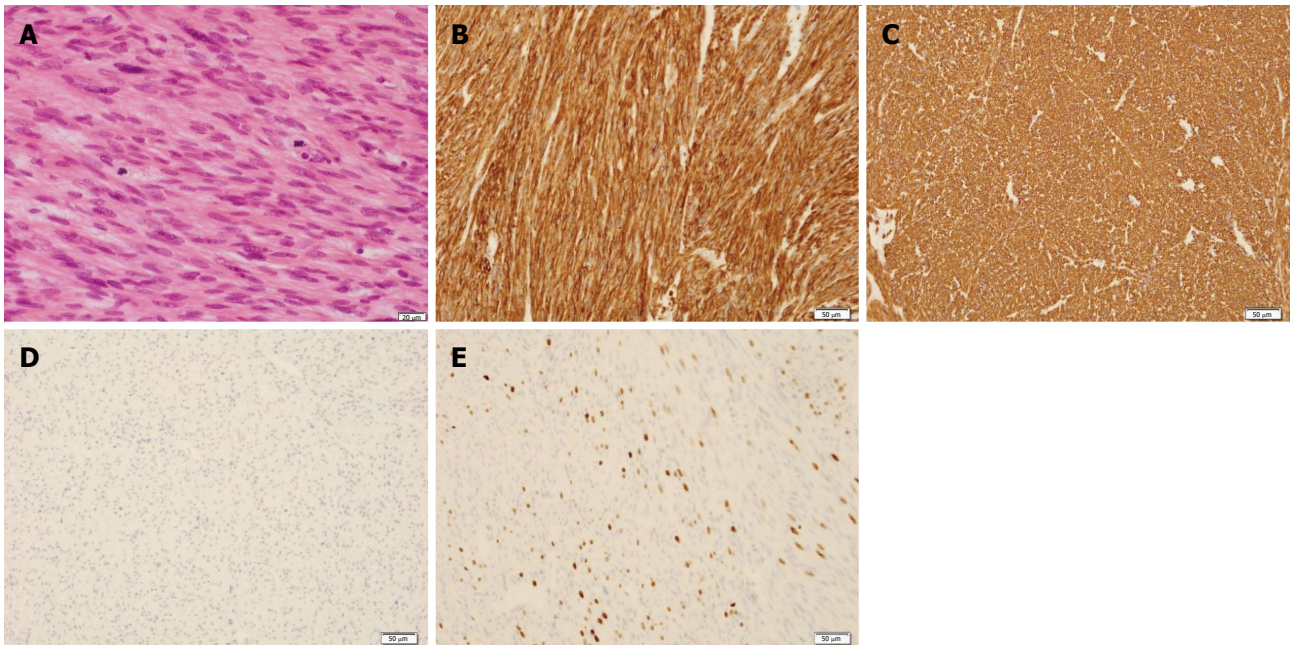

Figure 3 Pathological findings of the resected specimen. A: HE stain showed the proliferation of spindle-shaped cells with ⩾ 10 MFs-/-10 HPFs and diffuse moderate-to-severe atypia without coagulative tumor cell necrosis; B: Immunohistochemical findings were strongly positive for SMA; C: Immunohistochemical findings were strongly positive for desmin; D: Immunohistochemical findings were strongly positive for negative for c-kit; E: Approximately 35% ki-67 positive cells were seen in the lesion. Scale bars: 20 μm (A), 50 μm (B-E).

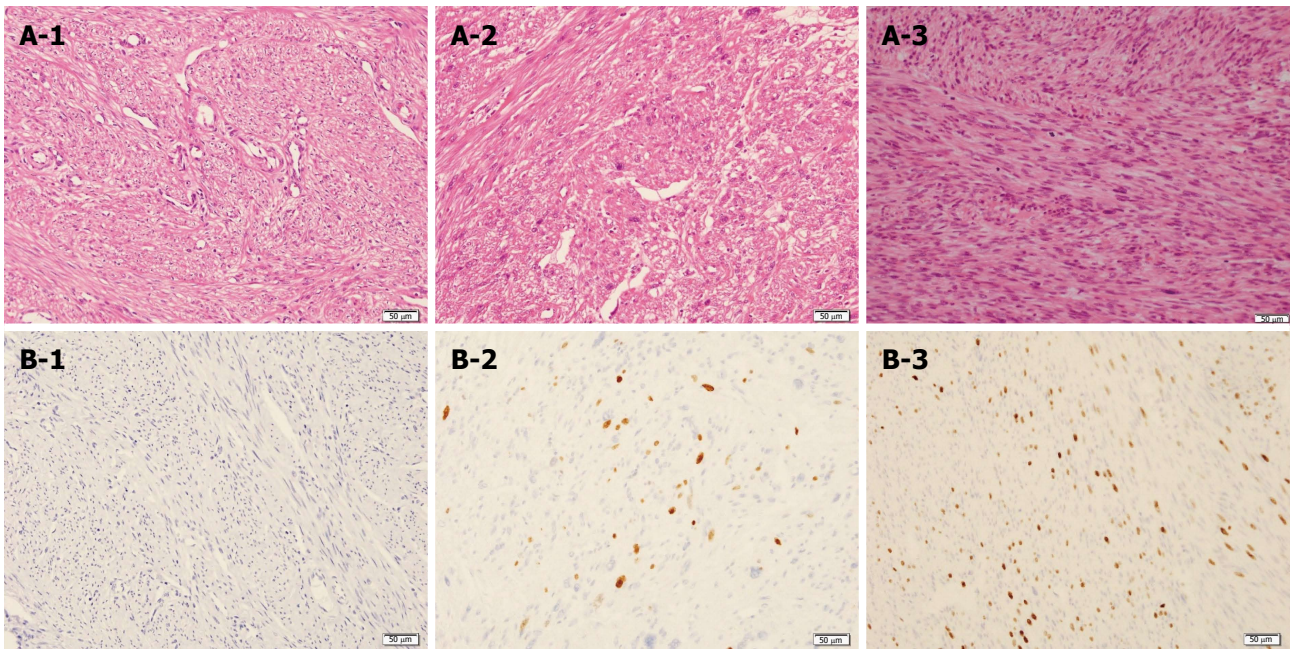

Figure 4 Pathological reconfirmation of uterine leiomyoma and broad ligament with STUMP and liver lesion with leiomyosarcoma. MF was absent in an HE stain of the uterine leiomyoma (A-1). The number of MFs was 1-5 MFs / 10 HPFs in the broad ligament (A-2) and more than 10 MFs/10 HPFs in the liver mass (A-3). Ki-67-positive cells were not seen in the uterine leiomyoma (B-1). The MIB-1 index was approximately 5% of the broad ligament and approximately 35% of the liver mass (B-2) (B-3). The pathological findings of MFs and the MIB-1 index showed malignant transformation from STUMP to leiomyosarcoma. Scale bars: 50 μm.

in a broad ligament that developed into metastatic hepatic leiomyosarcoma over a period of 6 years.

She was discharged 8 d after the operation without any complications, and she was not received adjuvant systemic treatment. However, follow-up CE-CT and EOB-MRI studies showed recurrent metastatic hepatic leiomyosarcoma in the edge of Segment 5 (12 mm × 9 mm) 7 mo after the initial surgery. We performed pure laparoscopic partial resection with these procedures safely in both primary and secondary resections because of the minimum postoperative abdominal adhesions. No evident disease recurrence has been seen in the 4 mo

since the secondary surgery.

DISCUSSION

SMTs are broadly categorized into 6 major histological types: leiomyoma, mitotically active leiomyoma, cellular leiomyoma, atypical leiomyoma, STUMP and leiomyosarcoma. SMTs are diagnosed based on the assessment of three histopathological characteristics: Mitotic count activity (MFs/10 HPFs), the presence or absence of coagulative tumor cell necrosis, and the degree of cytological atypia, and the histopathological features determine the difference between benign leiomyomas and malignant leiomyosarcomas[5]. In addition, Mayerhofer *et al*[6] indicated that the expression of ki-67 in SMTs may be a useful immunohistochemical parameter for distinguishing between malignant histology and borderline histology. However, it is difficult to categorize them clearly because there are various opinions regarding the pathological diagnostic criteria of SMTs.

There is general acceptance of the notion that STUMP has characteristics lying between benign leiomyomas and leiomyosarcomas[5,7]. In fact, the WHO classification also indicates that STUMP is difficult to unequivocally diagnose as benign or malignant[2]. The criteria used for the diagnosis of STUMP were the presence of coagulative necrosis, low mitotic count (less than or equal to 10 per 10 HPFs) and the absence of atypia, or a mitotic count greater than 10 per 10 HPFs, focal mild to moderate atypia in the absence of coagulative necrosis, or a mitotic count equal to or less than 10 per 10 HPFs, and focal moderate-to-severe atypia in the absence of coagulative necrosis[8]. It is known that STUMP occurs in broad ligaments as well as the uterus. STUMP in broad ligaments is very rare, with only a few cases reported in the medical literature[8]. A previous study reported based on molecular analysis that most atypical leiomyoma including STUMP and leiomyosarcoma had a similar origin and may represent different stages of tumorigenesis[9]. Leiomyosarcoma is considered to arise *de novo* or it may develop from preexisting atypical leiomyoma, while the mechanism of malignant transformation has not been determined conclusively. Zhang *et al*[9] demonstrated that *p53* mutation and *PTEN* deletions were significantly higher in leiomyosarcoma, atypical leiomyoma and STUMP compared with other uterine SMTs.

The common sites of metastatic hepatic leiomyosarcoma are the stomach (31%), retroperitoneum (19%), small bowel (15%) and vena cava (4%)[10]. Also, few hepatic leiomyosarcomas are known to have occurred in the female genital system. Among the soft tissue sarcomas, the most common site of leiomyosarcoma was the retroperitoneum[11]. However, these are generally distant metastases of leiomyosarcoma rather than distant metastases of other SMTs without leiomyosarcoma. Few studies have reported that the lung and liver are two common sites of metastases of STUMP[2,8,12,13]. We searched all common literature search engines (PubMed, Medline, Google Scholar and Embase). To our knowledge, only 2 cases involving malignant transformation of metastatic hepatic leiomyosarcoma associated with STUMP have been reported[12].

It is very difficult to make a definitive diagnosis of leiomyosarcoma preoperatively. Also, primary or metastatic hepatic leiomyosarcoma falls into the preoperative diagnostic dilemma because of the non-specific diagnostic imaging and poor clinical presentation[14]. In addition, although several studies have shown the outcome of metastatic hepatic leiomyosarcoma, definitive evidence is lacking. Because metastases from leiomyosarcoma are usually not sensitive to chemotherapy or chemoembolization, the outcome is often poor, with only short survival. Without treatment, the median survival of patients with liver metastases is no more than 14 mo[10]. Currently, the best proven approach to metastatic hepatic leiomyosarcoma is a radical cure excision involving the complete removal of all tumors. Lang *et al*[10] reported that the 5-year survival rate was 13% for all patients and 20% after R0 resection for metastatic hepatic leiomyosarcoma. Recent studies have indicated that liver resection is superior to chemoradiotherapy as a treatment for metastatic hepatic leiomyosarcoma[15-17]. However, standard treatments for metastatic hepatic leiomyosarcoma have not been established. Two agents have been considered to be active in soft tissue sarcomas in general, doxorubicin and ifosfamide[18,19]. In recent years, trabectedin, a marine-derived drug, exhibited activity in patients with metastatic soft tissue sarcoma after the failure of conventional chemotherapy[20]. Radiotherapy has been shown to improve local control and local recurrence but without any improvement in overall survival[21]. A molecular targeted study also demonstrated that the expression of LMP2 significantly blocked the tumorigenesis of leiomyosarcoma *in vitro*[22].

Laparoscopic hepatic surgery produces relatively less peritoneal trauma and blood loss than conventional laparotomy and may result in decreased perioperative complications[23,24]. Therefore, it has recently been considered a better operative approach and has increasingly been performed worldwide. Moreover, ultrasound-guided laparoscopic liver resection with vessel-sealing devices using the crush clamping method and Pringle maneuver is a minimally invasive, safe and effective procedure for patients with primary and metastatic hepatic tumor[24-26]. A recent study reported that laparoscopic intraoperative ultrasound examination had a higher detection rate regarding liver metastases, when compared to CE-CT and MRI[26]. Therefore, it enables a more accurate R0 resection. The Pringle maneuver is the simplest method of vascular occlusion by clamping of the hepatoduodenal ligament

to occlude total inflow to the liver. This method ensures safety and prevent major accidental blood loss during laparoscopic liver resection[25]. The crush clamping method for hepatic parenchymal transection is well-known. Moreover, a combination technique with vessel-sealing devices in laparoscopic liver resection have been applied. This technique shows lower blood loss, shorter transection time, and reduces rates of post-hepatectomy complications[24]. We also performed laparoscopic partial resection with these procedures safely in both primary and secondary resections. However, excessive intraoperative blood loss can occur during laparoscopic liver resection because active bleeding is difficult to control, as the conversion to laparotomy takes time[25]. In addition, laparoscopic partial resections of segment 7 and segment 8 on the dorsal liver head side are considered relatively difficult because of the anatomical features[27]. Therefore, it is necessary to carefully identify the surgical indications for laparoscopic liver resection.

In conclusion, we documented a STUMP in a broad ligament that developed into metastatic hepatic leiomyosarcoma over a period of 6 years. An ultrasound-guided pure laparoscopic partial liver resection was safely performed. There are few case reports and case series regarding metastatic liver leiomyosarcoma, however, this is an extremely rare case that described malignant transformation from primary STUMP to metastatic hepatic leiomyosarcoma. This case provides important evidence regarding the treatment for metastatic hepatic leiomyosarcoma associated with STUMP.

ARTICLE HIGHLIGHTS

Case characteristics

Metastatic hepatic leiomyosarcoma associated with STUMP in a broad ligament was treated by laparoscopic partial liver resection.

Clinical diagnosis

Preoperative imaging tests and past medical history strongly suggested that the lesion was an atypical malignant tumor.

Differential diagnosis

Hepatocellular carcinoma, Hepatic hemangioma, Metastatic hepatic tumor.

Laboratory diagnosis

Mild dysfunction of aspartate aminotransferase and alanine aminotransferase consistent with suspected NASH.

Imaging diagnosis

CE-CT and EOB-MRI showed a heterogeneous mass enhancement, and FDG-PET strongly suggested malignant liver tumor.

Pathological diagnosis

We diagnosed the condition as metastatic hepatic leiomyosarcoma.

Treatment

The patients were treated with ultrasound-guided pure laparoscopic partial liver resection with vessel-sealing devices using the crush clamping method and Pringle maneuver.

Experiences and lessons

We share this case to provide important knowledge regarding the appropriate treatment method for metastatic hepatic leiomyosarcoma.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical epidemiology of chronic viral hepatitis B: A Tuscany real-world large-scale cohort study

Cristina Stasi, Caterina Silvestri, Roberto Berni, Maurizia Rossana Brunetto, Anna Linda Zignego, Cristina Orsini, Stefano Milani, Liana Ricciardi, Andrea De Luca, Pierluigi Blanc, Cesira Nencioni, Donatella Aquilini, Alessandro Bartoloni, Giampaolo Bresci, Santino Marchi, Franco Filipponi, Piero Colombatto, Paolo Forte, Andrea Galli, Sauro Luchi, Silvia Chigiotti, Alessandro Nerli, Giampaolo Corti, Rodolfo Sacco, Paola Carrai, Angelo Ricchiuti, Massimo Giusti, Paolo Almi, Andrea Cozzi, Silvia Carloppi, Giacomo Laffi, Fabio Voller, Francesco Cipriani

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Institutional review board statement: This study was reviewed and approved by the Institutional Review Board.

Informed consent statement: The demographic data of the study were appropriately encrypted in the database and the exported file (containing the "anonymized" data) was sent from the system to the Regional Health Agency of Tuscany *via* a secure channel.

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Abstract

AIM

To build a regional database of chronic patients to define the clinical epidemiology of hepatitis B virus (HBV)-infected patients in the Tuscan public health care system.

METHODS

This study used a cross-sectional cohort design. We evaluated chronic viral hepatitis patients with HBV referred to the outpatient services of 16 hospital units. Information in the case report forms included main demographic data, blood chemistry data, viral hepatitis markers, instrumental evaluations, and eligibility for treatment or ongoing therapy and liver transplantation.

RESULTS

Of 4015 chronic viral hepatitis patients, 1096 (27.3%) were HBV infected. The case report form was correctly completed for only 833 patients (64% males, 36% females; mean age 50.1 ± 15.4). Of these HBV-infected patients, 73% were Caucasian, 21% Asian, 4% Central African, 1% North African and 1% American. Stratifying patients by age and nationality, we found that 21.7% of HBV-infected patients were aged < 34 years (only 2.8% were Italian). The most represented routes of transmission were nosocomial/dental procedures (23%), mother-to-child (17%) and sexual transmission (12%). The most represented HBV genotypes were D (72%) and A (14%). Of the patients, 24.7% of patients were HBeAg positive, and 75.3% were HBeAg negative. Of the HBV patients 7% were anti-HDV positive. In the whole cohort, 26.9% were cirrhotic (35.8% aged < 45 years), and 47% were eligible for or currently undergoing treatment, of whom 41.9 % were cirrhotic.

CONCLUSION

Only 27.3% of chronic viral hepatitis patients were HBV infected. Our results provide evidence of HBV infection in people aged < 34 years, especially in the foreign population not protected by vaccination. In our cohort of patients, liver cirrhosis was also found in young adults.

Key words: Hepatitis B virus infection; Liver fibrosis; Cirrhosis; Public health; Epidemiology

Core tip: Although the introduction of a vaccine against hepatitis B virus (HBV) has been highly effective in reducing the incidence and prevalence of HBV infection in many countries, an estimated 257 million people worldwide are chronically infected. In 2015, WHO published the guidelines for prevention, care and treatment of HBV infected people promoting treatment based on noninvasive assessment. This real-world large-scale cohort study provides appropriate planning for public health programs, as well as the specific characteristics of patients, thus contributing to the successful, efficient translation of new knowledge to management and treatment of HBV patients to eradicate HBV.

Stasi C, Silvestri C, Berni R, Brunetto MR, Zignego AL, Orsini C, Milani S, Ricciardi L, De Luca A, Blanc P, Nencioni C, Aquilini D, Bartoloni A, Bresci G, Marchi S, Filipponi F, Colombatto P, Forte P, Galli A, Luchi S, Chigiotti S, Nerli A, Corti G, Sacco R, Carrai P, Ricchiuti A, Giusti M, Almi P, Cozzi A, Carloppi S, Laffi G, Voller F, Cipriani F. Clinical epidemiology of chronic viral hepatitis B: A Tuscany real-world large-scale cohort study. *World J Hepatol* 2018; 10(5): 409-416 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i5/409.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i5.409

INTRODUCTION

While the introduction of an effective vaccine has been highly effective in reducing the incidence and prevalence of hepatitis B virus (HBV) infection in many countries, an estimated 257 million people worldwide are chronically infected[1].

HBV prevalence is > 8% in parts of sub-Saharan Africa. An intermediate prevalence (2%-8%) is present in some regions of the eastern Mediterranean, Central Asia, Southeast Asia, China, parts of South America and some European countries (Albania, Bulgaria, Romania, Turkey and Italy). There is a low prevalence (< 2%) in parts of North America and in some European countries (Belgium, the Czech Republic, Denmark, France), as well as Australia and New Zealand[2].

High levels of viremia, or the infection contracted at a young age (affecting mainly males) are associated with an increased risk of death or of developing hepatocellular carcinoma[3]. In 2015, hepatitis B resulted in 887000 deaths, mostly from complications (including cirrhosis and hepatocellular carcinoma)[1].

In 2015, WHO published guidelines for the prevention, care and treatment of persons with chronic HBV infection. These promote the use of simple, noninvasive tests to assess liver disease stage and eligibility for treatment. For adult patients in countries with limited resources, the test for ratio of aspartate aminotransferase to platelets (APRI) is recommended as a noninvasive diagnostic to assess the presence of cirrhosis (APRI score > 2), while transient elastography (*e.g.,* fibroscan) or fibrotest are recommended in countries where these tests are available and cost constraints are not significant. These guidelines are based on a public health approach to the use of antiviral drugs for the treatment of chronic HBV. Such an approach considers the feasibility and effectiveness of treatment, even in countries with limited resources-for example, in areas where diagnostic methods such as HBV DNA and liver biopsy are unable to be used[4].

According to the latest Italian recommendations[5], treatment with nucleoside or nucleotide analogues is indicated in the following instances: HBeAg-positive patients with moderate or severe fibrosis who have failed seroconversion after a cycle of Peg-IFN, for whom treatment with Peg-IFN is not indicated at diagnosis; in HBeAg-negative patients with moderate or severe fibrosis who have already been treated with Peg-IFN without success or those for whom treatment with PegIFN is not indicated at diagnosis; in HBeAg-positive or HBeAg-negative patients who (for family, work or social reasons) are unable to practice or do not accept Peg-IFN therapy; in HBeAg-positive or HBeAg-negative patients with compensated or decompensated cirrhosis, regardless of the likelihood of seroconversion, serum HBV DNA levels and ALT values.

The aim of this study was to build a regional database of patients diagnosed with chronic HBV infection in specialised outpatient services in our region to define the clinical epidemiology of HBV-infected patients in the Tuscan public health care system.

MATERIALS AND METHODS

This prospective observational study is fully in line with the objectives of the National Plan for the Prevention of Viral Hepatitis, in particular the Address Line 1 (LI 1)-Epidemiology, which seeks to "define the epidemiology of viral hepatitis B and C and to strengthen surveillance systems". Objective 3 is of particular focus: It outlines the importance of the quality of reporting system data and the monitoring of HBV and HCV as acute or chronic infections[6].

All patients with HBV-related chronic liver disease referred to the hepatology outpatient services of 16 hospital units from January 1, 2015, to December 31, 2015, and who agreed to participate in the study, were evaluated. These 16 hospital units were: Hepatology Unit, University Hospital of Pisa, Pisa, Italy; Centre for Systemic Manifestations of Hepatitis Viruses (MaSVE), Internal Medicine and Liver Unit, Department of Experimental and Clinical Medicine, Careggi University Hospital, Florence, Italy; Gastroenterology Research Unit, Department of Experimental and Clinical Biomedical Sciences Mario Serio, Careggi University Hospital, Florence, Italy; Infectious Disease Unit, Hospital of Lucca, Italy; Infectious Diseases Unit, Siena University Hospital, Siena, Italy; Infectious Disease Unit, S. Maria Annunziata Hospital, Florence, Italy; Infectious Disease Unit, Hospital of Grosseto, Italy; Infectious Disease Unit, Hospital of Prato, Italy; Infectious and Tropical Diseases Unit, Careggi

University Hospital, Florence, Italy; Gastroenterology and Metabolic Disorders, Cisanello University Hospital, Pisa, Italy; Gastroenterology Unit, Department of Translational Research and New Technologies in Medicine and Surgery, Cisanello University Hospital of Pisa, Pisa, Italy; Liver Surgery and Transplantation Unit, Cisanello University Hospital, Pisa, Italy; Internal Medicine Unit, San Jacopo Hospital, Pistoia, Italy; Infectious Diseases and Hepatology Unit, University Hospital of Siena, Siena, Italy; Gastroenterology Unit, San Giuseppe Hospital, Empoli, Italy; and Internal Medicine and Liver Unit, Careggi University Hospital, Florence, Italy.

Study design

The Regional Health Agency of Tuscany provided all units with a computerised clinical database to collect the main socio-demographic, clinical and treatment data for all patients with chronic HCV and HBV infection admitted at each centre. A record was created for each patient containing his or her main personal data, blood chemistry data, viral hepatitis markers, HCV-RNA and HBV-DNA viral load, histology and instrumental evaluation (FibroScan®, liver ultrasound), previous treatment (with outcome), eligibility for treatment, or therapy already in progress, and liver transplantation details.

Liver ultrasound and elastography are among the main diagnostic procedures used in patients with chronic hepatitis. Liver ultrasound allows clinicians to diagnose and monitor chronic liver diseases, including the diagnosis of liver tumours and the identification of signs of portal hypertension. Liver surface irregularities detected by ultrasound techniques define the presence of micro- and macronodules and are considered the most sensitive and reproducible signs of liver cirrhosis[7]. Elastography characterises the properties and mechanical response of tissues, measuring their stiffness[8]. Elastography is currently used to determine clinical priorities based on the estimated fibrosis stage. In HBV naïve patients with normal ALT, the cut-offs used were < 6 kPa (no significant fibrosis), 6-9 kPa (grey area), and > 9 kPa (severe fibrosis/cirrhosis). In HBV-naïve patients with elevated ALT (but < 5 × ULN), the cut offs used were < 6 kPa (no significant fibrosis), 6-12 kPa (grey area), and > 12 kPa (severe fibrosis/cirrhosis)[9].

To estimate the number of patients with more severe liver disease, we considered those patients with a Child-Pugh score (used only to assess the severity of liver cirrhosis), and/or with a liver stiffness either > 9 kPa (normal ALT) or > 12 kPa (elevated ALT)[9] at elastography, and/or with a histological diagnosis of cirrhosis (METAVIR score equal to F4 or Ishak equal to S5-6), and/or with a nodular structure of the liver identified by ultrasound examination. Duplicate cases were excluded.

The demographic data were appropriately encrypted in the database. The software not only allowed the research team to include guided and controlled information using a combo-box but also to export files ready to be sent.

The exported file (containing the "anonymized" data) was sent from the system to the Regional Health Agency of Tuscany *via* a secure channel (SSL). After logging in and connecting to the Agency's web page, the user was then able to upload the file.

The research topic was described to the patients. The local Ethics Committee approved the study. Each participant gave written informed consent prior to the study, in accordance with the principles of the Declaration of Helsinki (Sixth Revision, Seoul 2008).

Statistical analysis

All data were validated, checking for out-of-range values and values that were logically inconsistent were handled. All results are expressed as mean ± SD. The numerical comparison of continuous data was performed using the Student's *t*-test for unpaired and for paired samples with Bonferroni correction, after checking similar variances in the groups by Levine's test for equality of variances. To avoid the potential bias related to the missing data, the analysis of the variables was conducted only on the completed fields.

Statistical significance was set at a value of $P < 0.05$. Statistical analysis was obtained using statistical software Stata 12 (College Station, TX, United States).

RESULTS

Demographic and epidemiological features

A total of 23 hepatology units were invited to participate in the study. Data were obtained from 16 hospital units for a total of 4654 cases of chronic HBV and HCV infections. Diagnoses were specified for 4015 (86.3%) patients, of whom 1096 (27.3%) were HBV infected (Figure 1). However, the case report form was correctly completed for only 833 patients (64% males, 36% females; mean age 50.1 ± 15.4). For HBV patients, 73% were Caucasian, 21% Asian, 4% Central African, 1% North African and 1% American. Of these HBV-infected patients, 106 (12.7%) were referred to the clinics for the first time during the period in question. Table 1 shows the patients stratified by age and gender.

Analysis of the calendar year of diagnosis and of the first access to specialised services reveals that about 98.1% of these cases occurred after 1990.

Stratifying patients by age and nationality, we found that 21.7% of HBV-infected patients were aged < 34 years (only 2.8% were Italian). Table 2 shows the most represented routes of transmission were nosocomial/dental procedures (23%), mother-to-child (17%) and sexual transmission (12%).

Clinical data

The most represented HBV genotypes were D (72%) and A (14%), shown in Table 3, seen in 135 HBV patients.

The total population displayed the following parameters: mean ALT = 59.84 ± 108.49 IU/L; mean AST

Table 1 Percentage of hepatitis B virus infected patients stratified by age group *n* (%)

Age group	Males	Females	Total
⩽ 34	54 (18.3)	46 (27.9)	100 (21.7)
35-44	60 (20.3)	26 (15.7)	86 (18.7)
45-54	68 (23.1)	33 (20.0)	101 (22.0)
55-64	58 (19.7)	27 (16.4)	85 (18.5)
65-74	36 (12.2)	22 (13.3)	58 (12.6)
> 75	19 (6.4)	11 (6.7)	30 (6.5)
Total	295 (100)	165 (100)	460 (100)
Missing			479

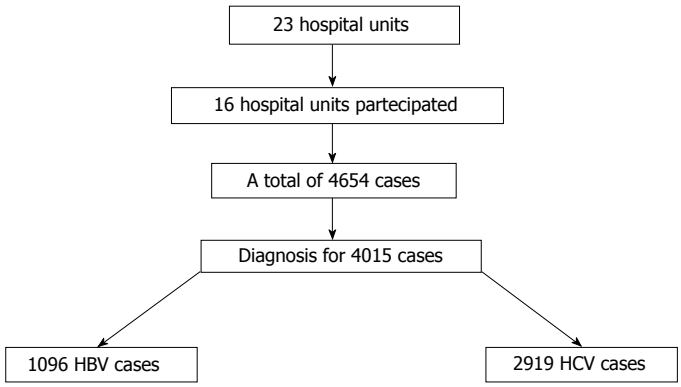

Figure 1 A total of 23 hepatology units were invited to enrol patients in the study, but 16 hepatology units have participated for a total of 4654 cases of chronic viral hepatitis. The diagnoses were specified for 4015 patients, of whom 1096 (27.3%) were hepatitis B virus infected. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

= 48.20 ± 87.02 IU/L; mean Platelets = 195.23 ± 72.13 cells/L. The number of patients with PLT < 100000 was 31. In the population, 24.7% of HBV patients were HBeAg positive, 75.3% were HBeAg negative, and 7% were anti-HDV positive.

In non-treated HBeAg and anti HBeAg positive patients, the HBV DNA levels were $7.26 \times 10^7 \pm 1.71 \times 10^8$ and 8358838 ± 2.71×10^7 UI/mL, respectively.

In non-treated HBeAg and anti-HBeAg-positive patients, the ALT levels were 42.05 ± 29.70 U/L and 33.96 ± 33.49 U/L, respectively.

Staging of liver disease

A total of 89 HBV patients underwent liver biopsy. Of these, 66 also had elastography, with 45% of total liver biopsies performed before 2007 (*i.e.*, before liver elastography was used in clinical practice). Of 89 patients underwent liver biopsy, 70% were evaluated with an Ishak score (supplementary material), providing a better definition of liver fibrosis compared to METAVIR score. Twenty-three patients underwent liver biopsy alone, while 283 underwent elastography, with the latter performed exclusively after 2008.

Ultrasound examination revealed liver structure alteration in 29.4% of cases, and the presence of a nodular structure in 12%.

In the whole cohort, 26.9% of patients were cirrhotic (35.8% were aged < 45 years). The Child-Pugh score was specified in 156 patients. Most cirrhotic patients were classified as Child-Pugh Class A (*n* = 150, 96.2%), with an early stage severity of liver cirrhosis, with 4 classified as Child B (2.5%) and 2 as Child C (*n* = 1.3%). One hundred and six patients displayed signs of portal hypertension, which mostly occurred in those < 45 years (26%). Eight patients received liver transplantations. Forty-seven percent of patients were either eligible for or currently undergoing treatment, of whom 41.9% were cirrhotic. Dividing the cohort into treated (*n* = 162) and untreated HBeAg-positive patients (*n* = 182), we found significant differences between liver stiffness values (7.83 ± 3.56 *vs* 5.3 ± 2.3, *P* = 0.02). Similar differences were observed (7.84 ± 5.05 *vs* 4.82 ± 1.88, *P* < 0.001) when dividing the cohort into treated (*n* = 163) and untreated anti-HBeAg-positive patients (*n* = 181).

DISCUSSION

To our knowledge, this study involves the largest cohort of HBV patients in Italy. HBV infection was responsible for 27.3% of cases of chronic liver disease, whereas a previous study by Sagnelli *et al*[10] found an HBV prevalence of 20.2%.

We also found HBV-infected patients in those aged < 34 who would normally be covered by vaccination. Analysis of the nationality of HBV-infected patients highlights the prevalence of infection in the non-Italian population (only 2.8% of Italians were infected). Similar data was found in a previous study conducted in an industrialised city in the Tuscany region[11]. Further efforts are needed to improve vaccination coverage for

Table 2 Distribution of hepatitis B virus transmissions-multiple-choice analysis *n* (%)

Routes of transmission	Distribution
Intravenous drug users	15 (3.5)
Coagulation factors/blood transfusions	26 (6.0)
Sexual transmission	53 (12.3)
Piercing and tattooing	9 (2.1)
Vertical transmission	73 (16.9)
Nosocomial/dental cure	101 (23.4)
Undefined	255 (59.0)

Table 3 Distribution of the major hepatitis B virus genotypes *n* (%)

Genotypes	Distribution
A	19 (14.1)
B	4 (3.0)
C	4 (3.0)
D	97 (71.9)
E	5 (3.6)
F	6 (4.4)
Total	135 (100)

non-infected and non-vaccinated immigrants who are susceptible to infection.

According to national data on risk factors associated with the HBV infection in the Integrated Epidemiological System of Viral Acute Hepatitis (SEIVA), nosocomial/dental procedures (23%) are some of the most represented risk factors for HBV transmission, followed by mother-to-child (17%) and sexual transmission (12%). Recently, cosmetic treatment with percutaneous exposure (28% of patients), sexual exposure (24.7%), and dental therapy (11.5%) have been recognized as the main risk factors in acute viral hepatitis B in Italy. The risk of maternal-infant transmission is related to the mother's HBV replicative status (90% correlation with HBeAg-positive mothers compared to 10%-20% for HBeAg-negative mothers)[12]. Many studies[13-16] have highlighted that pregnant women in particular with high levels of HBV DNA (more elevated in HBeAg positive patients compared to HBeAg-negative patients) have an increased risk of transmitting infection. As recently shown by Sagnelli *et al*[17], the screening of pregnant women to detect circulating HBsAg and prophylaxis procedures for new-born babies, the universal vaccination against HBV infection introduced in 1991, and national media campaigns against HIV infection are considered the main reasons for the significant reduction in this type of transmission. Heterosexual (and, to a greater extent, homosexual) activity remain significant means of transmission. A study of Zuccaro *et al*[14] for an HBV cohort of 103 patients in 15 Italian hospital units showed a high prevalence of sexual transmission. In a study by Hahné *et al*[15], HBsAg prevalence estimates in men who have sex with men (MSM) ranged from < 1% to 4% in 3 of the 34 countries. This prevalence was 22 times higher than that of the general population for countries with available data[15].

As reported by Liaw *et al*[16], HBV genotype D is predominant in Mediterranean countries. In line with this finding, our study found that the most represented HBV genotype were D (72%), followed by A (14%). As concerns the potential bias of missing data, the statistical advantage of data that are missing completely at random is that the estimated parameters are not biased by the absence of the data.

To our knowledge, this is one of the few Italian studies to consider both infection rates and liver fibrosis stage based on international guidelines for a large cohort of patients.

We found a higher prevalence of HBeAg positive patients (24.7%) compared to Sagnelli *et al*[10] (7.2%) and a lower anti-HDV prevalence (7% *vs* 11.9%). It is well established that ongoing HBV replication or the presence of HBeAg may accelerate the progression of liver fibrosis. In a study of the natural course of HBV infection during the HBeAg-positive phase, Chu *et al*[18] showed that the annual incidence of cirrhosis was 0.5%, and the cumulative probability of cirrhosis after 17 years was 12.6%. Hence, age at anti-HBe seroconversion and hepatitis relapse were independent risk factors for cirrhosis. In particular, deferred HBeAg seroconversion (patients > 40 years) were associated with an increased risk of cirrhosis[18]. Moreover, HDV coinfection is associated with an accelerated progression towards liver cirrhosis, decompensation in existing cases of cirrhosis, and an increased risk of hepatocellular carcinoma[19].

In our cohort, almost all untreated patients were represented by inactive HBV carriers (HBV DNA < 2000 IU/mL), and by patients in an immune-tolerant phase (HBV DNA > 20000 IU/mL) with liver stiffness measurements < 6 kPa and normal ALT. These results are in agreement with national guidelines for treatment[5]. A study of 361 patients with inactive HBeAg-negative chronic hepatitis B patients demonstrated that liver fibrosis progression is rare given serum HBV DNA < 20000 IU/mL and normal ALT[21]. Brunetto *et al*[20] demonstrated that after a mean follow-up of six years, anti-HBe positive chronic hepatitis B progressed to cirrhosis in 45% of patients, with end-stage complications occurring in 24% of those presenting with cirrhosis. These outcomes seem to be associated with older age and persistent viral replication or hepatitis exacerbations in chronic hepatitis or cirrhotic patients. A recent study by Olivieri *et al*[22] showed that HBeAg-negative HBsAg-carriers with baseline HBV-DNA ⩽ 20000 IU/mL, and normal transaminases were associated with transition to inactive carrier status in 43% of low-viremic active carriers, and to occult HBV infection in 20% of inactive carrier, within five years.

We found that almost all patients with liver stiffness > 6 kPa were treated. There are several instances of evidence showing that antiviral therapy stops the progression of liver fibrosis and induces fibrosis regression. A recent study by Stasi *et al*[23] conducted in anti-HBeAg-positive patients before and during antiviral treatment using liver stiffness measurements with

transient elastography at different time points to assess changes in liver fibrosis showed a statistically significant reduction in stiffness values at 18 and 24 mo from those observed prior to therapy.

In the whole cohort, 26.9% were cirrhotic patients; it should be stressed here that 35.8% of these were aged < 45 years. The majority of cirrhotic patients showed signs of portal hypertension (43%). This data was different to that of Sagnelli *et al*[10] for a cohort of 513 HBV patients, 24% of whom had liver cirrhosis.

In conclusion, our data showed that 27.3% of chronic viral hepatitis patients were HBV infected. Evidence of HBV infection in people aged < 34 years was apparent, especially in the foreign population not protected by vaccination. The main routes of transmission were consolidated by data reported in several other studies. In our cohort of patients, we found an elevated prevalence of HBeAg-positive patients. In line with other Italian data, we found a high prevalence of liver cirrhosis in young adults. Antiviral treatment seems to be fully in line with national guidelines. Further efforts are necessary to increase vaccine coverage in immigrant populations.

ARTICLE HIGHLIGHTS

Research background

While the introduction of an effective vaccine has been highly effective in reducing the incidence and prevalence of hepatitis B virus (HBV) infection in many countries, an estimated 257 million people worldwide are still chronically infected. In 2015, WHO published guidelines for the prevention, care and treatment of persons with chronic HBV infection. These promote the use of simple, noninvasive tests to assess liver disease stage and eligibility for treatment.

Research motivation

Few data are currently available concerning the real-word large-scale staging and treatment of HBV patients followed in specialised outpatient services. The knowledge of the specific characteristics of patients could lead to appropriate planning for public health programs, thus contributing to the successful, efficient translation of this knowledge to management and treatment of HBV patients to eradicate HBV.

Research objectives

The main objective of this study was to build a regional database of patients diagnosed with chronic HBV infection in specialised outpatient services in our region to define the clinical epidemiology of HBV-infected patients in the Tuscan public health care system.

Research methods

This prospective observational study is fully in line with the objectives of the National Plan for the Prevention of Viral Hepatitis, in particular with those outlining the importance of the quality of reporting system data and of the monitoring of HBV and hepatitis C virus as acute or chronic infections. This study used a cross-sectional cohort design to evaluate all patients with HBV-related chronic liver disease referred to the hepatology outpatient services of 16 hospital units from January 1, 2015, to December 31, 2015. A computerised clinical database was used to collect the main socio-demographic, clinical and treatment data for all patients with chronic HBV infection admitted at each centre. The exported file (containing the "anonymized" data) was then sent to the Regional Health Agency of Tuscany *via* a secure channel (SSL).

Research results

The results of this study demonstrated that 27.3% of chronic viral hepatitis patients were HBV infected. Of these HBV-infected patients, 73% were Caucasian, 21% Asian, 4% Central African, 1% North African and 1% American. Stratifying patients by age and nationality, we found that 21.7% of HBV-infected patients were aged < 34 years. Among these only 2.8% were Italian. The most represented routes of transmission were nosocomial/dental procedures (23%), mother-to-child (17%) and sexual transmission (12%). The most represented HBV genotypes were D (72%) and A (14%). Of the patients, 24.7% were HBeAg positive, and 75.3% were HBeAg negative. Of the HBV patients, 7% were anti-HDV positive. In the whole cohort, 26.9% were cirrhotic (35.8% aged < 45 years), and 47% were eligible for or currently undergoing treatment, of whom 41.9% were cirrhotic.

Research conclusions

The informatization of clinical data and the real-word large-scale staging and treatment of these kinds of patients would allow health planners to assign specific healthcare resources to targeted populations. This observational research may provide a public health opportunity to screen and treat specific groups of patients. In particular, to eradicate HBV infection further efforts are necessary to increase vaccine coverage in immigrant populations.

Research perspectives

Based on the data collected in the course of this investigation, it is necessary to implement a series of preventive measures and strengthen the entire care process to eliminate, or at least reduce, risk factors for HBV infection and progression toward advanced liver fibrosis stages and to adopt care protocols suitable for immigrant populations.

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ORIGINAL ARTICLE

Retrospective Study

Isolated hepatic non-obstructive sinusoidal dilatation, 20-year single center experience

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Abstract

AIM

To characterize isolated non-obstructive sinusoidal dilatation (SD) by identifying associated conditions, laboratory findings, and histological patterns.

METHODS

Retrospectively reviewed 491 patients with SD between 1995 and 2015. Patients with obstruction at the level of the small/large hepatic veins, portal veins, or right-sided heart failure were excluded along with history of cirrhosis, hepatic malignancy, liver transplant, or absence of electrocardiogram/cardiac echocardiogram. Liver histology was reviewed for extent of SD, fibrosis, red blood cell extravasation, nodular regenerative hyperplasia, hepatic

peliosis, and hepatocellular plate atrophy (HPA).

RESULTS

We identified 88 patients with non-obstructive SD. Inflammatory conditions (32%) were the most common cause. The most common pattern of liver abnormalities was cholestatic (76%). Majority (78%) had localized SD to Zone Ⅲ. Medication-related SD had higher proportion of portal hypertension (53%), ascites (58%), and median AST (113 U/L) and ALT (90 U/L) levels. Nineteen patients in our study died within one-year after diagnosis of SD, majority from complications related to underlying diseases.

CONCLUSION

Significant proportion of SD and HPA exist without impaired hepatic venous outflow. Isolated SD on liver biopsy, in the absence of congestive hepatopathy, requires further evaluation and portal hypertension should be rule out.

Key words: Sinusoidal dilatation; Sinusoidal obstruction syndrome; Hepatic plate atrophy

Core tip: We identified 88 patients with diagnosis of non-obstructive sinusoidal dilatation (SD) over the period of twenty years. Inflammatory conditions (32%) were the most common cause identified. Medication related SD was associated with higher proportion of portal hypertension, ascites, and elevated transaminases. The finding of non-obstructive SD on liver biopsy should prompt a review of patient's medical history and drug exposure. Additionally, portal hypertension should be rule out either clinically, endoscopically, or radiographically. There does not appear to be any relationship between histological patterns and medical conditions, which may suggest overlapping biological pathways in the development of non-obstructive sinusoidal dilatation.

Sunjaya DB, Ramos GP, Braga Neto MB, Lennon R, Mounajjed T, Shah V, Kamath PS, Simonetto DA. Isolated hepatic non-obstructive sinusoidal dilatation, 20-year single center experience. *World J Hepatol* 2018; 10(5): 417-424 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i5/417.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i5.417

INTRODUCTION

Hepatic sinusoidal dilatation (SD) is usually attributed to either hepatic venous outflow obstruction at the level of small or large hepatic veins, supra-hepatic inferior vena cava, or right-sided heart failure. A proportion of patients demonstrate SD in the absence of post-sinusoidal venous outflow impairment or portal vein thrombosis and the clinical significance of this finding is unclear[1]. Histologically, non-obstructive SD is often characterized by distended sinusoidal spaces, most evident in zone Ⅲ and, sometimes accompanied by hepatocellular plate atrophy, and/or red blood cell (RBC) extravasation (Figure 1). These findings are non-specific and have been reported with systemic inflammatory states, hematological malignancies, granulomatous disease, medications, and inflammatory bowel disease[2-10]. Sinusoidal dilatation may also be seen on wedge biopsies of the liver obtained intra-operatively. The long-term outcomes of patients with SD are not known. Moreover, there is no clear guidance on how such patients are to be investigated.

The purpose of this study was to better characterize non-obstructive SD by: (1) identifying associated conditions, such as: Vascular disorder, neoplastic disease, inflammatory disease, infections, surgery, or medications; (2) describing the long-term outcomes of these patients; and (3) identifying distinct laboratory or histological patterns that may identify the potential cause or disease association of the SD.

MATERIALS AND METHODS

We identified 491 patients from the Mayo Clinic, Rochester Minnesota electronic medical record between 1995 and 2015 with histological findings consistent with SD on high quality liver biopsy. SD was defined as sinusoidal lumen greater than one liver cell plate wide, observed in several lobules in a high-quality liver specimen devoid of artefactual tearing[9]. We defined high quality liver specimen by the presence of seven or more portal tracts from either needle or wedge biopsy. Patients with confirmed obstruction at the level of the small hepatic veins (veno-occlusive disease or sinusoidal obstruction syndrome), large hepatic veins/inferior vena cava (Budd-Chiari syndrome), portal vein thrombosis, evidence of vascular infiltration (sickle cell, hemophagocytic syndrome, or malignancy) or right-sided heart failure (moderate to severe tricuspid regurgitation, constrictive pericarditis, restrictive cardiomyopathy, or elevated right ventricle systolic pressure on echocardiogram) were excluded from the study (Figure 2). In addition, patients with cirrhosis, hepatic malignancy, liver transplant recipients, or absence of electrocardiogram or echocardiogram on medical records (to rule out heart failure) were also excluded from our study. Liver transplant recipients were excluded from the study due to the high likelihood of anastomotic vascular complications resulting in sinusoidal dilatation in this group.

The remaining cases were investigated for associated medical conditions. The electronic records were reviewed for clinical, laboratory values and imaging data (Supplementary Table 1). Imaging studies include abdominal ultrasound, abdominal/ pelvis CT with or without contrast, and abdominal/ pelvis MRI if available. Patients were classified into 1 out of 4 possible categories: inflammatory/auto-immune disorder, malignancy, medication, or undefined based on review of clinical history, histological findings, history of medication exposure, and laboratory findings. Patients with diagnosis of long standing inflammatory/ auto-immune disorder in

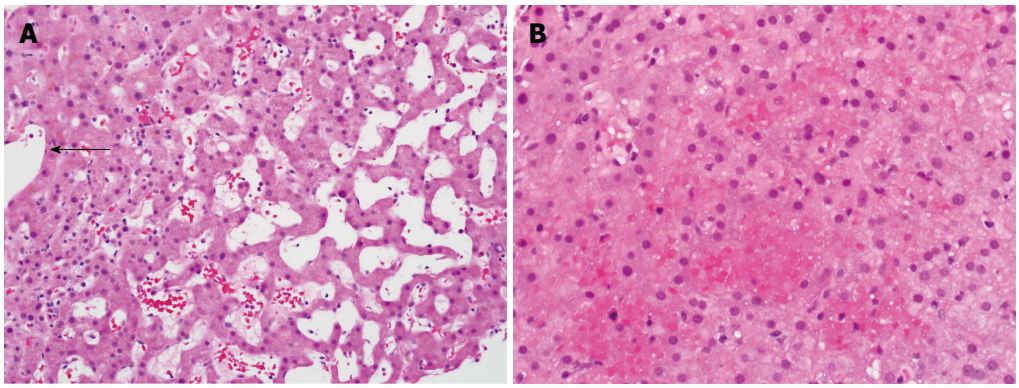

Figure 1 Sinusoidal dilatation and hepatocellular plate atrophy in zone 3 (arrow marks hepatic vein branch) (20 ×) (A); Zone 3 congestion and red blood cell extravasation (20 ×) (B).

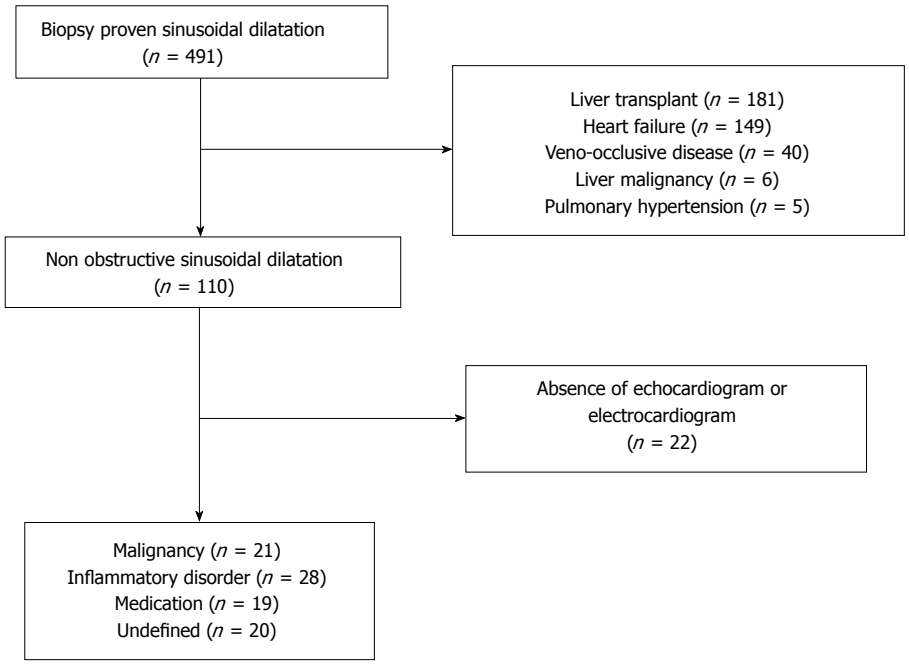

Figure 2 Flow chart of the screening process.

the absence of other plausible etiology were categorized to the inflammatory/auto-immune category. Patients with history of malignancy without previous exposure to medications associated with SD (including chemotherapy drugs) were assigned to the malignancy group. Patients with exposure to medications known to be associated with SD, such as oxaliplatin or estrogen, in the absence of other plausible etiology were categorized into the medication-related category. Patients without history of inflammatory/auto-immune disorder, malignancy, or exposure to medications associated with SD were assigned to the undefined category.

We reviewed the quality of liver biopsies by evaluating for the number of samples, size of biopsies, and number of portal tracts. Standard histologic staining such as: trichrome and reticulin, were performed on all samples. Special staining, such as PAS- diastase, Congo red, and copper, were performed on select samples based on the degree of clinical or histologic suspicion. The following information was recorded: (1) Extent of SD; (2) extent of fibrosis; (3) RBC extravasation; and presence of (4) hepatocellular plate atrophy. For this study, RBC extravasation was defined as the presence of red blood cells in the space of Disse. Extent of fibrosis was staged using the METAVIR system, which assigns 0 = no fibrosis, 1 = portal fibrosis without septa, 2 = portal fibrosis with few septa, 3 = numerous septa without cirrhosis or 4 = cirrhosis. Histopathological data on nodular regenerative hyperplasia and peliosis hepatis were also obtained. Nodular regenerative hyperplasia was defined as the presence of regenerative nodules on reticulin stain, and no or minimal fibrosis on trichrome staining[11]. Peliosis hepatis was defined as presence of round or oval cavities randomly distributed between areas of normal hepatic parenchyma[12].

Although early sinusoidal obstruction syndrome

Table 1 Study demographics *n* (%)

Variables	*n* = 88
Age	56 (6-85)
Gender	
Female	48 (54.5)
Ethnicity	
Caucasian	75 (85.2)
Mortality within 1 yr	19 (21.6)
Portal hypertension	31 (35.2)
Radiological Findings	
Hepatic nodule(s)	15 (17.0)
Splenomegaly	35 (39.8)
Ascites	29 (33.0)
Pattern of liver injury	
Hepatocellular	8 (9.1)
Cholestatic	67 (76.1)
Mixed	9 (10.2)
Normal	4 (4.5)
Primary medical condition	
Malignancy	21 (23.9)
Inflammatory disorder	28 (31.8)
Medication	19 (21.6)
Undefined	20 (22.7)

(SOS) cannot be entirely ruled out in patients exposed to chemotherapy drugs, those with typical histologic features of SOS including centrilobular fibrosis and hepatocyte necrosis were not included in this study. Therefore, we defined possible SOS based on liver injury arising within 20 d of chemotherapy exposure with at least two of the following: (1) Rise of serum bilirubin above 2.0 mg/dL; (2) hepatomegaly and/or right upper quadrant tenderness; and (3) sudden weight gain (> 2% of body weight) attributable to fluid accumulation.

The presence of portal hypertension was identified by either: (1) hepatic vein pressure gradient > 6 mmHg; (2) splenomegaly and thrombocytopenia (< 150000); (3) Serum Ascites Albumin Gradient > 1.1 g/dL; or (4) porto-systemic venous collaterals identified on ultrasound, CT, or MRI scans.

The pattern of liver test abnormality was categorized as either as: (1) hepatocellular; (2) cholestasis; (3) mixed; or (4) normal. Normal was defined as the absence of liver test abnormality. The pattern of liver injury was classified using the R factor score[13]. An R value of > 5.0 is used to define hepatocellular injury, R < 2.0 as cholestatic injury, and R between 2.0 to 5.0 as mixed hepatocellular-cholestatic injury. If serum ALT level was not available for R factor score calculation, we utilized our best clinical judgment based on serum AST, alkaline phosphatase, and bilirubin level.

Overall mortality and death within one year of diagnosis of non-obstructive SD were collected.

Statistical review of this study was performed by a biomedical statistician from the Mayo Clinic division of biomedical statistics and informatics. Dichotomous data were expressed as frequency (percentage). Continuous data were expressed as median and range. Kruskal-Wallis test was used to compare continuous variables between different groups. For nominal variables, chi-square test was used. All tests were two-sided and a *P* value of $\leqslant$ 0.05 was considered statistically significant. Analysis were done using SPSS version 20.0.

RESULTS

We identified a total of 88 patients with non-obstructive SD, which accounts for 17.9% of all cases with histologic evidence of SD in our center (Figure 2). Abnormal liver enzymes and presence of ascites were the most common indications for the liver biopsy. Needle biopsy accounts for majority of samples (97%). The median number of tissue sample collected was 2 (1-10) with a median number of 8 portal tracts identified and a median biopsy dimension of 1.5 cm (0.5-3.9). The majority of patients were female (55%) and Caucasian (85%) with a median age of 56 years old (6-85) at diagnosis. The most common medical conditions associated with SD were inflammatory conditions or autoimmune disorder (32%) followed by malignancy (24%) and medications (22%). We were not able to identify a potential etiology for twenty patients (23%) (Table 1). The list of complete medical conditions identified in our cohort can be found on Table 2. The most common autoimmune or inflammatory conditions identified were granulomatous hepatitis (*n* = 4), mixed connective tissue disease (*n* = 3), and inflammatory bowel disease (*n* = 3). Hematological malignancies and myeloproliferative diseases, accounted for the majority of the neoplasms in our study cohort. Oxaliplatin based chemotherapy (*n* = 7) was the most common medication identified in our study cohort followed by purine analogs (*n* = 3). Oral contraceptive use was identified as a probable cause of non-obstructive SD in two patients.

The most common radiological findings associated with SD were splenomegaly (40%) followed by ascites (33%), and hyperechoic liver lesion or hepatic nodule(s) (17%). Portosystemic collateral veins were identified in five patients (6%). Medication related non-obstructive SD had a significantly higher proportion of ascites (58%, *P* = 0.044) than the other associated clinical conditions (Table 3).

The median serum AST was 43 U/L, ALT was 44 U/L, total bilirubin 0.9 mg/dL, direct bilirubin 0.4 mg/dL, alkaline phosphatase 271 IU/L, GGT 123 U/L, serum protein 6.4 g/dL, albumin 3.2 g/L, and INR 1.1. Median ESR and CRP were 45.5 mm/h and 5.1 mg/L respectively. ESR and CRP were collected in a subset of patients where clinical suspicion for inflammatory disorder was high. We found medication-related non-obstructive SD was associated with higher median serum AST (113 U/L, *P* < 0.008) and ALT level (90 U/L, *P* = 0.002). Five of the thirteen patients (39%) in the medication related SD group had serum total bilirubin greater than 2.0 mg/dL. Four out of five patients in this subgroup had ascites and splenomegaly on imaging suggesting a possible diagnosis of early SOS. We also found lower median platelet counts in medication related SD group (80 x 10^3, *P* = 0.022), consistent with higher prevalence of non-cirrhotic portal hypertension (Table 4). Malignancy related

Table 2 Medical conditions associated with non-obstructive sinusoidal dilatation

Variables	*n*
Malignancy	
Hematological malignancies	
Leukemia	
AML	2
CLL	1
CML	1
Lymphoma	
B-cell lymphoma	4
T-cell lymphoma	1
Solid organ tumor	
Gastric adenocarcinoma	2
Angiosarcoma	1
Myeloproliferative disorder	9
Inflammatory condition	
Granulomatous hepatitis	4
Connective tissue disorder	3
Ulcerative colitis	2
Castleman's disease	2
Polyarthritis nodosa	2
Other[1]	15
Medications	
Oxaliplatin	7
Purine Analogs	3
Other[2]	9

[1]Acute inflammatory demyelinating polyradiculopathy, autoimmune hepatitis, amyloidosis, CINCA syndrome, Crohn's disease, IgA nephropathy, non-alcoholic steatohepatitis, primary biliary cirrhosis, psoriatic arthritis, and sarcoidosis; [2]Alcohol, atorvastatin, allopurinol, cyclophosphamide, decitabine, ethinyl estradiol and norgestimate, vincristine, and ibritumomab tiuxetan. AML: Acute myelocytic leukemia; CLL: Chronic lymphoblastic cytic leukemia; CML: Chronic myelocytic leukemia.

non-obstructive SD had higher median total bilirubin (1.6 mg/dL, $P = 0.008$).

The most common pattern of liver abnormalities was cholestasis (76%) followed by mixed (10%) and hepatocellular (8%) (Table 5). We did not identify a difference in the pattern of liver test abnormalities between varying causes of non-obstructive SD ($P = 0.54$). The majority of patients (78.4%) had SD localized to Zone Ⅲ. Fibrosis was found in 37 patients and 17 of them were limited to portal fibrosis without septa involvement (stage 1/4) (Table 6). Patients with autoimmune or inflammatory diseases had higher proportion of fibrosis on liver biopsy (39%) followed by medication (32%) and malignancy (19%) (Table 6). Hepatocellular plate atrophy and RBC extravasation were found in 58% and 32% of the study population respectively. Patients with autoimmune or inflammatory disorder also had higher proportion of hepatocellular plate atrophy (75%) followed by malignancy (52%) and medication related (42%). Nodular regenerative hyperplasia and hepatic peliosis were identified on histopathology in nine patients (10%) and one patient (1%) respectively. Lymphocytic infiltration was identified in twenty-four patients (27%) (Table 6).

The median follow up for all patients was 437 d (0-5616 d). Thirty seven patients (42%) died during the study follow up. Nineteen patients in our study died within one year after diagnosis of SD. The one-year mortality was highest in the medication related group (47%) followed by malignancy group (19%) and inflammatory group (14%). Ten patients died from complications of their respective underlying disease, such as: High burden of malignancy or infections related to immunosuppression. Four patients died from conditions unrelated to the cause of non-obstructive SD and in five patients the cause of death was not identified. Fifty out of sixty-nine patients (72%) that survived had at least 1 year of follow-up with a median follow-up time of 1943 d (370-5616 d).

Our cohort included 20 patients with unspecified cause of non-obstructive SD. The median age of diagnosis in this subgroup was 53 years (range: 16-85 years) with a median follow up of 636 d (range: 11-4120 d). Six patients (30.0%) from this subgroup died with median time to death of 2711 d (range: 146-5518 d) and 2 patients (10%) died within one-year of SD diagnosis.

DISCUSSION

The main findings of this study are that a significant proportion of SD occurs in the absence of impaired hepatic venous outflow. Twenty-eight percent (19/69) of patients with follow-up of at least one year died within one year after non-obstructive SD diagnosis, which might reflect poor clinical status or high disease burden in this study population. Medication related non-obstructive SD was associated with elevated serum AST and ALT levels but lower platelet counts compared to other causes. This observation correlates with a higher prevalence of non-cirrhotic portal hypertension in this group. A subset of these patients may have developed early SOS in the setting of recent chemotherapy exposure, such as oxaliplatin, despite the lack of typical histologic features. No patients had a history of stem cell transplantation. We did not identify a relationship between the extent of SD, hepatocellular plate atrophy, lymphocytic infiltration, or RBC extravasation with a specific etiology of non-obstructive SD. Our findings suggest that non-obstructive SD likely occurs through a common pathway associated with various medical conditions. Several studies have suggested the role of both IL-6 and VEGF overexpression in the development of SD[2,3]. Furthermore, the same IL-6 and its soluble receptor (sIL-6R) have been shown to be upregulated in chronic inflammatory or autoimmune conditions. In our cohort, one patient had a markedly elevated serum IL-6 level (249.1 pg/mL, normal range: 0-12.2 pg/mL). This was obtained as part of his extensive rheumatological work up. Unfortunately, this patient was not included in our final analysis due to the absence of echocardiogram to exclude right-sided heart failure, although he had no suggestive cardiac symptoms. Furthermore, the median serum CRP in our cohort was significantly elevated suggesting a role of chronic inflammatory state in the development of SD.

The prevalence of SD without hepatic venous outflow impairment has been reported in several small

Table 3 Clinical and radiological features stratified by conditions *n* (%)

	Malignancy (*n* = 21)	Inflammatory state (*n* = 28)	Medication (*n* = 19)	Not specified (*n* = 20)	*P*
Hepatic nodules	4 (19)	4 (14)	4 (21)	3 (15)	0.922
Portal hypertension	10 (48)	8 (29)	11 (58)	6 (30)	0.144
Ascites	7 (33)	5 (18)	11 (58)	6 (30)	0.04
Splenomegaly	9 (43)	11 (39)	9 (47)	6 (30)	0.719

Table 4 Laboratory findings stratified by conditions

	Malignancy	Inflammatory state	Medication	Not specified	*P*
AST (U/L)	46 (14-120)	43 (9-222)	113 (21-2351)	36 (14-80)	0.008
ALT (U/L)	36 (15-283)	46 (6-237)	90 (23-785)	28 (13-84)	0.002
Total bilirubin (mg/dL)	1.6 (0.3-28)	0.6 (0.2-9.1)	1.3 (0.4-12.8)	0.5 (0.3-8)	0.008
Direct bilirubin (mg/dL)	0.9 (0.1-24)	0.3 (0.1-6.2)	0.6 (0.1-8.2)	0.3 (0.1-5.8)	0.141
Alk Phosphotase (IU/L)	323 (71-1616)	355 (50-2288)	313 (104 - 1905)	135 (50-801)	0.057
GGT (U/L)	140 (82-1856)	106 (51-1066)	233 (50-418)	125 (N/A)	0.881
Albumin (g/L)	3.2 (1.7-4.3)	3.3 (2.1-4.1)	3.1 (2.5-4.5)	3.6 (1.2-4.3)	0.826
INR	1.2 (1-2.4)	1.1 (0.9-1.7)	1.1 (0.9-1.9)	1.1 (0.9-1.5)	0.106
Total protein (g/dL)	5.8 (1.6-9.2)	6.4 (4.7-8.6)	5.8 (4.8-8.2)	7.1 (5.2-10)	0.111
Platelets (10^3)	161 (25-907)	187 (10-708)	80 (17-339)	188 (78-352)	0.022
ESR (mm/h)	59 (3-132)	43 (0-121)	24 (13-117)	35 (6-127)	0.687

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; ESR: Erythrocyte sedimentation rate.

case series[1,14]. Bruguera *et al*[14] reported the a 2.9% incidence of non-obstructive SD on consecutive liver biopsy, while Kakar *et al*[1] reported one in three patients with diagnosis of SD occurred in the absence of venous outflow impairment. In our study, we found that 18% of all cases of sinusoidal dilatation occurred in the absence of venous outflow obstruction, or hepatic malignancy. This supports the previous studies indicating that non-obstructive SD may be more common than expected. The clinical significance of non-obstructive SD on histopathology is unclear, although we identified a high one-year mortality rate in our cohort. The majority of patients that died had coexisting malignancy (58%) and/or an autoimmune/inflammatory condition (29%). Seventy nine percent of patients died from either high burden of disease or complications of their underlying medical condition, such as malignancy. This brings forward a question of whether or not asymptomatic patients with abnormal liver enzymes and non-obstructive SD on biopsy require further evaluation for occult malignancy or inflammatory conditions. In our cohort of patients with undefined cause of SD, the one year mortality rate was low and interestingly all patients died from systemic infections. Future studies should evaluate the utility of screening for inflammatory/autoimmune condition or malignancy in patients with abnormal liver enzymes without an obvious cause of non-obstructive SD.

We were also interested in determining whether or not there is distinct biochemical, radiological, or histological patterns associated with specific medical conditions in patients with non-obstructive SD. We found that medication associated non-obstructive SD, such as previous exposure to platinum-based chemotherapy or purine analogs, were associated with higher median serum AST (113 U/L, $P = 0.008$) and ALT (90 U/L, $P = 0.002$) levels. This was consistent with previously reported findings highlighting the hepatotoxic nature of both oxaliplatin and 5-FU even after cessation of treatment[10,15-20].

We did not identify a relationship between the presence of hepatic nodules on imaging with nodular regenerative hyperplasia or peliosis hepatis on histopathology.

The strengths and weaknesses of our study merit further discussion. The major strength of our study was that this is the largest study to date on isolated non-obstructive SD. Previous studies were limited to case reports or small case series[1,14]. In addition, we have lon-

Table 5 Pattern of liver injury stratified by conditions

	Malignancy	Inflammatory state	Medication	Not specified	*P*
Liver injury					0.536
Hepatocellular	1	2	4	1	
Cholestasis	16	21	14	16	
Mixed	3	4	1	1	
Normal	1	1	0	2	

Table 6 Histology features stratified by conditions

	Malignancy (*n* = 21)	Inflammatory state (*n* = 28)	Medications (*n* = 19)	Not specified (*n* = 20)	Total	*P*
Zone						0.82
Ⅲ	17 (81)	22 (79)	16 (84)	14 (70)	69	
Ⅱ and Ⅲ	1 (5)	3 (11)	1 (5)	3 (15)	8	
Ⅰ, Ⅱ, and Ⅲ	1 (5)	0 (0)	1 (5)	0 (0)	2	
Other	2 (10)	3 (11)	1 (5)	3 (15)	9	
METAVIR score						0.60
0	15 (71)	14 (50)	13 (68)	9 (45)	51	
1	3 (14)	6 (21)	3 (16)	5 (25)	17	
2	1 (5)	4 (14)	1 (5)	3 (15)	9	
3	0 (0)	1 (4)	2 (11)	2 (10)	5	
Nodular regenerative hyperplasia	1(5)	2 (7)	4 (21)	2 (10)	9	0.33
Hepatic peliosis	1 (5)	0	0	0	1	0.36
Lymphocytic infiltration	6 (29)	8 (29)	6 (32)	4(20)	24	0.77
RBC extravasation	8 (38)	8 (29)	7 (37)	5 (25)	28	0.76
Hepatocellular plate atrophy	11 (52)	21 (75)	8 (42)	11 (55)	51	0.13

gitudinal data, up to 10 years after the initial diagnosis in majority of the patients. There were several limitations to our study: (1) As a tertiary center, a significant proportion of our cohort was referred for a second opinion of abnormal liver enzymes and had significant medical comorbidities, which may affect our one-year mortality rates and duration of follow up; (2) follow up data was not available in all patients, but the majority (69 out of 88 patients) had at least one-year follow up; (3) there were a large proportion of undefined causes of non-obstructive sinusoidal dilatation, which reflects the need for high quality prospective studies on this condition; and (4) we utilized our best clinical judgment based on available clinical data and histologic findings when selecting the primary etiology of non-obstructive SD in the setting of multiple medical conditions and/or history of medication exposure.

In conclusion, the finding of non-obstructive SD on liver biopsy should prompt a review of patient's medical history and drug exposure. Additionally, portal hypertension should be rule out either clinically, endoscopically or radiographically. There does not appear to be a relationship between histological patterns and medical conditions, which may suggest overlapping biological pathways in the development of non-obstructive sinusoidal dilatation.

ARTICLE HIGHLIGHTS

Research Background

A proportion of patients demonstrate (SD) in the absence of post-sinusoidal venous outflow impairment or portal vein thrombosis and the clinical significance of this finding is unclear. Long-term outcomes of patients with SD are not known. Moreover, there is no clear guidance on how such patients are to be investigated.

Research motivation

To better understand the clinical relevance and long-term outcomes of patients with non-obstructive SD.

Research objectives

To better characterize isolated non-obstructive SD by identifying associated conditions, laboratory findings, and histological patterns.

Research methods

Retrospective chart review of patients with isolated non-obstructive SD.

Research results

Inflammatory conditions (32%) were the most common cause identified. The most common pattern of liver abnormalities was cholestatic (76%). The majority (78%) had localized SD localized to Zone III. Medication-related SD had higher proportion of portal hypertension (53%), ascites (58%), and median AST (113 U/L) and ALT (90 U/L) levels. Nineteen patients in our study died within one-year after diagnosis of SD. Ten patients died from complications related to underlying diseases associated with SD.

Research conclusions

Significant proportion of SD may exist without impaired hepatic venous outflow. There does not appear to be any relationship between histological patterns and medical conditions. High one-year mortality rate in our cohort may suggest relationship between clinical status and development of SD. Isolated SD on liver biopsy, in the absence of congestive hepatopathy, requires further evaluation and portal hypertension should be rule out.

Research perspectives

Future studies should evaluate the utility of screening for inflammatory/autoimmune condition or malignancy in patients with non-obstructive SD

without an obvious cause.

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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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ORIGINAL ARTICLE

Retrospective Study

Paracentesis in cirrhotics is associated with increased risk of 30-day readmission

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Abstract

AIM

To determine the readmission rate, its reasons, predictors, and cost of 30-d readmission in patients with cirrhosis and ascites.

METHODS

A retrospective analysis of the nationwide readmission database (NRD) was performed during the calendar year 2013. All adults cirrhotics with a diagnosis of ascites,

spontaneous bacterial peritonitis, or hepatic encephalopathy were identified by ICD-9 codes. Multivariate analysis was performed to assess predictors of 30-d readmission and cost of readmission.

RESULTS

Of the 59597 patients included in this study, 18319 (31%) were readmitted within 30 d. Majority (58%) of readmissions were for liver related reasons. Paracentesis was performed in 29832 (50%) patients on index admission. Independent predictors of 30-d readmission included age < 40 (OR: 1.39; CI: 1.19-1.64), age 40-64 (OR: 1.19; CI: 1.09-1.30), Medicaid (OR: 1.21; CI: 1.04-1.41) and Medicare coverage (OR: 1.13; CI: 1.02-1.26), > 3 Elixhauser comorbidity (OR: 1.13; CI: 1.05-1.22), nonalcoholic cirrhosis (OR: 1.16; CI: 1.10-1.23), paracentesis on index admission (OR: 1.28; CI: 1.21-1.36) and having hepatocellular carcinoma (OR: 1.21; CI: 1.05; 1.39). Cost of index admission was similar in patients readmitted and not readmitted (*P*-value: 0.34); however cost of care was significantly more on 30 d readmission ($30959 ± 762) as compared to index admission ($12403 ± 378), *P*-value: < 0.001.

CONCLUSION

Cirrhotic patients with ascites have a 33% chance of readmission within 30-d. Younger patients, with public insurance, nonalcoholic cirrhosis and increased comorbidity who underwent paracentesis are at increased risk of readmission. Risk factors for unplanned readmission should be targeted given these patients have higher healthcare utilization.

Key words: Cirrhosis; Readmission rates; Paracentesis; Ascites

Core tip: Cirrhotic patients with ascites have a 33% chance of 30-d readmission. Factors associated with 30-d readmission include age < 64 years, Medicaid and Medicare insurance, increased comorbidities, nonalcoholic cirrhosis, hepatocellular carcinoma and paracentesis during index admission. Based on identification of these predictors and significant cost involvement, there is need to find ways to counteract them and reduce 30-d readmission rate.

Sobotka LA, Modi RM, Vijayaraman A, Hanje AJ, Michaels AJ, Conteh LF, Hinton A, El-Hinnawi A, Mumtaz K. Paracentesis in cirrhotics is associated with increased risk of 30-day readmission. *World J Hepatol* 2018; 10(6): 425-432 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i6/425.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i6.425

INTRODUCTION

The prevalence of cirrhosis in the United States has increased from 400000 to 600000 individuals in the past decade[1,2]. Approximately, 5%-7% of patients with compensated cirrhosis develop decompensation each year[3,4]. Decompensation of cirrhosis is marked by complications such as ascites, spontaneous bacterial peritonitis (SBP), hepatic encephalopathy, esophageal varices, and/or jaundice[2,3,5]. Patients with decompensated cirrhosis have worse outcomes with a median life expectancy of 2 years compared to 12 years in patients with compensated disease[3].

Ascites is one of the early signs of portal hypertension and decompensated cirrhosis[5]. The development of ascites is an ominous sign with a mortality rate of 50% in 2 years after initial development[6]. Patients with symptomatic, treatment refractory, or ascites complicated by SBP have an even higher mortality rate, estimated around 50% in 6 mo[7,8]. Paracentesis is a low risk procedure and recommended in all patients with refractory or symptomatic ascites on hospital admission to diagnose SBP and relieve symptoms[9,10]. Recent national studies have shown reduced inpatient short-term mortality in those who underwent paracentesis during hospitalization[10,11]. However, increased length of hospital stay and hospital charges were also reported in paracentesis group[10].

Given the economic burden of readmissions, the Patient Protection and Affordable Care Act instituted the Readmission Reduction Program that required the Centers for Medicare and Medicaid to reduce payment for hospitals with higher readmission rates[12]. Therefore, it is crucial to identify factors that predict 30-d readmissions in patients with decompensated cirrhosis and ascites given risk of frequent readmission and mortality. Readmission rates and mortality in cirrhotic patients have been reported in the North American Consortium for the Study of End-Stage Liver Disease cohort and insurance claim database[13,14]. Patients with decompensated cirrhosis and ascites are at higher risk of hospital readmission with recent studies reporting a readmission rate around 50%[13,14]. Moreover, presence of ascites and paracentesis was found to be independent predictors for readmission and increased 90 d and overall mortality[14]. However, there is no national report on the incidence of 30-d readmission rates and its predictors in patient population with ascites and/or HE. The aim of this study is to use Nationwide Readmission Database (NRD) to evaluate 30-d readmission rates, its reasons, predictors and cost of readmission.

MATERIALS AND METHODS

Data source

A retrospective NRD study was performed from January 1st 2013 to December 1st 2013. NRD contains publically available data from 35 million hospitalizations over 21 geographically distributed states and offers insight into over 100 clinical and hospital variables[15]. National readmission rates from all payers and uninsured are provided in this analysis. The Ohio State University Data

and Specimen Policy and Human Subjects Research Policy does not require informed consent for research conducted using public available data set as they do not involve "human subject."

Study sample

Utilizing International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes, patients with a diagnosis of ascites (789.5, 789.69), SBP (567.23), or hepatic encephalopathy (572.2) and a diagnosis of cirrhosis (571.2, 571.5, 571.6) were included in this study. Patients were excluded from this study if they were under the age of 18, left against medical advice, transferred from a different facility, experienced mortality during the index admission, or were pregnant. Patients with missing length of stay between admissions were also excluded from this study. Moreover, if a patient was admitted more than once in 30 d, only the first readmission was included.

Covariates

During index admission, multiple variables were evaluated to determine association with 30-d readmission. Patient demographics included age, gender, primary insurance payer, and annual income. Hospital demographics included size and type-urban-teaching, urban non-teaching and rural. Other variables of interest were identified using the appropriate ICD-9 codes and included comorbidities, evaluated by the Elixhauser co-morbidity scale and features of liver decompensation defined as the presence of esophageal varices, hepatorenal syndrome, and hepatocellular carcinoma. Etiology of cirrhosis was also determined by ICD-9 codes and was divided broadly as alcoholic *vs* non-alcoholic liver disease (Supplementary Appendix 1). Each patient was evaluated in order to determine if a paracentesis was performed on index admission. The procedure was identified using the proper procedural code (Supplementary Appendix 1).

Outcomes of interest

We studied the 30-d readmission rate, reasons for readmission, predictors of 30-d readmission and cost with an emphasis on the effect of paracentesis in patients with cirrhosis and ascites. Reasons for readmission were divided into liver *vs* non-liver related based on the primary diagnosis on the 30-d readmission then we specifically evaluated the top 10 liver related reasons for readmission. We also studied the length of stay, cost during index admission and the difference of cost of index admission and readmission at 30 d.

Statistical analysis

All analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, United States) on weighted data and accounted for the complex survey design. Chi-square test was used to compare proportions and *t*-test was used to compare means. A multivariate logistic regression model was fit to identify independent predictors for 30-d readmission where results are presented as odds ratios (OR) with 95% confidence intervals (CI). Variables included in the model were determined through stepwise selection where paracentesis, age, gender, type of insurance, income, Elixhauser comorbidity score, hospital size and type, etiology of cirrhosis and features of liver decompensation were eligible for inclusion.

RESULTS

Patient and hospital characteristics

There were 59597 patients included in this study with 18319 (33%) readmitted within 30 d. Mean age of patients in our study group was 59 ± 0.12 years. On univariate analysis, patients' ⩽ 64 years (66% *vs* 65%, *P* value 0.004) were more likely to be readmitted within 30 d. Patients with 30-d readmission were also more likely to have Medicaid (24% *vs* 20%, *P*-value < 0.001) or Medicare (45% *vs* 44%, *P*-value < 0.001) as their primary insurance provider. Readmitted patients were more likely to have > 3 comorbidities (78% *vs* 76%, *P*-value 0.003), nonalcoholic cirrhosis (54% *vs* 48%, *P*-value < 0.001) and hepatocellular carcinoma (4% *vs* 3%, *P*-value 0.002). All other patient and hospital information including gender, income, features of decompensation, hospital size and type were not significantly different between patients that were readmitted within 30-d compared to patients that were not (Table 1).

Reason for readmission

Reasons for 30-d readmission were grouped into liver related *vs* non liver related. Most readmissions (58%) were liver related with the number one reason for 30 d readmission being hepatic encephalopathy (Figure 1).

Effect of paracentesis

A total of 29832 (50%) patients underwent paracentesis during their index admission. Paracentesis during index admission was significantly higher in patients readmitted (*n* = 9918; 54%) as compared to those not readmitted within 30 d (*n* = 19914; 48%), *P*-value < 0.001 (Table 1).

Predictors of 30-d readmission

On multivariate analysis, patients under the age of 40 years (OR: 1.39; CI: 1.19-1.64, *P*-value: < 0.001) and those between 40 and 64 (OR: 1.18; CI: 1.08 - 1.30, *P*-value: < 0.001) were more likely to be readmitted than patients ⩾ 65 years (Table 2). Other independent predictors of 30-d readmission included: Medicaid (OR: 1.20; CI: 1.08-1.33; *P*-value: < 0.001) or Medicare insurance (OR: 1.13; CI: 1.02-1.26; *P*-value <0.001) *vs* private insurance, > 3 comorbidities on the Elixhauser comorbidity scale (OR: 1.13; CI: 1.05-1.22; *P*-value: 0.001), nonalcoholic cirrhosis (OR: 1.16; CI: 1.10-1.23; *P*-value: < 0.001) and hepatocellular carcinoma (OR: 1.21, CI: 1.05-1.39; *P*-value: 0.010). Most importantly, a paracentesis during index admission was also an independent predictor of 30-d readmission (OR: 1.28;

Table 1 Index admission characteristics for adult patients with decompensated cirrhosis

	Overall *n* = 59597		No readmission within 30 d *n* = 41279		30-day readmission *n* = 18319		*P*-value
Age (mean, SE)	59.15	0.12	59.41	0.13	58.58	0.17	< 0.001
Age, yr							0.004
< 40	2636	4.42	1727	4.18	909	4.96	
40-64	38865	65.21	26798	64.92	12067	65.88	
⩾ 65	18096	30.36	12754	30.90	5342	29.16	
Gender							0.679
Male	36582	61.38	25302	61.30	11280	61.58	
Female	23015	38.62	15976	38.70	7039	38.42	
Type of insurance							< 0.001
Medicare	26282	44.18	18150	44.05	8132	44.48	
Medicaid	12784	21.49	8424	20.44	4360	23.85	
Private	11957	20.10	8383	20.34	3575	19.55	
Other	8465	14.23	6250	15.17	2216	12.12	
Income (Zip Code)							0.392
1-37999	18523	31.76	12722	31.49	5801	32.35	
38000-47999	16491	28.27	11394	28.20	5097	28.43	
48000-63999	13613	23.34	9542	23.62	4071	22.70	
64000+	9702	16.63	6740	16.68	2962	16.52	
AHRQ-Elixhauser Index							0.003
< 3	13981	23.46	9923	24.04	4058	22.15	
⩾ 3	45616	76.54	31356	75.96	14260	77.85	
Hospital size							0.646
Small	6345	10.65	4435	10.75	1910	10.43	
Medium	13725	23.03	9555	23.15	4169	22.76	
Large	39527	66.32	27288	66.11	12239	66.81	
Type of hospital							0.020
Urban non-teaching	22770	38.21	15896	38.51	6875	37.53	
Urban teaching	30504	51.18	20879	50.58	9625	52.54	
Rural	6322	10.61	4504	10.91	1819	9.93	
Etiology of cirrhosis							< 0.001
Alcoholic	34242	57.45	24072	58.32	10170	55.52	
Non-alcoholic	25356	42.55	17207	41.68	8149	44.48	
In-hospital procedures							
Paracentesis	29832	50.06	19914	48.24	9918	54.14	< 0.001
Features of liver decompensation							
Esophageal varices	272	0.46	201	0.49	71	0.39	0.313
Portal hypertension	22074	37.04	15264	36.98	6810	37.17	0.794
Hepatorenal syndrome	2734	4.59	1817	4.40	917	5.00	0.055
Hepatocellular carcinoma	2274	3.82	1471	3.56	803	4.38	0.002
Index admission mortality[1]							
None	59566	94.41	--	--	--	--	
Mortality	3526	5.59	--	--	--	--	
Calendar year mortality							< 0.001
None	53603	89.97	38960	94.42	14643	79.95	
Mortality	5978	10.03	2304	5.58	3673	20.05	
Length of stay (mean, SE)	5.69	0.08	5.66	0.10	5.77	0.10	0.345
Cost (mean, SE)	12488	363	12403	378	12680	421	0.391

[1]Out of a total of 63092 patients as the index admission mortality exclusion was not applied.

CI: 1.21-1.36; *P*-value: < 0.001) (Table 2).

Length of stay during index admission

The average length of stay during index admission was 5.69 ± 0.08 d. Length of stay was not significantly different between patients that were readmitted within 30 d (5.77 ± 0.10 d) and patients that were not (5.66 ± 0.10 d), *P*-value 0.34 (Table 1). Length of stay was also not an independent predictor of readmission on multivariate analysis (Table 2).

Cost of 30-d readmission and calendar year hospitalization

Cost of index hospitalization was similar (mean: $12403 ± 378 *vs* mean: $12680 ± 421, *P*-value = 0.391), however, cost of 30-d readmission (mean: $18120 ± 476) was higher than the cost of index admission (mean: $12403 ± 378), *P*-value: < 0.001). Cumulative total hospital cost for all admissions in calendar year was also significantly greater for patients readmitted within 30 d (mean: $51472 ± 1265) compared to patients not readmitted within 30 d (median: $23765 ± 595) (Figure 2).

DISCUSSION

In this study based on the Nationwide Readmission Database, approximately 1/3rd of patients with cirrhosis complicated by ascites and/or hepatic encephalopathy

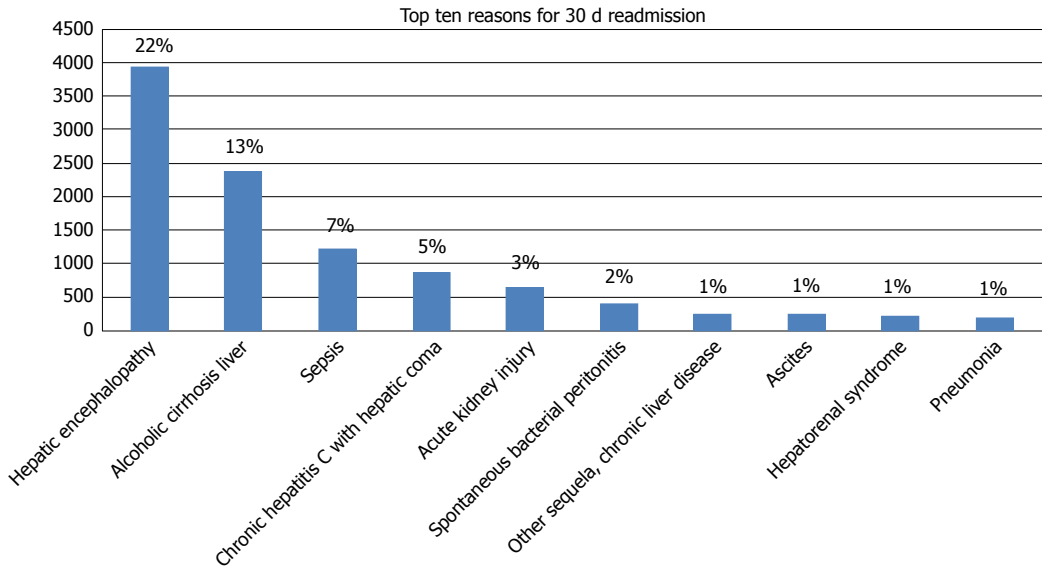

Figure 1 Top ten reasons for 30 d readmission in patients with cirrhosis and ascites.

Table 2 Multivariable logistic regression model for 30-d readmission

	OR (95%CI)		*P*-value
Age, yr			< 0.001
< 40	1.42	(1.22, 1.66)	
40-64	1.19	(1.09, 1.30)	
⩾ 65	Reference		
Type of insurance			< 0.001
Private	Reference		
Medicare	1.13	(1.02, 1.26)	
Medicaid	1.20	(1.08, 1.33)	
Other	0.83	(0.75, 0.92)	
AHRQ-elixhauser index			0.001
< 3	Reference		
⩾ 3	1.13	(1.05, 1.22)	
Etiology of cirrhosis			< 0.001
Alcoholic	Reference		
Non-alcoholic	1.16	(1.10, 1.23)	
Paracentesis	1.28	(1.21, 1.36)	< 0.001
Hepatocellular Carcinoma	1.21	(1.05, 1.39)	0.010

Terms included in the model were determined through stepwise selection where all variables shown in Table 1 (the full logistic regression model) were eligible for inclusion.

were readmitted within 30 d. Most patients were readmitted with liver related reasons. Half of the admitted patients underwent paracentesis. Independent predictors of 30-d readmission included younger age, Medicaid and Medicare insurance, encephalopathy cirrhosis, increased comorbidities, hepatocellular carcinoma and paracentesis during index admission. Patients that were readmitted within 30 d contributed to increased healthcare utilization. These predictors of 30-d readmission should be recognized in patients with decompensated cirrhosis and strategies designed to minimize readmissions as it has significant impact on healthcare utilization.

Diagnostic paracentesis to rule out SBP is part of quality indicators developed for the care of patients with cirrhosis admitted to the hospital with ascites and HE and is considered a safe procedure[16]. However, large volume paracentesis (LVP) is needed in certain situations for diagnostic and therapeutic purposes to relieve symptoms of tense ascites. Over the course of cirrhosis, patients may also require LVP when they develop diuretic refractory or diuretic resistant ascites, resulting in increased frequency of procedural intervention and rates of complication which could prompt readmission and increased cost[17]. Patients who undergo frequent LVP are subject to complications, such as post paracentesis circulatory dysfunction, which leads to faster re-accumulation of ascites, hyponatremia, renal impairment, and shorter survival. Given these complications, patients that undergo LVP would have higher rates of 30-d readmission and cost, which was noted in this study[18]. Despite these findings, it is important to note that performing a paracentesis is a quality indicator in cirrhotic patients with ascites or encephalopathy to evaluate for SBP; albeit paracentesis performance may be associated with increased 30-d readmission[10].

We identified that patients under the age of 64 were more likely to be readmitted within 30 d compared to older patients, which is consistent with previous studies[14]. This may seem counterintuitive, but reported in previous studies on patients with cirrhosis and in other chronic diseases, including chronic obstructive pulmonary disease (COPD)[19,20]. It is hypothesized that this is influenced by the "survivor effect" where younger patients admitted to the hospital typically have more severe disease compared to older patients[21]. Younger patients also tend to be better candidates for surgical intervention and the complications from these procedures may result in an increased risk of readmission[22].

Our results regarding Medicaid and Medicare as a predictor of 30-d readmission are in line with other studies showing a similar trend of increased readmission

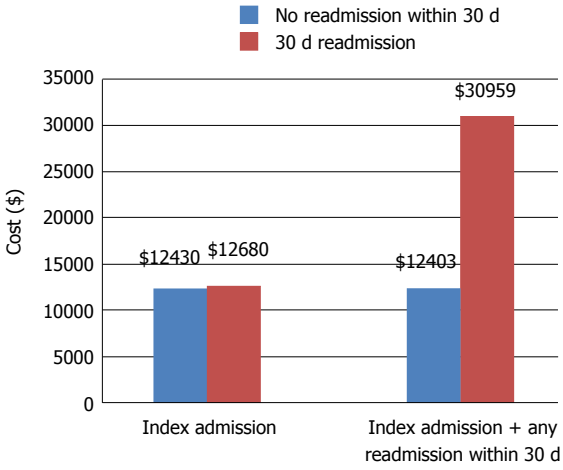

Figure 2 Cost of 30-d readmission was more than the cost of index admission in patients with cirrhosis and ascites.

rates in patients with government funded insurances[21]. Multiple reasons for early readmission in Medicaid population are proposed including inability to schedule prompt hospital follow up, poor compliance with follow up appointment due to lack of support person or transportation, *etc*[23].

Other factors associated with readmission in patients with cirrhosis and ascites included higher number of comorbid conditions. Patients with multiple, complex medical conditions are more likely to return to medical care as they usually belong to a lower socioeconomic scale and are in poor general health[24].

Unplanned readmission at 30 d in patients with cirrhosis and ascites places a large financial burden on the healthcare system. This study shows that an unplanned readmission within 30 d increases the cost of management by more than 100%. In fact, unplanned 30 d readmission cost almost double the cost of index admission and the majority of the expense in the calendar year, further emphasizing the need to focus on modifiable factors that are associated with readmission in order to reduce cost of care[25,26]. Hospital readmission have been proven to be more expensive in previous literature as many of these patients are readmitted for hospital acquired infections, complications from previous admissions or poor discharge planning[27].

Recognizing factors associated with readmission and increased cost is crucial in order to reduce subsequent readmissions, hospital costs, morality, and ultimately improve quality of life. While age is a non-modifiable risk factors and insurance provider is challenging to modify, these patients should be targeted for interventions that are proven to reduce readmission rates, including a call from healthcare provider, early outpatient follow up and providing patients with enough supply of medications prior to discharge[28,29]. Patients that undergo frequent paracentesis should be evaluated early for other interventions, such as a transjugular intrahepatic portosystemic shunt (TIPS) or liver transplantation in order to prevent frequent readmission and costs associated with frequent large volume paracentesis[30]. Arrangement of outpatient clinic or day unit paracentesis may also be helpful in avoiding readmission. Further interventions to reduce readmission rates should be researched in order to improve hospital outcomes in this vulnerable and complicated patient population.

Utilizing the NRD provides a major advantage when evaluating factors associated with readmission and long-term outcomes, as this database allows individual/unique patients to be followed longitudinally over the course of a calendar year. This cannot be performed with the Nationwide Inpatient Sample database. Limitations of this study must be kept in mind while reviewing the results; NRD is an administrative database, which is dependent on ICD-9 coding; however, the validity of these codes has been determined in previous studies. Ascites is influenced by other factors such as hypoalbuminemia, portal hypertension, HCC with portal vein thrombosis; however laboratory results cannot be determined from the NRD and other factors are subject to the accuracy of ICD-9 codes. In addition, the indication for paracentesis could not be determined and we are unable to differentiate between diagnostic and large volume paracentesis as both have similar ICD-9 codes. In our clinical experience, most patients undergo a large volume paracentesis at the time of a diagnostic paracentesis; therefore we assume most patients in this study had a LVP. Disease severity in NRD is dependent on coding accuracy for features of decompensation rather than MELD score or Child turcotte Pugh score which limits the accuracy in predicting disease severity. Given that this study is based on administrative nature of database, we were unable to determine the causality of paracentesis with 30 d and subsequent readmission. In addition, this study only evaluates patients during hospitalization therefore, outpatient mortality is not included in this study.

The prevalence of cirrhosis and its complications such as ascites, encephalopathy, and SBP are ever-increasing with a large economic burden in the United States. A significant part of the burden is related to increased readmission rates in this vulnerable patient population with projected 30-d readmission rate around 33%. Though a paracentesis is indicated in this group of patients to rule out SBP and for symptomatic relief, paracentesis was also associated with increased 30-d readmission and cost; therefore, strategies in this patient population to minimize 30-d readmission and unnecessary cost should be designed. Further research should be conducted to determine ways to reduce readmission rates and cost in this population.

ARTICLE HIGHLIGHTS

Research background

Patients with decompensated cirrhosis secondary to ascites or hepatic encephalopathy are at high risk of complication and readmission. Previous studies have determined that performing a paracentesis in these patients will improve inpatient mortality; however, the effect of performing a paracentesis on

30-d readmission has not been studied.

Research motivation

Given the economic burden of readmissions, we aimed to determine the readmission rate in patients with decompensated cirrhosis with ascites and encephalopathy. Identifying factors associated with readmission are crucial to preventing unnecessary hospital admission and healthcare spending.

Research objective

The objective for this study included determining 30-d readmission rate in patients with cirrhosis with ascites or encephalopathy, reasons for readmission, factors associated with readmission and cost of readmission.

Research methods

We performed a retrospective database analysis utilizing the Nationwide Readmission Database. All adult patients with a diagnosis of cirrhosis and ascites or encephalopathy were included. Multivariate analysis was performed to assess predictors of 30-d readmission and cost of readmission.

Research results

The 30 d readmission rate in patients with cirrhosis and ascites or encephalopathy was 31% and the majority of patients were readmitted for liver related issues (58%). Paracentesis was performed on 50% of patients during the index admission. Factors associated with readmission included age under 64, Medicaid or Medicare insurance provider, greater than 3 Elixhauser comorbidities, nonalcoholic cirrhosis, hepatocellular carcinoma and undergoing a paracentesis on index admission. Cost of index admission between patients that were readmitted within 30 d and those that were not readmitted were similar; however cost of care was significantly higher for the readmission compared to the index admission.

Research conclusion

This study determined the readmission rate and economic burden of 30-d readmission in patient with cirrhosis and ascites or encephalopathy. We also highlighted multiple factors associated with readmission, specifically undergoing a paracentesis that were associated with 30 d readmission. Modifying factors associated with readmission during index admission could reduce unplanned readmissions, decrease the economic burden associated with readmission and decrease patient morbidity and mortality.

Research perspectives

Further directions for this research include implementing intervention to modify factors associated with readmission in order to determine the effect on readmission, cost and patient mortality.

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SYSTEMATIC REVIEWS

Systematic review of the outcomes of surgical resection for intermediate and advanced Barcelona Clinic Liver Cancer stage hepatocellular carcinoma: A critical appraisal of the evidence

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Author contributions: Koh YX, Tan HL, Lye WK, Kam JH, Chiow AKH, Tan SS, Choo SP, Chung AYF and Goh BKP were all critically involved in the design of the study, article review, manuscript drafting and final approval of the version to be published; all authors are in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Abstract

AIM

To perform a systematic review to determine the survival outcomes after curative resection of intermediate and advanced hepatocellular carcinomas (HCC).

METHODS

A systematic review of the published literature was performed using the PubMed database from 1st January 1999 to 31st Dec 2014 to identify studies that reported outcomes of liver resection as the primary curative treatment for Barcelona Clinic Liver Cancer (BCLC) stage B or C HCC. The primary end point was to determine the overall survival (OS) and disease free survival (DFS) of liver resection of HCC in BCLC stage B or C in patients with adequate liver reserve (*i.e.*, Child's A or B status). The secondary end points were to assess the morbidity and mortality of liver resection in large HCC (defined as lesions larger than 10 cm in diameter) and to compare the OS and DFS after surgical resection of solitary *vs* multifocal HCC.

RESULTS

We identified 74 articles which met the inclusion criteria and were analyzed in this systematic review. Analysis of the resection outcomes of the included studies were grouped according to (1) BCLC stage B or C HCC, (2) Size of HCC and (3) multifocal tumors. The median 5-year OS of BCLC stage B was 38.7% (range 10.0-57.0); while the median 5-year OS of BCLC stage C was 20.0% (range 0.0-42.0). The collective median 5-year OS of both stages was 27.9% (0.0-57.0). In examining the morbidity and mortality following liver resection in large HCC, the pooled RR for morbidity [RR (95%CI) = 1.00 (0.76-1.31)] and mortality [RR (95%CI) = 1.15 (0.73-1.80)] were not significant. Within the spectrum of BCLC B and C lesions, tumors greater than 10 cm were reported to have median 5-year OS of 33.0% and multifocal lesions 54.0%.

CONCLUSION

Indication for surgical resection should be extended to BCLC stage B lesions in selected patients. Further studies are needed to stratify stage C lesions for resection.

Key words: Barcelona Clinic Liver Cancer; Hepatocellular carcinoma; Hepatectomy; Milan criteria

Core tip: This is a systematic review of the current literature reporting the surgical outcomes of liver resection for Barcelona Clinic Liver Cancer (BCLC) Stage B and C hepatocellular carcinomas (HCC). Based on this review, there is robust evidence that indications for primary surgical resection of HCC should be extended to include BCLC stage B lesions in selected patients. There is a need for further studies that stratify BCLC stage C lesions and potentially extend surgical indications for resectable lesions.

Koh YX, Tan HL, Lye WK, Kam JH, Chiow AKH, Tan SS, Choo SP, Chung AYF, Goh BKP. Systematic review of the outcomes of surgical resection for intermediate and advanced Barcelona Clinic Liver Cancer stage hepatocellular carcinoma: A critical appraisal of the evidence. *World J Hepatol* 2018; 10(6): 433-447 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i6/433.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i6.433

INTRODUCTION

Hepatocellular carcinoma (HCC) remains a significant disease burden worldwide today[1]. Appropriate treatment for HCC is complex because radical oncological clearance and preservation of adequate liver function need to be carefully balanced. Several staging systems have been developed to guide management of HCC[2-7].

Surgical resection for HCC within the "Milan Criteria" or Barcelona Clinic Liver Cancer (BCLC) stage A is the widely accepted standard of care[8]. However, surgical treatment for BCLC stage B (intermediate) or C (advanced) lesions remains controversial[4-7]. Presently, the European Association for the Study of Liver Disease (EASL) and the American Association for the Study of Liver Disease (AASLD) guidelines do not recommend surgical resection for these patients[4,7,9].

However, despite the recommendations from these two large reputable organizations, many international high-volume tertiary centers, especially centers in Asia, still routinely perform surgical resection for large solitary lesions, multifocal lesions and lesions with macrovascular invasion[10-16]. Critical appraisal of both Western and Asian literature is needed to resolve the controversies.

The aim of this study was to perform a systematic review and summarize the current literature to determine the long-term survival outcomes after curative resection of intermediate and advanced HCCs.

MATERIALS AND METHODS

A systematic review of the published literature was performed using the PubMed database from 1st January 1999 to 31st Dec 2014 to identify studies that reported outcomes of liver resection as the primary curative treatment for BCLC stage B or C HCC.

The primary end point was to determine the overall survival (OS) and disease free survival (DFS) of liver resection of HCC in BCLC stage B or C in patients with adequate liver reserve (*i.e.*, Child's A or B status) and in good general status (PS 0-2). The secondary end points were to assess the morbidity and mortality of liver resection in large HCC (defined as lesions larger than 10 cm in diameter) and to compare the OS and DFS after surgical resection of solitary *vs* multifocal HCC.

The Medical Subject Heading (MeSH) major topic was "hepatocellular carcinoma". The keywords used were "liver tumor", "hepatoma", "liver neoplasm", "liver cancer", "Barcelona Clinic Liver Cancer", "multifocal" and "vascular invasion". The keywords used for surgical resection were "hepatectomy", "liver resection", "liver

surgery", "partial hepatectomy", "hemi-hepatectomy", "sectionectomy", "segmentectomy", "non-anatomical resection", "anatomical resection", "curative surgery" and "surgical procedures". The keywords used for liver reserve were "Child A/B", "Child Pugh A/B", "early liver disease" and "early liver cirrhosis". Key references of the short-listed studies were also searched manually.

Two authors conducted the search independently, with the search results obtained by both authors discussed with the senior author Goh BK. The final list of studies to be short-listed was decided by consensus between all three authors. This study was conducted in accordance to the PRISMA guidelines[17].

Data extraction

All short-listed studies were assessed independently according to a modified Newcastle-Ottawa scale. The three main factors assessed were: (1) selection of the patients; (2) comparability of the study groups; and (3) outcome assessment. The scoring scale ranged from 0-9 and studies of score 6 or greater were considered high quality and included in this study. The following data was extracted from the included studies: first author, year of data collection, year of publication, country of origin, characteristics of study population, number of patients, clinico-pathological characteristics, OS and DFS.

Inclusion criteria

The inclusion criteria were: (1) studies reporting surgical resection of lesions fulfilling the criteria of BCLC stage B (intermediate) or BCLC stage C (advanced) HCC, studies reporting surgical resection for large HCC, multifocal HCC and HCC with vascular invasion; (2) evaluation of at least one of the clinico-pathological or survival characteristics mentioned in the "parameters and outcomes of interest" section below; and (3) for studies reported by the same institution (and/or) authors with overlapping cohorts, only the study with the larger sample size or the one with higher quality was included. Major resection was defined as resection of 3 segments or more whereas minor resection involved 2 segments or less[18].

Studies which described adjunctive treatments such as radiofrequency ablation (RFA), selective internal radiation therapy (SIRT), trans- arterial chemoembolization (TACE) and infusional chemotherapy were also included.

Exclusion criteria

All studies that did not meet the inclusion criteria were excluded. In addition, the following exclusion criteria were used: (1) studies that did not report the survival outcomes of surgically resected HCC; (2) studies that focused on transplant, RFA, TACE and SIRT; (3) studies that focused on DNA, biochemical and proteomic analysis of HCC; (4) studies that focused on radiological imaging techniques; (5) studies reporting patients with Child-Pugh grade C or unknown status; (6) studies reporting tumor rupture, extra-hepatic metastases and/or lymph node metastases; (7) studies which included palliative (R2) resections; and (8) studies written in languages other than English.

Definitions, parameters and outcomes of interest

The most updated BCLC staging criteria was used as the reference staging system[4,7,9]. Adequate liver function was defined as Child-Pugh grade A or B. The main outcomes of interest were the OS and DFS. Clinico-pathological characteristics including age, gender, Child-Pugh status, hepatitis status, tumor size, number of nodules, extent of macrovascular invasion, extent of liver resection, post-operative morbidity, mortality and recurrent disease were recorded.

Statistical methods

If the data on the OS or DFS was not provided explicitly in a study, the information was derived from the survival graphs if present, or calculated from the primary data using a measurement method as described by Lim *et al*[8]. The 1, 3 and 5 year OS and DFS were summarized graphically using bubble plots, with the sample size of each cohort relative to the size of the bubble.

The inverse variance (IV) method was used to pool the RR across studies. A fixed 0.5 zero-cell correction was used when the number of events for one of the groups was zero. Pairwise comparisons of subtypes were done. If there were no events in an outcome of interest for both groups that were compared, the study was excluded from the meta-analysis for the specific outcome.

Heterogeneity between the studies was evaluated using the chi-squared test of heterogeneity. A random effects model was used. Sensitivity analyses were performed by excluding each study individually from the pool of studies combined for each outcome. Pooled results from these subgroups were computed and compared with the pooled results from the set of studies without these exclusion criteria. All statistical analyses were conducted using SAS 9.3 (SAS Institute, Cary, NC, United States) and Review Manager 5 (Nordic Cochrane, Copenhagen, Denmark).

RESULTS

The systematic review identified 1908 articles, from which 130 articles were selected for full text review. Seventy-four articles met the inclusion criteria and were analyzed in this systematic review[10-14,19-87]. Fifty-six articles were excluded for the following reasons[88-143]: Three because they were not published in the English language[88-90], 11 because other treatment modalities were used as primary treatment[91-101], 19 because of overlapping cohorts[102-120], nine due to incomplete data[121-129], two due to inclusion of palliative liver resection[130-131], and 12 because the study populations included patients with other types of hepatic malignancies[132-143] (Supplementary Figure 1). Analysis of the resection outcomes of the included studies were

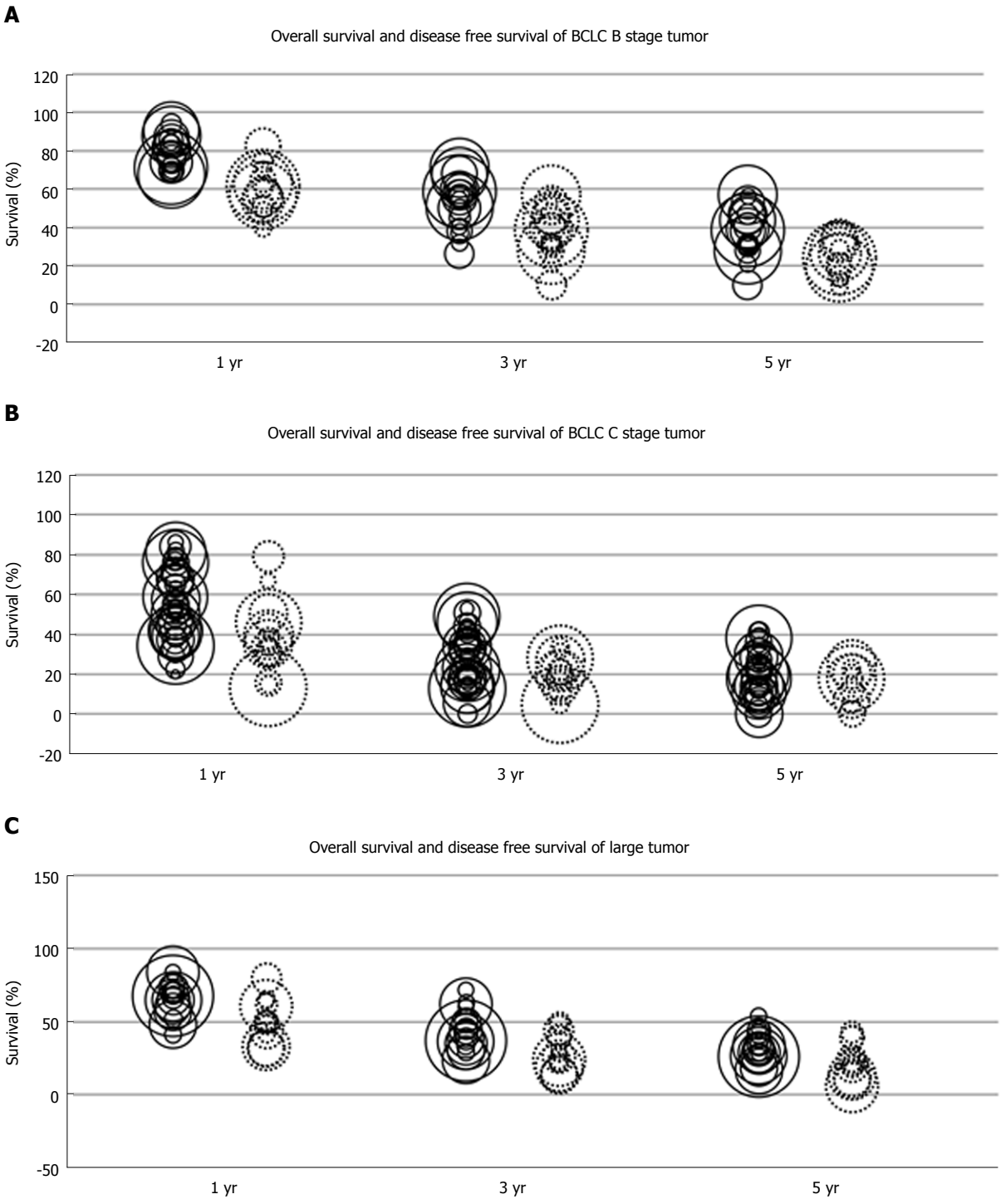

Figure 1 Bubble plot of overall survival and disease-free survival of BCLC B, C and large tumors.

grouped according to (1) BCLC stage B or C HCC; (2) Size of HCC; and (3) multifocal tumors.

BCLC Stage B or C HCC

Studies which classified HCC according to BCLC Staging System[4-7] utilized the following common definitions: Stage B - single tumor more than 5 cm in diameter; 2 to 3 tumors of which at least one is more than 3 cm in diameter; or more than 3 tumors of any diameter; Stage C - any tumor with radiologically evident and histologically proven macrovascular invasion, N1 disease or M1 disease.

The baseline characteristics of the patients are presented in Table 1. There are 6103 BCLC stage B cases in 19 studies and 3449 BCLC stage C cases in 32 studies. The clinical outcomes are summarized in Table 2. The study recruitment periods extended from 1982 to 2011. Figure 1 are bubble plots showing OS and DFS, with bubble size indicating relative sample size. The median 5-year OS of BCLC stage B was

38.7% (range 10.0-57.0); while the median 5-year OS of BCLC stage C was 20.0% (range 0.0-42.0). The collective median 5-year OS of both stages was 27.9% (0.0-57.0).

Size of HCC

HCC analyzed according to size criterion were categorized as: Large HCC - greater than or equal to 10 cm in diameter; and Small HCC - less than 10 cm in diameter.

The baseline characteristics are presented in Table 3. There are 2437 cases of large HCC in 21 studies and 5436 cases of small HCC in 14 studies. The clinical outcomes are summarized in Table 4. The study recruitment periods extended from 1964 to 2011. Supplementary Figure 2A and B are forest plots showing the morbidity and mortality respectively of the included studies. The pooled RR for morbidity [RR (95%CI): 1.00 (0.76-1.31)] and mortality [RR (95%CI): 1.15 (0.73-1.80)] were not significant. The median 5-year OS of large HCC was 33.0 % (range: 16.7-79.0) and the median 5-year OS of small HCC was 52.3% (range 21.0-89.2).

Multifocal HCC

The baseline characteristics are presented in Supplementary Table 1. There are 1095 cases in 9 studies. The clinical outcomes are summarized in Supplementary Table 2. The study recruitment periods extended from 1992 to 2011. Supplementary Figure 3 displays the bubble plot showing overall and disease free survival rate of the included studies, with bubble size indicating relative sample size. The median 5-year OS was 54.0% (29.9-75.5). Supplementary Figure 4 was plotted to show 5 year OS for those studies with sample size greater or equal to 100 against the midpoint of recruitment period. The trend line was fitted using weighted least squares regression with sample size as weight. An uptrend with weighted slope 0.38 was seen in the plot.

DISCUSSION

BCLC stage B or C HCC

Presently, a major controversy in the management of HCC is the role of surgical resection for intermediate (BCLC stage B) and advanced (BCLC stage C) stage HCC. According to EASL and the AASLD guidelines, surgical resection is not offered for BCLC stages B and C HCC because of the poor 5-year overall survival rates[7,9].

However, the results of this systematic review demonstrate that the median 5 year OS after surgical resection for BCLC B is 38.9% (range: 10.0%-57.0%). These outcomes are clearly not attainable by other modalities such as TACE which only confers a 40% two year survival and median survival of 20 mo for similar lesions[144].

This systematic review demonstrates that for BCLC C lesions, surgical resection results in uniformly poor results with a median 5-year OS of 20% (range: 0%-42.0%). However, proponents of resection argue that the tumor thrombus has the potential to cause portal vein obstruction, intractable ascites, esophageal variceal bleeding and liver failure[66-68]. This frequently leads to an even more rapid demise of these patients.

In general, the prognosis for tumor thrombus located within the main trunk has been reported to be poorer as compared to more distal lesions[58,62,68]. Some authors have advocated for portal vein resection for 1st order portal vein tumor thrombus with minimal bifurcation involvement, citing results of 5-year OS over 20% which was superior to thrombectomy alone[53,75]. However, statistical analysis could not be performed due lack of stratification based on the extent of PV invasion, heterogeneous surgical procedures and different extents of hepatic vein and inferior vena cava involvement.

Size of HCC

The median 5 year OS in these large lesions was 33% (range: 16.7%-79.0%). The 10 cm arbitrary cut-off used by many studies represents the more advanced cases in the spectrum of BCLC B HCC[32-43]. In addition, BCLC C lesions that were > 10 cm, usually with worse prognosis, were not excluded from the analysis in these studies, confounding the results. Despite this, the relatively favorable survival still indicates that surgical resection is beneficial for selected lesions within the combined spectrum of large HCCs[32-43].

Multifocal HCC

The EASL guidelines do not recommend surgical resection as first line therapy for all multifocal lesions[4,7,9]. On the other end of the spectrum, the APASL guidelines support resection of all lesions regardless of multifocality[2]. The APASL guidelines are supported by the fact that that over 65% OS has been reported after surgical resection for multifocal HCC within the "Milan criteria"[8]. In addition, studies from large specialized liver centers have showed favorable 5-year OS of over 50% after surgical resection for multifocal HCC[13,77,82]. This was further improved to between 55%-75% 5-year OS when performed in combination with RFA for bilobar lesions[76,78,80]. In this review, the median 5-year OS for all surgically resected multifocal HCC analyzed in this systematic review is 54.0% (range: 29.9%-75.5%), supporting surgical resection as the primary management of multifocal HCC.

It is important to highlight that surgical series of the aforementioned groups represent the entire spectrum of tumors beyond the "Milan criteria", and the wide range of survival reported for these lesions can be attributed to the heterogeneity of tumors encompassing large solitary and multifocal lesions of various sizes. Differentiation of the outcomes of purely single or multifocal HCC within this heterogeneous selection and is often not pursued in many studies and making interpretation of the data difficult.

Based on this review, it is evident that arbitrary classifications by the current guidelines do not adequately

Table 1 Characteristics of patients classified as BCLC stage B or C hepatocellular carcinoma

Ref.	Year	*n*	Male (%)	Cirrhosis (%)	HBV (%)	HCV (%)	Median tumor diameter (cm)
BCLC stage B							
Régimbeau *et al*[25]	1999	94	75 (79.8)	37 (39.4)	35 (37.2)	10 (10.6)	12.0
Hanazaki *et al*[19]	2001	133	105 (78.9)	NS	NS	NS	8.6
Ng *et al*[10]	2005	380	278 (73.2)	380 (100.0)	281 (73.9)	20 (5.3)	NS
Chen *et al*[26] (TP1)	2006	959	816 (85.1)	717 (74.8)	776 (80.9)	NS	14.9
Chen *et al*[26] (TP2)	2006	1143	968 (84.7)	897 (78.5)	940 (82.2)	NS	11.1
Cho *et al*[26]	2007	230	46 (20.0)	35 (15.2)	40 (17.4)	5 (2.2)	7.1
Vitale *et al*[31]	2009	124	NS	NS	NS	NS	NS
Yang *et al*[11]	2009	260	228 (87.7)	198 (76.2)	239 (91.9)	NS	9.6
Zhou *et al*[29] (SX)	2009	56	49 (87.5)	50 (89.3)	55 (98.2)	0 (0.0)	9.5
Zhou *et al*[29] (TCSX)	2009	52	48 (92.3)	49 (94.2)	51 (98.1)	0 (0.0)	9.0
Delis *et al*[27]	2010	66	45 (68.2)	NS	36 (54.5)	15 (22.7)	8.4
Lin *et al*[30]	2010	93	75 (80.6)	NS	60 (64.5)	22 (23.7)	8.0
Ramacciato *et al*[28]	2010	51	37 (72.5)	44 (86.3)	NS	NS	8.2
Xu *et al*[21]	2010	165	NS	NS	NS	NS	NS
Wei *et al*[22]	2011	51	NS	NS	NS	NS	NS
Zhou *et al*[20]	2011	85	74 (87.1)	65 (76.5)	68 (80.0)	6 (7.1)	NS
Chang *et al*[32]	2012	318	263 (82.7)	97 (30.5)	201 (63.2)	57 (17.9)	7.4
Hsu *et al*[34]	2012	268	213 (79.5)	NS	176 (65.7)	48 (17.9)	NS
Ma *et al*[23]	2012	178	158 (88.8)	79 (44.4)	140 (78.7)	41 (23.0)	NS
Torzilli *et al*[12]	2013	737	586 (79.5)	360 (48.8)	158 (21.4)	208 (28.2)	6.0
Zhong *et al*[120]	2013	660	NS	NS	NS	NS	NS
BCLC Stage C							
Ohkubo *et al*[79]	2000	47	41 (87.2)	NS	20 (42.6)	11 (23.4)	NS
Wu *et al*[57] (SX 1st bifurcation)	2000	15	13 (86.7)	NS	14 (93.3)	2 (13.3)	10.8
Wu *et al*[57] (SX 1st)	2000	97	83 (85.6)	NS	67 (69.1)	25 (25.8)	8.8
Minagawa *et al*[58]	2001	18	NS	NS	NS	NS	5.3
Poon *et al*[59]	2003	20	18 (90.0)	NS	17 (85.0)	NS	8.6
Fan *et al*[60] (SX, CHT)	2005	84	76 (90.5)	NS	NS	NS	10.5
Fan *et al*[60] (SX)	2005	24	20 (83.3)	NS	NS	NS	NS
Pawlik *et al*[10]	2005	102	87 (85.3)	NS	NS	NS	10
Chen *et al*[62] (SX 1st)	2006	286	248 (86.7)	NS	172 (60.1)	NS	7.7
Chen *et al*[62] (SX Main)	2006	152	135 (88.8)	NS	95 (62.5)	NS	8.1
Ikai *et al*[64]	2006	78	57 (73.1)	NS	24 (30.8)	36 (46.2)	NS
Le Treut *et al*[63]	2006	26	22 (84.6)	NS	NS	NS	9
Kamiyama *et al*[66] (RTSX)	2007	15	13 (86.7)	NS	NS	NS	6.47
Kamiyama *et al*[66] (SX)	2007	28	25 (89.3)	NS	NS	NS	11
Takizawa *et al*[65]	2007	12	8 (66.7)	NS	NS	NS	8.24
Ban *et al*[69]	2009	45	NS	NS	NS	NS	NS
Inoue *et al*[70] (TB)	2009	20	19 (95.0)	NS	6 (30.0)	12 (60.0)	NS
Inoue *et al*[70] (EN)	2009	29	26 (89.7)	NS	10 (34.5)	15 (51.7)	NS
Kondo *et al*[68] (SX, Main)	2009	5	NS	NS	NS	NS	NS
Kondo *et al*[68] (SX, 1st-3rd)	2009	43	NS	NS	NS	NS	NS
Peng *et al*[67] (TC)	2009	51	46 (90.2)	NS	31 (60.8)	5 (9.8)	9.04
Peng *et al*[67] (SX)	2009	53	50 (94.3)	NS	40 (75.5)	3 (5.7)	8.39
Vitale *et al*[31]	2009	48	NS	NS	NS	NS	NS
Shi *et al*[71]	2010	406	361 (88.9)	NS	354 (87.2)	3 (0.7)	NS
Xu *et al*[21]	2010	95	NS	NS	NS	NS	NS
Lin *et al*[72] (TP1)	2011	21	NS	NS	NS	NS	NS
Lin *et al*[72] (TP2)	2011	47	NS	NS	NS	NS	NS
Peng *et al*[14]	2011	201	187 (93.0)	NS	172 (85.6)	4 (2.0)	NS
Wei *et al*[22]	2011	17	NS	NS	NS	NS	NS
Chang *et al*[32]	2012	160	140 (87.5)	60 (37.5)	112 (70.0)	20 (12.5)	7.5
Huang *et al*[56] (SX)	2012	54	40 (74.1)	NS	41 (75.9)	2 (3.7)	21.4
Huang *et al*[56] (SXTC)	2012	62	42 (67.7)	NS	50 (80.6)	0 (0.0)	20.5
Liu *et al*[74]	2012	65	54 (83.1)	NS	NS	NS	NS
Ma *et al*[23]	2012	46	41 (89.1)	25 (54.3)	41 (89.1)	0 (0.0)	NS
Li *et al*[75]	2013	13	11 (84.6)	NS	NS	NS	10.2
Nitta *et al*[77]	2013	35	28 (80.0)	NS	7 (20.0)	21 (60.0)	7
Roayaie *et al*[78]	2013	164	132 (80.5)	NS	61 (37.2)	70 (42.7)	90
Tang *et al*[76]	2013	186	166 (89.2)	NS	159 (85.5)	23 (12.4)	9.53
Torzilli *et al*[12]	2013	297	228 (76.8)	169 (56.9)	61 (20.5)	100 (33.7)	6.0
Zhong *et al*[120]	2013	248	NS	NS	NS	NS	NS

TP: Time period; SX: Surgery; TC: Trans- arterial chemoembolization; CHT: Chemotherapy; TB: Thrombectomy; EN: *En.bloc*, RT: Radiotherapy.

Table 2 Clinical outcomes of liver resection in BCLC stage B or C hepatocellular carcinoma

Ref.	Recruitment period	*n*	Overall survival (%)			Median OS (mo)	Disease free survival (%)			Median DFS (mo)
			1-yr	3-yr	5-yr		1-yr	3-yr	5-yr	
BCLC stage B										
Régimbeau *et al*[25]	1984-1996	94	69.0	45.0	31.0	NS	51.0	35.0	21.0	NS
Hanazaki *et al*[19]	1983-1997	133	70.0	38.0	28.0	NS	65.0	26.0	20.0	NS
Ng *et al*[10]	1982-2001	380	74.0	50.0	39.0	36.9	54.0	38.0	26.0	15.6
Chen *et al*[26] (TP1)	1990-2003	959	67.8	50.7	27.9	16.0	56.5	34.7	18.9	10.0
Chen *et al*[26] (TP2)	1990-2003	1143	71.2	58.8	38.7	19.0	61.5	38.6	23.8	17.0
Cho *et al*[26]	1998-2001	230	85.0	59.3	52.9	NS	58.3	40.0	31.7	NS
Vitale *et al*[31]	2000-2007	124	85.0	56.0	NS	NS	NS	NS	NS	NS
Yang *et al*[11]	1992-2002	260	87.0	55.5	38.2	45.5	82.4	51.0	35.0	36.7
Zhou *et al*[29] (SX)	2001-2003	56	69.6	32.1	21.1	NS	39.2	21.4	8.9	NS
Zhou *et al*[29] (TCSX)	2001-2003	52	73.1	40.4	30.7	NS	48.9	25.5	12.8	NS
Delis *et al*[27]	2002-2008	66	69.0	37.0	32.0	36.0	60.0	33.0	29.0	29.0
Lin *et al*[30]	2001-2007	93	83.0	49.0	30.0	27.6	NS	NS	NS	NS
Ramacciato *et al*[28]	2000-2006	51	NS	NS	56.1	68.0	NS	NS	41.3	NS
Xu *et al*[21]	1991-2004	165	75.6	57.4	40.2	NS	NS	NS	NS	NS
Wei *et al*[22]	2003-2007	51	84.3	54.9	NS	NS	70.2	45.4	NS	NS
Zhou *et al*[20]	1995-2002	85	93.8	56.2	47.0	56.0	74.3	34.4	14.8	36.0
Chang *et al*[32]	1991-2006	318	81.2	59.4	46.5	NS	55.8	39.4	31.9	6.0
Hsu *et al*[34]	2002-2010	268	82.0	68.0	46.0	NS	NS	NS	NS	NS
Ma *et al*[23]	1998-2011	178	77.0	26.0	10.0	27.9	49.0	18.0	NS	16.8
Torzilli *et al*[12]	1990-2009	737	88.0	71.0	57.0	NS	63.0	38.0	27.0	NS
Zhong *et al*[120]	2000-2007	660	91.0	67.0	44.0	NS	NS	NS	NS	NS
BCLC stage C										
Ohkubo *et al*[79]	1985-1997	47	53.9	33.2	23.9	NS	31.2	17.9	NS	NS
Wu *et al*[57] (SX 1st bifurcation)	1990-1998	15	80.0	44.0	26.4	NS	67.0	32.0	21.1	NS
Wu *et al*[57] (SX 1st)	1990-1998	97	68.0	34.0	28.5	NS	51.0	22.0	20.4	NS
Minagawa *et al*[58]	1989-1998	18	82.0	42.0	42.0	40.8	NS	NS	NS	7.8
Poon *et al*[59]	1989-2000	20	30.0	13.3	13.3	6.0	15.0	5.0	5.0	2.9
Fan *et al*[60] (SX, CHT)	1997-2002	84	29.3	15.6	NS	15.1	NS	NS	NS	NS
Fan *et al*[60] (SX)	1997-2002	24	22.7	0.0	NS	10.1	NS	NS	NS	NS
Pawlik *et al*[10]	1984-1999	102	45.0	17.0	10.0	11.0	NS	NS	NS	NS
Chen *et al*[62] (SX 1st)	1990-2003	286	58.7	22.7	18.1	18.8	NS	NS	NS	NS
Chen *et al*[62] (SX Main)	1990-2003	152	39.5	5.7	0.0	10.1	NS	NS	NS	NS
Ikai *et al*[64]	1990-2002	78	45.7	21.7	10.9	8.9	NS	NS	NS	NS
Le Treut *et al*[63]	1988-2004	26	38.5	20.0	13.0	9.0	NS	NS	NS	NS
Kamiyama *et al*[66] (RTSX)	1990-2006	15	86.2	43.5	34.8	19.6	NS	NS	NS	NS
Kamiyama *et al*[66] (SX)	1990-2006	28	39.0	13.1	13.1	9.1	NS	NS	NS	NS
Takizawa *et al*[65]	1992-2003	12	63.6	53.0	26.0	26.0	NS	NS	NS	NS
Ban *et al*[69]	1992-2008	45	69.6	37.4	22.4	20.0	30.4	21.2	0.0	NS
Inoue *et al*[70] (TB)	1995-2006	20	58.0	46.0	39.0	NS	34.0	34.0	23.0	NS
Inoue *et al*[70] (EN)	1995-2006	29	65.0	41.0	41.0	NS	38.0	22.0	18.0	NS
Kondo *et al*[68] (SX, Main)	1996-2004	5	20.0	NS	NS	NS	NS	NS	NS	NS
Kondo *et al*[68] (SX, 1st-3rd)	1996-2004	43	54.0	33.0	27.0	NS	NS	NS	NS	NS
Peng *et al*[67] (TC)	1996-2004	51	50.9	33.8	21.6	13.0	NS	NS	NS	NS
Peng *et al*[67] (SX)	1996-2004	53	33.3	17.0	8.5	9.0	NS	NS	NS	NS
Vitale *et al*[31]	2000-2007	48	55.0	44.0	0.0	NS	NS	NS	NS	NS
Shi *et al*[71]	2001-2003	406	34.4	13.0	NS	NS	13.3	4.7	NS	NS
Xu *et al*[21]	1991-2004	95	37.5	18.2	14.2	NS	NS	NS	NS	NS
Lin *et al*[72] (TP1)	1996-2006	21	77.0	19.0	5.0	21.0	NS	NS	NS	NS
Lin *et al*[72] (TP2)	1996-2006	47	76.0	51.0	36.0	36.0	NS	NS	NS	NS
Peng *et al*[14]	2002-2007	201	42.0	14.1	11.1	20.0	NS	NS	NS	NS
Wei *et al*[22]	2003-2007	17	52.9	29.4	NS	NS	35.2	17.6	NS	NS
Chang *et al*[32]	1990-2009	34	45.0	20.0	20.0	NS	NS	NS	NS	NS
Huang *et al*[56] (SX)	1991-2006	160	57.6	33.8	29.1	NS	35.3	27.2	25.0	NS
Huang *et al*[56] (SXTC)	1998-2008	54	71.0	35.0	11.0	NS	NS	NS	NS	NS
Liu *et al*[74]	1998-2008	62	71.0	24.0	6.0	NS	NS	NS	NS	NS
Ma *et al*[23]	2000-2009	65	84.0	NS	NS	17	79.0	NS	NS	14.0
Li *et al*[75]	1998-2011	46	37.0	16.0	NS	16.9	16.0	NS	NS	7.7
Nitta *et al*[77]	1997-2009	13	53.8	15.4	NS	NS	NS	NS	NS	NS
Roayaie *et al*[78]	2006-2008	35	78.0	37.4	32.7	NS	45.0	11.8	11.8	NS
Tang *et al*[76]	1992-2010	164	50.0	23.0	14	13.1	40.0	20.0	18.0	8.1
Torzilli *et al*[12]	2006-2008	186	40.1	13.6	NS	10.0	NS	NS	NS	NS
Zhong *et al*[120]	1990-2009	297	76.0	49.0	38.0	NS	46.0	28.0	18.0	NS
Ohkubo *et al*[79]	2000-2007	248	81.0	46.0	20.0	NS	NS	NS	NS	NS

TP: Time period; SX: Surgery; TC: Trans- arterial chemoembolization; CHT: Chemotherapy; TB: Thrombectomy; EN: *En.bloc*, RT: Radiotherapy.

Table 3 Characteristics of patients classified as large or small hepatocellular carcinoma

Ref.	Year	*n*	Male (%)	Cirrhosis (%)	HBV (%)	HCV (%)	Median tumor diameter (cm)
Large HCC							
Poon *et al*[36]	2002	120	99 (82.5)	32 (26.7)	103 (85.8)	NS	13.8
Yeh *et al*[38]	2003	211	164 (77.7)	63 (29.9)	163 (77.3)	16 (7.6)	13.9
Zhou *et al*[37]	2003	621	NS	NS	NS	NS	NS
Liau *et al*[41]	2005	82	48 (58.5)	8 (9.8)	NS	NS	14.7
Nagano *et al*[40]	2005	26	19 (73.1)	5 (19.2)	14 (53.8)	3 (11.5)	14.8
Pawlik *et al*[10]	2005	300	222 (74.0)	NS	188 (62.7)	NS	NS
Lee *et al*[43]	2007	100	77 (77.0)	NS	NS	NS	12.5
Pandey *et al*[44]	2007	166	143 (86.1)	80 (48.2)	130 (78.3)	2 (1.2)	13.0
Shah *et al*[42]	2007	24	NS	NS	9 (37.5)	1 (4.2)	13.1
Young *et al*[45]	2007	42	29 (69.0)	2 (4.8)	NS	NS	14.0
Shimada *et al*[46]	2008	85	72 (84.7)	NS	27 (31.8)	19 (22.4)	12.0
Taniai *et al*[47]	2008	29	26 (89.7)	12 (41.4)	6 (20.7)	17 (58.6)	13.5
Choi *et al*[50]	2009	50	34 (68.0)	13 (26.0)	33 (66.0)	1 (2.0)	NS
Miyoshi *et al*[49]	2009	22	19 (86.4)	5 (22.7)	NS	NS	12.0
Ng *et al*[48]	2009	44	33 (75.0)	NS	15 (34.1)	3 (6.8)	12.4
Yamashita *et al*[51]	2011	53	48 (90.6)	NS	18 (34.0)	22 (41.5)	13.2
Truant *et al*[35]	2012	52	38 (73.1)	23 (44.2)	6 (11.5)	NS	14.0
Allemann *et al*[55]	2013	22	NS	9 (40.9)	4 (18.2)	2 (9.1)	13.5
Ariizumi *et al*[54]	2013	107	NS	NS	NS	NS	NS
Shrager *et al*[52]	2013	130	98 (75.4)	NS	56 (43.1)	23 (17.7)	14.2
Yang *et al*[53]	2013	258	212 (82.2)	171 (66.3)	195 (75.6)	NS	13.2
Small HCC							
Miyoshi *et al*[49]	2009	230	160 (69.6)	114 (49.6)	NS	NS	3.4
Allemann *et al*[55]	2013	79	NS	61 (77.2)	10 (12.7)	13 (16.5)	4.9
Poon *et al*[36]	2002	368	295 (80.2)	203 (55.2)	311 (84.5)	NS	5.4
Yeh *et al*[38]	2003	778	776 (99.7)	591 (76.0)	616 (79.2)	305 (39.2)	4.5
Zhou *et al*[37]	2003	2039	NS	NS	NS	NS	NS
Liau *et al*[41]	2005	111	80 (72.1)	40 (36.0)	NS	NS	6.1
Nagano *et al*[40]	2005	143	112 (78.3)	81 (56.6)	17 (11.9)	87 (60.8)	3.3
Shah *et al*[42]	2007	165	NS	NS	73 (44.2)	36 (21.8)	4.7
Young *et al*[45]	2007	43	30 (69.8)	10 (23.3)	NS	NS	5.0
Taniai *et al*[47]	2008	291	225 (77.3)	156 (53.6)	135 (46.4)	78 (26.8)	3.7
Choi *et al*[50]	2009	447	344 (77.0)	244 (54.6)	331 (74.0)	26 (5.8)	NS
Yamashita *et al*[51]	2011	412	328 (79.6)	NS	60 (14.6)	311 (75.5)	3.8
Truant *et al*[35]	2012	37	28 (75.7)	26 (70.3)	1 (2.7)	NS	4.7
Yang *et al*[53]	2013	293	236 (80.5)	201 (68.6)	216 (73.7)	NS	6.7

HCC: Hepatocellular carcinoma.

measure the extent of tumor burden, or prognosticate the continuum of outcomes after resection in the wide spectrum of tumors beyond the "Milan criteria". The "up-to-seven" criteria described by Mazzaferro *et al*[145] which is a better surrogate measure of tumor burden, could be useful for selection of patients with appropriately sized large solitary HCC or multifocal HCC with an acceptable number of lesions to undergo surgery[145,146].

As evidenced by the results of this systematic review, long-term survival results after surgical resection are acceptable and represent the best possible therapeutic option for selected BCLC stage B HCC. This review showed that resection beyond criteria advised by the AASLD and EASL guidelines, has achieved survival exceeding that accorded by non-curative methods such as TACE and sorafenib which typically confers a median OS between 8-12 mo[147-152].

There are several limitations of this systematic review. Firstly, the studies in this review comprise a group of highly selected patients who underwent surgical resection. They do not represent the entire spectrum of patients with BCLC stage B or C HCC and will be biased towards patients who are more suitable surgical candidates. Secondly, there exists a myriad of neo-adjuvant and adjuvant treatment protocols included in these studies. However, the evidence does not show definitive benefit in terms of survival and thus the effect is not likely to be significant[153-155].

In conclusion, the results of the current systematic review provides evidence that indications for surgical resection of HCC should be extended to include selected BCLC stage B lesions and further studies should seek to identify the optimal criteria for the consideration of the criteria for liver resection.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) remains a significant disease burden worldwide today. Appropriate treatment for HCC is complex because radical oncological clearance and preservation of adequate liver function need to be carefully balanced. Several staging systems have been developed to guide

Table 4 Clinical outcomes of liver resection in large or small hepatocellular carcinoma

Ref.	Recruitment period	*n*	Overall survival (%)			Median OS (mo)	Disease free survival (%)			Median DFS (mo)
			1-yr	3-yr	5-yr		1-yr	3-yr	5-yr	
Large HCC										
Poon *et al*[36]	1991-2000	120	60.6	37.8	27.5	18.8	32.0	14.1	9.5	5.5
Yeh *et al*[38]	1982-2001	211	48.1	24.0	16.7	NS	32.9	18.8	12.7	NS
Zhou *et al*[37]	1964-1999	621	68.0	37.3	26.2	NS	NS	NS	NS	NS
Liau *et al*[41]	1985-2002	82	73.0	49.0	33.0	32.0	80.0	44.0	24.0	22.0
Nagano *et al*[40]	1985-2001	26	41.0	29.3	29.3	10.1	65.4	49.0	NS	29.0
Pawlik *et al*[10]	1981-2000	300	64.9	36.7	26.9	20.3	NS	NS	NS	NS
Lee *et al*[43]	1997-2003	100	66.0	44.0	31.0	NS	43.0	26.0	20.0	NS
Pandey *et al*[44]	1995-2006	166	65.0	35.0	28.6	20.0	NS	NS	NS	NS
Shah *et al*[42]	1993-2004	24	69.0	63.0	54.0	NS	41.0	23.0	NS	8.4
Young *et al*[45]	1994-2006	42	70.0	45.0	45.0	NS	62.0	49.0	43.0	NS
Shimada *et al*[46]	1988-2004	85	NS	NS	31.5	NS	NS	NS	NS	NS
Taniai *et al*[47]	1987-2006	29	51.9	33.6	33.6	NS	48.4	21.5	21.5	NS
Choi *et al*[50]	1996-2006	50	70.0	50.2	40.2	NS	49.0	38.6	38.6	9.0
Miyoshi *et al*[49]	1987-2004	22	71.8	60.3	45.2	20.5	53.3	29.1	18.2	12.0
Ng *et al*[48]	1990-2008	44	66.4	38.1	27.8	21.5	49.6	23.9	19.1	10.7
Yamashita *et al*[51]	1995-2007	53	74.0	43.0	35.0	NS	50.0	40.0	24.0	NS
Truant *et al*[35]	2000-2010	52	NS	NS	43.3	NS	NS	NS	39.3	NS
Allemann *et al*[55]	1997-2009	22	84.0	72.0	45.0	27.0	64.0	28.0	27.0	10.0
Ariizumi *et al*[54] (S)	1990-2008	NS	81.0	60.0	47.0	14.3	41.0	18.0	12.0	NS
Ariizumi *et al*[54] (M)	1990-2008	NS	88.0	83.0	79.0	38.5	76.0	54.0	48.0	NS
Shrager *et al*[52]	1992-2010	130	56.9	30.2	18.8	17.0	31.8	13.4	11.5	6.7
Yang *et al*[53]	2002-2011	258	84.0	62.0	33.0	NS	61.0	24.0	6.0	NS
Small HCC										
Miyoshi *et al*[49]	1987-2004	230	89.3	74.6	60.4	48.2	68.0	43.7	26.7	20.0
Allemann *et al*[55]	1997-2009	79	75.0	42.0	21.0	24.0	50.0	18.0	14.0	15.0
Poon *et al*[36]	1991-2000	368	83.3	64.2	51.6	62.8	64.6	41.8	28.2	25.4
Yeh *et al*[38]	1982-2001	778	81.4	57.3	39.5	NS	61.2	40.7	32.1	NS
Zhou *et al*[37]	1964-1999	2039	85.0	65.1	54.3	NS	NS	NS	NS	NS
Liau *et al*[41]	1985-2002	111	80.0	58.0	39.0	40.0	70.0	49.0	31.0	28.0
Nagano *et al*[40]	1985-2001	143	93.1	74.5	44.7	53.4	80.0	46.5	31.0	33.9
Shah *et al*[42]	1993-2004	165	88.0	70.0	53.0	NS	76.0	53.0	43.0	38.0
Young *et al*[45]	1994-2006	43	82.0	63.0	57.0	NS	71.0	54.0	48.0	NS
Taniai *et al*[47]	1987-2006	291	81.0	61.4	45.0	NS	74.6	37.1	25.4	NS
Choi *et al*[50]	1996-2006	447	91.3	77.2	65.9	NS	72.7	53.1	45.4	35.0
Yamashita *et al*[51]	1995-2007	412	89.0	67.0	54.0	NS	72.0	45.0	37.0	NS
Truant *et al*[35]	2000-2010	37	NS	NS	89.2	NS	NS	NS	60.7	NS
Yang *et al*[53]	2002-2011	293	83.0	66.0	39.0	NS	56.0	26.0	9.0	NS

HCC: Hepatocellular carcinoma; M: Multi nodules; S: Single nodule.

management of HCC.

Research motivation

Surgical resection for HCC within the "Milan Criteria" or Barcelona Clinic Liver Cancer (BCLC) stage A is the widely accepted standard of care. However, surgical treatment for BCLC stage B (intermediate) or C (advanced) lesions remains controversial. Presently, the European Association for the Study of Liver Disease (EASL) and the American Association for the Study of Liver Disease (AASLD) guidelines do not recommend surgical resection for these patients. However, despite the recommendations from these two large reputable organizations, many international high-volume tertiary centers, especially centers in Asia, still routinely perform surgical resection for large solitary lesions, multifocal lesions and lesions with macrovascular invasion. Critical appraisal of both Western and Asian literature is needed to resolve the controversies.

Research objectives

The aim of this study was to perform a systematic review and summarize the current literature to determine the long-term survival outcomes after curative resection of intermediate and advanced HCCs.

Research methods

We conducted a systematic review of the published literature using the PubMed database from 1st January 1999 to 31st Dec 2014 to identify studies that reported outcomes of liver resection as the primary curative treatment for BCLC stage B or C HCC. The primary end point was to determine the overall survival (OS) and disease free survival (DFS) of liver resection of HCC in BCLC stage B or C in patients with adequate liver reserve (*i.e.*, Child's A or B status) and in good general status (PS 0-2). The secondary end points were to assess the morbidity and mortality of liver resection in large HCC (defined as lesions larger than 10 cm in diameter) and to compare the OS and DFS after surgical resection of solitary *vs* multifocal HCC.

Research results

We included a total of 74 articles in this systematic review. Analysis of the resection outcomes of the included studies were grouped according to: (1) BCLC stage B or C HCC; (2) Size of HCC; and (3) multifocal tumors. The median 5-year OS of BCLC stage B was 38.7% (range 10.0-57.0); while the median 5-year OS of BCLC stage C was 20.0% (range 0.0-42.0). The collective median 5-year OS of both stages was 27.9% (0.0-57.0). In examining the morbidity and mortality following liver resection in large HCC, the pooled RR for morbidity [RR (95%CI): 1.00 (0.76-1.31)] and mortality [RR (95%CI): 1.15 (0.73-1.80)] were not significant. Within the spectrum of BCLC B and C lesions, tumors greater than 10 cm were reported to have median 5-year OS of 33.0% and multifocal lesions 54.0%.

Research conclusions

In conclusion, the results of the current systematic review provides evidence

that indications for surgical resection of HCC should be extended to include selected BCLC stage B lesions and further studies should seek to identify the optimal criteria for the consideration of the criteria for liver resection.

Research perspectives

As evidenced by the results of this systematic review, long-term survival results after surgical resection are acceptable and represent the best possible therapeutic option for selected BCLC stage B HCC. This review showed that resection beyond criteria advised by the AASLD and EASL guidelines, has achieved survival exceeding that accorded by non-curative methods such as TACE and sorafenib which typically confers a median OS between 8-12 mo. Further studies should seek to identify the optimal criteria for the consideration of the criteria for liver resection.

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LETTERS TO THE EDITOR

Responsibility of hepatitis C virus in the development of hepatocellular carcinoma: From molecular alterations to possible solutions

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Abstract

There are several causes of hepatocellular carcinoma (HCC), but certainly the hepatitis C virus (HCV) is one of the most common. The HCV is able to contribute, both directly and indirectly, to the development of HCC. Determining early HCV clearance before an advanced liver disease develops, is absolutely necessary as this prevents the initiation of the cascade of events induced by HCV that may result in the development of HCC. The early treatment of the infection and the clearance of HCV represents today, in the age of the direct antiviral agents (DAAs), an extraordinary opportunity for true prevention of the development of HCV-related HCC.

Key words: Hepatitis C virus; Hepatocellular carcinoma;

Inflammation; Fibrosis; Insulin-resistance; Oxidative stress; Direct acting antivirals

Core tip: The hepatitis C virus (HCV) is able to contribute, both directly and indirectly, to the development of hepatocellular carcinoma (HCC). The early treatment of the infection and the clearance of HCV represents today, in the age of the direct antiviral agents, an extraordinary opportunity for true prevention of the development of HCV-related HCC.

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TO THE EDITOR

I read with great interest the paper by Mohammad Irshad, Priyanka Gupta and Khushboo Irshad, published in *World J Hepatol* on 28 December 2017; 9(36): 1305-1314 titled "Molecular basis of hepatocellular carcinoma (HCC) induced by hepatitis C virus infection"[1].

Among all human cancers, the hepatocellular carcinoma (HCC) is one of the most frequent[2-6]. There are several causes of HCC (asian males > 40 years, asian females > 50 years, africans, family history of HCC, hepatitis B chronic infection, non-alcoholic steatohepatitis, occupational exposure to chemicals), but certainly the hepatitis C virus (HCV) is one of the most common[7,8].

In recent years, many efforts have been made to obtain an early diagnosis of HCC, through: (1) the use of serum HCC biomarkers, such as: Alpha fetoprotein (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3), des-gamma-carboxyl prothrombin (DCP), glypican-3 (GPC-3), osteopontin (OPN), squamous cell carcinoma antigen-immunoglobulin M complex (SCCA-IgM), alpha-1-fucosidase (AFU), chromogranin A (CgA), human hepatocytes growth factor (HGF), insulin-like growth factor (IGF); (2) through the computerized axial tomography (CT); and (3) the nuclear magnetic resonance with hepatospecific contrast agent (MR). The use of these tools, often in combination, allows an early diagnosis of HCC especially in the context of close follow-up protocols[9-11].

However, there remains the great problem of understanding the mechanisms that determine the development of HCC in subjects with chronic HCV infection[12,13]. Moreover, even if an exact diagnosis of image and histology of HCC is often obtained, a molecular typing of the alterations that determine HCC is not routinely carried out, also because these are not yet fully known[6].

Irshad *et al*[1], in a very clear and precise way, show that chronic HCV infection is able to determine a progressive fibrosis with transition to cirrhosis, through the mechanisms of inflammation, the activation of stellate cells and the proliferation of hepatocytes. Hepatic cirrhosis and cell proliferation are risk factors for HCC.

Nevertheless, we should also take into account the alteration of the hepatic microenvironment in a pro-oncogenic sense and of the intestinal microbiome. The HCV also determine insulin resistance, hepatic steatosis, oxidative stress and all these events are associated with genetic instability[13-15].

Furthermore, the HCV, which is an RNA virus and does not integrate into the host genome, also has a direct role in the development of HCC, through the interaction of its proteins (HCV core, E1, E2, NS3 and NS5A) with various cell pathways that produce different effects as preconditions for the induction of HCC[16-18].

The data provided by the manuscript of Irshad *et al*[1] are very interesting because they set up a new panorama in chronic HCV infection, underline the role of HCV in the development of HCC and arouse some considerations.

Since the HCV is able to contribute, both directly and indirectly, to the development of HCC, it is now absolutely a priority to treat all subjects with chronic HCV infection, regardless of the degree of liver disease and the presence or absence of any co-morbidities[8,19].

Nowadays, the therapy is based on the use of direct antiviral agents (DAAs) that guarantee the disappearance of the infection, intended as Sustained Virologic Response (SVR), in over 95% of cases, with no significant side effects, which are instead reported during interferon and ribavirin therapy[20-23].

In the scientific community, the paper by Reig *et al*[24] published in *Journal of Hepatology* 2016; 65: 719-726, has provoked great concern because the authors concluded that an unexpected and high percentage of HCC recurrence had occurred in their patients after obtaining the clearance of HCV with DAAs therapy. Fortunately, this statement was "reshaped" by subsequent research that demonstrated, in a large cohort of subjects treated with DAAs, the risk of early recurrence from HCC was comparable and not higher than that observed in patients not treated with DAAs. On the other hand, we must not forget that the rate of early recurrence of HCC remains elevated in patients with advanced liver disease despite the HCV clearance, since liver cirrhosis is a itself risk factor for the development and recurrence of HCC[25].

The research by Ikeda *et al*[26] in *Digestive Diseases and Sciences* 2017 Oct; 62(10), by Kanwal *et al*[27] in *Gastroenterology* 2017 Oct; 153(4) and of Petta *et al*[28] in *Alimentary Pharmacology and Therapeutics* 2017 Jan; 45(1), have clearly shown that Direct-Acting Antivirals therapy reduces the frequency of HCC relapse when performed after initial HCC therapy and that obtaining

SVR is associated with the reduction of HCC. However, in patients with cirrhosis, even if SVR is obtained, the risk of HCC remains present. In fact, these subjects require continuous surveillance[26-28].

Determining early HCV clearance before an advanced liver disease develops, is absolutely necessary as this prevents the initiation of the cascade of events induced by HCV which may result in the development of HCC.

The emphasis made by Irshad *et al*[1] on the prominent role of HCV in hepatic tumorigenesis is very important, both in order to intercept possible new pathways of HCC development that could be used for the development of drugs against specific molecular targets of HCC, both because it reinforces our idea, shared by other researchers, that the early treatment of the infection and the clearance of HCV represents today, in the age of the DAAs, an extraordinary opportunity for true prevention of the development of HCV-related HCC.

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REVIEW

Contemporary role of liver biopsy in hepatocellular carcinoma

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Abstract

A correct diagnosis of hepatocellular carcinoma (HCC) in cirrhotic patients with focal liver lesions is one of the most important issues nowadays. Probably one of the oldest debates in the hepatology community is whether to perform liver biopsy (LB) in all cirrhotic patients with focal liver lesions. We now face a time when oncology is moving towards personalized medicine. According to the current European Association for the study of Liver diseases HCC guidelines, LB has only a minor role in the management of HCC. However, the current recommendations were made more than five years ago. As time has passed, the development of high-throughput molecular technologies has helped reveal the main molecular mechanism involved in HCC development and progression. Several subtypes of HCC, with both molecular and histological characterization, have been described. Importantly, some of these subtypes have prognostic impact. In the context of personalized treatment, the role of LB will be carefully reconsidered. Until then, it is mandatory to know the various techniques of LB, their performances, complications and limitations. The balance of risk and benefit defines many of the decisions that we make as providers of medical care. In this review, we discuss not only the risks associated with LB, but also the benefits of biopsy in various clinical scenarios. Not long from now, the role of LB will be reconsidered. It is possible that we will go back in time and once again use biopsy for HCC diagnosis. Then again, we may move back to the future to try to improve the use of liquid biopsy in the follow-up of HCC patients after various treatment modalities.

Key words: Molecular classification; Bleeding; Seeding; Liver biopsy; Hepatocellular carcinoma

Core tip: We now face a time when oncology is moving towards personalized medicine. The development of high-throughput molecular technologies has allowed us to define the main molecular mechanism involved in hepatocellular carcinoma (HCC) development and progression. Several subtypes of HCC have been described using both molecular and histological characterization. In the context of histological HCC sub-classes, each with distinct molecular patterns and prognostic impacts, the need for liver biopsy in HCC management becomes a necessity. In this era of personalized medicine, knowing the strengths of each sampling technique is of the utmost importance.

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INTRODUCTION

The correct identification, either malignant or benign, of focal liver lesions is one of the most important issues in cirrhotic patients. Nodular lesions are frequently discovered during an ultrasound screening of these patients. Recent progress in ultrasound has led to an earlier discovery of these lesions. Moreover, the application of contrast agents has gained more and more attention. Compared to other imaging modalities, contrast enhanced ultrasound (CEUS) can be performed immediately after conventional ultrasound (US), providing a simple, easy-to-perform and immediately available dynamic imaging tool[1]. The use of CEUS might therefore shorten the diagnostic and therapeutic work-up of hepatocellular carcinoma (HCC) patients. The large applicability of CEUS for the diagnosis of HCC in cirrhosis was questioned due to the risk of false-positive diagnoses in cases of cholangiocarcinoma. This has caused the American College of Radiology to release a diagnostic scheme for the characterization of focal liver lesions in patients at risk for HCC, named CEUS LI-RADS®[2]. In a multicenter Italian study, the use of CEUS LI-RADS in small HCC showed that the LR-5 category was 98.5% predictive of HCC, with no risk of misdiagnosing pure cholangiocarcinoma[3].

Despite all the latest improvements in liver imaging, correctly identifying these lesions remains a challenge, especially when dealing with small focal lesions.

According to the AASLD and EASL guidelines, the images in certain situations may not be characteristic, or the results from two imaging techniques may be conflicting (a liver biopsy (LB) is required in these cases)[4]. In addition, the information from tumor tissue may provide prognostic data that are useful in the selection of therapy.

TECHNIQUES, PERFORMANCE, COMPLICATIONS

The invasive techniques used for the morphological diagnosis of HCC are ultrasound-guided fine-needle aspiration (FNA) and needle-core biopsy. The performance of these techniques is somewhat similar in the morphological diagnosis of HCC. Cytology sensitivity varies between 69%-95% across different studies (consistently lower in well-differentiated HCC), while specificity varies between 70%-100%[5–11]. The diagnostic accuracy of the method is lower in lesions < 3 cm (50%-83%) *vs* large ones (85%-95%)[7,12]. The smear cytology technique using Papanicolaou's method will decrease the number of both required passes and inadequate fragments. Flow cytometry and the various immunohistochemical techniques are extremely helpful in the characterization of neoplastic cells.

The difficulty of a correct differential diagnosis between a regenerative nodule and a well-differentiated HCC can only be overcome by using a relatively large tissue sample, which is obtainable only with the use of thick needles.

Core biopsy performed with large needles (1.1-1.6 mm outer diameter) ensures the recovery of an adequate tissue fragment; it also allows for a better preservation of tissue architecture, providing more information on the tumor tissue and facilitating certain special staining techniques. These advantages are, however, counterbalanced by the high risk of complications.

The rate of successful sampling using large needles is 85%-98.5%. It may be diminished by certain factors: the small size of the target (smaller lesions are harder to approach in liver with significant fibrosis), the location of the lesion in deeper segments (posterior and superior segments, such as segments Ⅳ B, Ⅶ and Ⅷ), and the presence of necrotic areas within the tumor[11]. Additionally, lesions that are poorly visible or invisible using conventional ultrasound are another cause of liver biopsy failure.

The sensitivity of core needle biopsy for HCC diagnosis is 86%-96%, which is increased in the case of multiple passes[11,13-16]. The specificity ranges between 95%-100%, especially when also sampling from an extra-nodular area (non-neoplastic neighboring parenchyma)[9,12]. The accuracy of the method varies between 85% and 91%[11,13,14,16].

Micro-histology combines the safety profile of fine-needle aspiration with a higher quality of tissue samples (similar to that provided by core needle biopsy); it has a higher sensitivity and specificity than conventional cytology: 92.6% and 100% *vs* 81.3% and 97.6%, respectively[7,9,11,17]. In addition, micro-histology has a high accuracy (89.6%) in diagnosing nodules < 2 cm and varies according to size: 88.6% for nodules ⩽ 10 mm, 86.2% for nodules between 11-15 mm and 91.3% for nodules between 16-20 mm in diameter[17].

Some needles (Histocut) allow the recovery of tissue fragments for both cytology and micro-histology during the same pass. The cytology-micro-histology combination increases the sensitivity of HCC diagnosis: 89.8%-90% *vs* 80%-85.6% for cytology, and 61%-66.1% for micro-histology[7,9].

Using real-time contrast-enhanced harmonic ultrasound (SonoVue) to guide the biopsy will increase its diagnostic sensitivity by targeting: (1) the enhanced, vascular areas of the tumor in the arterial phase, particularly in the case of large tumors that often display central necrosis[18]; and (2) the nodules that are poorly visible or invisible on conventional ultrasound, which become clearly visible after contrast injection in both the arterial or late phase[18,19].

The negative predictive value of liver biopsy remains low, and malignancy cannot be excluded from one negative result alone. The management of these patients includes long-term imaging, follow-up, and re-biopsy. If a re-biopsy is taken into consideration, it is imperative to recall its low chance of success when performed immediately after the first biopsy, with only a 35% increase in positive diagnosis[20]. If a tumor was not found in the first biopsy, the chances of success are higher (50%) than in cases with a non-diagnostic result (necrosis) (25%)[20]. In these cases, especially in nodules < 2 cm, imaging follow-up is recommended. Performing liver biopsy for the diagnosis of HCC is not without risks**.** Hemorrhage is more frequent when using thick needles (1.1% *vs* 0.5% for fine needle biopsy) and when sampling an HCC (2.5%)[11,21]. Risk factors for bleeding include: hemostatic abnormalities, the degree of liver failure, age, the presence of ascites, or the technique used. The risk is generally considered to increase with each additional pass, with a larger needle diameter, and with a smaller area of interposed parenchyma[22]. The actual recommendations are to use a needle < 1.2 mm in diameter for a maximum of two passes, and an oblique approach, which would allow at least 1 cm between the lesion and the liver capsule[15,23].

The incidence of needle-tract seeding varies in the literature between 0% and 7.69%, with a mean of 3.16% and a median of 2.66% (Table 1); this value is lower (1.43%) when considering the global incidence. A meta-analysis published in 2007 has established the median incidence of tumor cell seeding to be 2.7%[24]. Apparently, the larger the needle diameter and the number of passes, or the lower the degree of tumor differentiation, the higher the risk of seeding. There are no studies, however, to confirm this supposition. Seeding can occur in the thoraco-abdominal wall or intraperitoneally, sometimes several years after the biopsy and even after performing liver transplantation. The risk of seeding is not reduced by using the coaxial technique[25]. The treatment of needle-tract seeding, especially if parietal, is surgical; after surgery, most patients experience no recurrences. The occurrence of seeding does not alter global survival rates, which only depend on the progression of either the primary tumor or cirrhosis[26].

Liver cells are generally found in the blood after both liver biopsy and liver resection, as attested by the presence of mRNA AFP in the serum. It is not exactly known whether these are normal or tumor cells. No association between this phenomenon and tumor cell seeding has been demonstrated to date.

Mortality after biopsy is higher when using thick (0.15%-0.19%) *vs* fine needles (0.008%)[24,25, 27–37].

CURRENT INDICATIONS OF LB IN THE DIAGNOSIS OF HCC

Presently, the indications of performing LB in patients with liver cirrhosis and HCC are highly regulated. The two extreme perspectives that include recommending either biopsy in all cases (as was the norm before the introduction of non-invasive criteria), or the avoidance of biopsy at all costs when having good diagnostic imaging studies, have both been abandoned. The main factors that indicate, adjust or limit the use of biopsy in HCC are presented in Table 2.

In the following paragraphs, we will make a critical appraisal of the indicators of LB in the diagnosis of HCC for each of the BCLC stages.

BCLC stages 0 and A (very early and early HCC)

Correlation with imaging techniques. Nodules measuring between 1 and 2 cm are difficult to characterize using non-invasive methods[38,39], since up to 33% are benign, while HCC nodules frequently have no distinctive pattern of behavior. Only 33% of HCC nodules meet the precise diagnostic criteria recommended by the AASLD (hypervascularization in the arterial phase and washout in the portal/parenchymal phase using two imaging techniques)[38]. It follows that 50%-70% of patients will require a biopsy in order to receive an exact diagnosis[38,39]. US-guided LB may not be justified in patients with decompensated cirrhosis, despite the nature of the nodule, and liver transplantation might be considered. In contrast, in patients with a small nodule and compensated cirrhosis, US-guided LB should be performed before surgical resection, which carries morbidity and mortality rates higher than those of biopsy itself[14]. It is difficult to assess the differential between a well-differentiated HCC and a dysplastic nodule when using a fragment sampled by LB. The use of molecular markers (GPC3, HSP70, and GS) will identify the exact nature of nodules with 57% sensitivity and 100% specificity[40]. Compared to LB, new imaging techniques such as Gd-EOB-DTPA magnetic resonance imaging (MRI) might be more accurate in the differential diagnosis between early HCC and dysplastic nodules. Hyperintensity at diffusion-weighted imaging (DWI) was shown

Table 1 The incidence of needle-tract seeding after hepatocellular carcinoma biopsy

Ref.	Year	Lesion	Needle	No. of biopsies	No. of seeding	%
Yamashita *et al*[35]	1995	HCC	0.8-1.2 mm Bard	125	1	0.80
Huang *et al*[11]	1996	HCC	1.4-2 mm	455	9	2
Kanematsu *et al*[36]	1997	HCC	FNB 0.8 mm	50	2	4
Ch Yu *et al*[15]	1997	HCC	1.2 mm gun	139	0	0
Chapoutot *et al*[37]	1999	HCC	1.0-1.2 mm	150	4	2.66
Kim *et al*[28]	2000	HCC	1.1 mm gun	205	7	3.40
Takamori *et al*[27]	2000	HCC	FNB	59	3	5
Durand *et al*[14]	2001	HCC	1.2 mm	137	2	1.60
Kosugi *et al*[29]	2004	HCC	n.a	372	6	1.61
Ng *et al*[30]	2004	HCC	FNA	91	1	1.09
Shuto *et al*[31]	2004	HCC	n.a	480	5	1.04
Wang *et al*[32]	2005	HCC	FNA	90	0	0
Saborido *et al*[33]	2005	HCC	FNA	26	2	7.69
Maturen *et al*[25]	2006	HCC	1.2 mm, coaxial	128	0	0
Colecchia *et al*[34]	2012	HCC	0.95 mm	81	0	0
Total				2588	42	1.62

n.a: Not available; HCC: Hepatocellular carcinoma; %: Percent; FNA: Fine needle aspiration.

Table 2 Factors influencing the use of liver biopsy in hepatocellular carcinoma

1 Poor accuracy of contrast-enhanced methods in the diagnosis of HCC, especially in small lesions
2 The risks of LB, which are more severe in patients with cirrhosis and coagulopathy
3 Inadequate sampling of HCC lesions, especially in cases with very small or very large ones
4 The complex system of staging, treatment, and patient allocation to various therapy regimens (BCLC); the correct assessment of prognosis is important in the allocation of therapy, and is based mainly on pathology data
5 Modern therapies sometimes have limited applicability (transplantation), cost and effectiveness (systemic treatment); information resulting from histological analysis is necessary in order to increase effectiveness and personalize treatment

LB: Liver biopsy; HCC: Hepatocellular carcinoma; BCLC: Barcelona clinic liver cancer staging.

to be a useful feature for differentiating hypovascular early HCC from dysplastic nodules, which appear as hypointense nodules in Gd-EOB-DTPA MRI[41]. A more recent study reported a sensitivity of 94.7% and a specificity of 99.3% in classifying high-grade dysplastic nodules, which appear hypointense in the hepatobiliary (HB) phase without arterial phase hyperintensity and without DWI restriction[42]. More importantly, the benign nodules appeared hyperintense in the HB phase, and HCC rarely develops from hyperintense hepatic nodules in the HB phase. This suggests that these type of nodules do not require treatment or more intensive follow-up[43].

The degree of tumor differentiation in nodules measuring 1-2 cm can be identified with 60% accuracy, however the sensitivity of the histological examination in assessing vascular micro-invasion is low, especially after fine-needle biopsy[34]. Since vascular micro-invasion defines the prognosis of patients allocated to various therapies, its estimation (using nodule size and the degree of differentiation) is of the utmost importance[34].

Identifying the exact nature of the cirrhotic nodules gains additional importance in the context of liver transplantation. Several situations where LB may play a central role can be defined. For instance, identifying an HCC in a patient already on the transplant list using imaging studies will increase his or her priority score. In the first years of using the MELD score, 7%-31% of stage 1 HCC patients who received transplants were found to have no HCC in the explanted liver[44,45]. Secondly, although HCC is the most frequent tumor type to develop in cirrhotic liver, other tumors are also possible (especially cholangiocarcinoma). It is currently believed that ⩽ 20% of nodules developing in a cirrhotic liver, with imaging behavior typical for HCC, will actually have another histological structure[16]. The incidence of cholangiocarcinoma has increased considerably in the past years, and the imaging appearance of small peripheral lesions is very similar (or even identical) with that of HCC. Since the risk of recurrence after transplant is much higher for these tumors than for HCC, other patient selection criteria are required, as well as a more aggressive pre-transplant treatment[16]. Thirdly, HCC may sometimes occur in patients with chronic liver disease prior to the development of cirrhosis. The risk for HCC development is lower in these patients, and consequently any newly discovered nodule, even if hy-

pervascular, should be biopsied.

The fourth situation when a pre-transplant liver biopsy is warranted is related to the importance of assessing the degree of tumor differentiation and vascular invasion. It has been clearly proven that HCC tumor differentiation is strongly correlated to survival, both after resection and transplantation. The risk of recurrence is higher for poorly- or moderately-differentiated *vs* well-differentiated tumors[16,46]. This is also applicable for tumors outside of the Milan criteria, but within the Up-to-seven criteria. This means that the patients with well-differentiated HCC, and without vascular invasion, have a very good prognosis (1- and 3-year survival rates of 84.2% and 67.4%, respectively)[47].

Vascular micro-invasion is difficult to ascertain by liver biopsy, and its risk can only be estimated at best. For instance, for a poorly differentiated tumor > 4 cm, the risk of vascular micro-invasion is 61%[46]. For well-differentiated tumors, the size and extent of vascular invasion do not appear to influence prognosis[46]. In situations where vascular micro-invasion cannot be estimated, the use of imagistic methods might be of real importance. Diffusion-weighted imaging (DWI), an emerging technique in hepatic MRI, provided a sensitivity of 93.5% and a specificity of 72.2% for the prediction of micro-vascular invasion during the preoperative evaluation of HCC[48]. Consequently, knowing the exact type of tumor appears to be very important for optimizing patient selection for transplantation[46,47].

In conclusion, choosing to perform a pre-transplant biopsy in patients with liver cirrhosis and HCC depends on the tumor stage and the severity of cirrhosis. For instance, in patients with compensated cirrhosis and HCC diagnosed with the Milan criteria, LB should be performed in order to correctly confirm or exclude an HCC, therefore avoiding the granting of additional MELD points. In patients with decompensated cirrhosis, liver biopsy is not indicated since transplantation is already an immediate necessity. For patients outside of the Milan but within the Up-to-seven criteria, liver biopsy is very useful in selecting patients with well-differentiated tumors who would benefit the most from transplantation[44,49].

An argument for the use of LB before resection concerns a poor correlation (sometimes below 50% for large biopsied tumors) between the degree of differentiation found on biopsy and on the resected tumor[34,50]. This can be explained by the high heterogeneity of larger tumors, which relates to their varying degrees of differentiation. Secondly, performing a biopsy before a resection will expose the patient to a higher risk of peritoneal metastases (12.5% *vs* 1.6%) and will decrease 5-year disease-free survival (24% *vs* 52%)[51]. However, some authors claim that fine-needle aspiration before resection does not affect either mortality or survival rates[30].

Thirdly, we must not ignore the risk of complications (seeding, bleeding), as well as the contraindications and limitations of LB (ascites, coagulopathy or isoechoic nodules). The negative predictive value of LB does not reach 100%, and a new biopsy or imaging follow-up is recommended in the case of negative results. This approach will prolong the time to resection and will expose the patient to additional risks[52]. The current approach states that LB should be indicated and performed only in tertiary centers that are equipped with state-of-the-art imaging techniques, high imaging expertise, interventional techniques, and pathology labs[47]. In other conditions, performing liver biopsy before resection should be avoided, except in cases where the biopsy result is expected to substantially alter the therapy[53].

BCLC intermediate and advanced stages

In each of these stages, the indication to perform LB is made based on the following issues: (1) choosing the optimal therapy from a variety of possible treatment courses. For instance, patients in the intermediate stage may benefit not only from chemoembolization, but also from curative options such as resection, percutaneous ablation or liver transplantation. Curative treatment is indicated in the presence of favorable prognostic factors, such as well-differentiated HCC or the lack of vascular micro-invasion[54]; (2) diagnosing a portal thrombus as benign using liver biopsy may suggest the need for liver transplantation or resection for a patient in an advanced stage; and (3) Considering the poor efficacy of current antiangiogenic therapies (Sorafenib), which can be attributed to its severe adverse effects and high cost, it is essential to exclude other tumors that may form in a cirrhotic liver (cholangiocarcinoma, mixed types - hepatocholangiocarcinoma) and that would require a different therapy[49]. The lack of histological confirmation in the Sharp studies, as well other similar ones, raises the question of whether or not some cases of hepatocholangiocarcinoma may have been wrongly diagnosed as HCC in the study groups.

Molecular testing is a staple nowadays in oncology. The selection of systemic treatments is made by considering the tumor molecular biology (as in breast or lung cancer). The concept of non-invasive diagnosis of HCC (which is the only tumor that does not require morphological examination) was established before the introduction of new therapeutic agents. Several authors speculate whether this lack of histological data may explain the limited efficacy of Sorafenib, considering that certain studies fail to prove the efficacy of other systemic therapies in HCC[55]. In the future, the multitude of studies performed on systemic therapy for HCC will have to make use of pathological, molecular and genetic information provided by the tissue fragment in order to accurately establish the prognosis and to individualize the therapy[49,53]. Current progress in molecular biology will soon allow for guided treatment based on the expression of tumor genes[53]. At present, molecular genetic

tests are costly, and their widespread use is limited by their ongoing validation and standardization, as well as by the lack of consensus in their guidelines[53].

LIVER BIOPSY IN THE CONTEXT OF PERSONALIZED MEDICINE

The role of liver biopsy for the management of patients with HCC is one of the most active debates in the liver cancer community[56,57]. Over the last decade, the emergence of high-throughput molecular technologies has provided the ability to interrogate the main molecular mechanism involved in HCC development and progression. HCC is best considered a highly heterogeneous entity that is composed of distinct transcriptomic subgroups with various genetic alterations[58,59]. Importantly, a high degree of heterogeneity can also be observed at the histological level. For instance, fibrolamellar carcinoma is already a well-accepted morphological and molecular subtype of HCC[60]. Furthermore, the chromophobe subtype shows a distinct morphology, while also utilizing a specific molecular mechanism to overcome replicative senescence, which is in contrast to the telomerase activation seen in most HCCs[61]. Several histological subtypes, which feature distinctive and recognizable morphological features, have also been reported, such as the steatohepatitic, cirrhotic, lymphoepithelioma-like, and inflammatory HCCs[61-63]. Indeed, the molecular mechanism behind these histological subtypes awaits clarification, however this is only a matter of time considering the rapid advancement of molecular technologies. It is estimated that 20%-30% of HCCs belong to a recognizable morphological/molecular subtype[57]. A recent paper, published in *Hepatology*, described another HCC subtype that displays distinct histological and molecular features[64]. The macrotrabecullar-massive HCC (MTM-HCC) was identified in 12% of the total cohort (16% of surgically-resected samples, 8.5% of liver biopsy samples). In multivariate analysis, the MTM-HCC subtype was an independent predictor of early and overall recurrence. From the molecular point of view, MTM- HCC was characterized by high expression of angiopoietin 2 and vascular endothelial growth factor A (VEGFA)[65]. Bi-specific, anti-angiopoietin 2 and anti-VEGFA antibodies may represent potent treatments for this subclass of HCC.

Taking into account this new, recently-described MTM-HCC subclass, we now have an estimated 36%-46% of HCCs that belong to a recognizable morphological or molecular subtype. For the remaining HCCs, molecular subtypes likely exist[66]. Tumor heterogeneity will not be fully reflected in all liver biopsies, however many HCCs can be sub-classified appropriately. The discovery of different histological subtypes, each with distinct molecular features, is still in its infancy. Until further evidence is revealed, no recommendations can be made regarding how to best treat different subtypes. For the time being, HCC should instead be considered as one disease. On the contrary, once all the signaling pathways for each HCC subtype have been described, liver biopsy will indeed be necessary for the correct identification of such signaling pathways. Moreover, the identification of distinct signaling pathways for different subtypes of HCC will allow for the development of new treatments. In this ideal and close-approaching scenario, liver biopsy will allow for the correct diagnosis of HCC subtypes, the corresponding upregulated signaling pathways, and the proper choice of specific molecules. This will ultimately open the path for personalized medicine.

The balance of risk and benefit defines many of the decisions that we make as providers of medical care. With respect to the use of liver biopsy in diagnosing HCC, the risks are well-defined and quantifiable. Common arguments against liver tumor biopsy have included the risk of bleeding and tumor seeding (Table 1). Up to 20% of focal liver lesions developed on a background of liver cirrhosis are not HCC (14), and almost 46% of HCCs have a distinct histological or molecular signature that might benefit from targeted therapies. We are all afraid of the invasive nature of liver biopsy, but must also consider the risks and benefits of treating a non-HCC patient as though they had HCC. What is the benefit of targeting a molecular pathway in a patient with HCC when that targeted pathway is not activated? We do not believe that the current guidelines are wrong, because the data that form the basis of the existing guidelines are against liver biopsy. Nevertheless, due to advancements in molecular biology, more and more molecular and histological classes of HCC have been, and will continue to be, described. We believe that there will come a time when diagnostic biopsies will be commonly performed. This will improve the diagnosis of HCC and increase our ability to provide better patient care in the future.

LIQUID BIOPSY: THE FUTURE OF LIVER BIOPSY

In the past few decades, several studies have demonstrated the utility of circulating cancer byproducts known as "liquid biopsy", which could provide accessible, accurate, and dynamic information to evaluate tumor progression. Circulating tumor cells (CTCs), circulating cell-free DNA, circulating miRNA, and circulating tumor associated microparticles (MPs) can all be united under the term "liquid biopsy". Compared to liver biopsy, liquid biopsy is a noninvasive method used for the identification of CTCs, circulating MPs, or circulating miRNA/DNA in the blood of patients with HCC. Moreover, it is well accepted by the patients, since only 1 mL of blood is enough for proper identification in flow cytometry or cell search systems. Similar to conventional biopsies, CTSs or MPs can be stained for various surface markers specific for HCC. A detailed description of all cancer

byproducts is beyond the scope of this review and has already been nicely reviewed elsewhere[67]. We will only provide some brief examples.

CTCs were detected in blood samples from 45 out of 69 HCC patients, compared to 0 out of 31 controls. Moreover, CTC numbers correlated significantly with tumor size, PVT and survival[68]. Others have found that patients with preoperative detectable EpCAM^{mRNA+} CTCs had significantly shorter TTR (median of 10.9 mo *vs* not reached) and higher recurrence rates (59.6% *vs* 25.7%) than those without detectable EpCAM^{mRNA+} CTCs[69]. Chan *et al*[70] confirmed the existence of typical DNA copy number variations in the peripheral blood of four HCC patients, which primarily disappeared after surgical resection. Circulating miRNA is probably the most studied form of liquid biopsy in HCC. Several miRNAs have been reported to have a role in diagnosis, prognosis and follow-up[67]. More recently, another form of liquid biopsy has gained particular attention. Circulating tumor microparticles that are positive for a combination of antigens, particularly AnexinV$^+$EpCAM$^+$ASGPR1$^+$ CD133$^+$, allowed for the distinction of liver malignancies (HCC or CCA) and cirrhosis from tumor-free individuals and, more importantly, from patients carrying other non-liver cancers. In addition, AnexinV$^+$EpCAM$^+$ASGPR1$^+$ microparticles were increased in liver cancer-bearing patients compared to patients with cirrhosis that lacked any detectable liver malignancy[71].

The term liquid biopsy has only recently been introduced, and the technology for cancer byproduct identification is still in its infancy. Until more and more data becomes available, liquid biopsy cannot be performed in daily practice and should instead be used for research intents. Time will decide the limits of liquid biopsies and whether it can replace conventional biopsies. The reported sensitivity and specificity of liquid biopsy in HCC is rather modest. Better performance was reported for liquid biopsy as a tool to monitor treatment outcomes. Indeed, a lot of work must be done in this field before we can draw any conclusions. Continuously improving the detection and characterization of CTCs, circulating free DNA, and MPs is of the utmost importance, since liquid biopsy has several advantages over conventional biopsy: (1) it is a non-invasive procedure; (2) it can be easily repeated over time, which offers a more complete portrait of the disease; (3) it could better reveal the genetic complexity of a highly heterogeneous tumor; and (4) it is much faster[72].

CONCLUSION

Presently, morphological examination during HCC diagnosis is very carefully adjusted, as one must consider the availability of non-invasive techniques and, on the other hand, the need for prognostic criteria and individualized therapy. Improving the biopsy technique (higher needle performance, more accurate guidance in the active, hypervascular areas of the tumor, and the use of techniques with a lower seeding risk) will increase the sensitivity of the procedure and decrease the complication rate. With recent advances in high-throughput molecular technologies, which have allowed for the identification of novel HCC subclasses with prognostic impact, the role of liver biopsy will gain increasingly more attention and reconsideration.

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MINIREVIEWS

MR with Gd-EOB-DTPA in assessment of liver nodules in cirrhotic patients

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Abstract

To date the imaging diagnosis of liver lesions is based mainly on the identification of vascular features, which are typical of overt hepatocellular carcinoma (HCC), but the hepatocarcinogenesis is a complex and multistep event during which, a spectrum of nodules develop within the liver parenchyma, including benign small and large regenerative nodule (RN), low-grade dysplastic nodule (LGDN), high-grade dysplastic nodule (HGDN), early HCC, and well differentiated HCC. These nodules may be characterised not only on the basis of their respective different blood supplies, but also on their different hepatocyte function. Recently, in liver imaging the introduction of hepatobiliary magnetic resonance imaging contrast agent offered the clinicians the possibility to obtain, at once, information not only related to the vascular changes of liver nodules but also information on hepatocyte function. For this reasons this new approach becomes the most relevant diagnostic clue for differentiating low-risk nodules (LGDN-RN) from high-risk nodules (HGDN/early HCC or overt HCC) and consequently new diagnostic algorithms for HCC have been proposed. The use of hepatobiliary contrast agents is constantly increasing and gradually changing the standard of diagnosis of HCC. The main purpose of this review is to

underline the added value of Gd-EOB-DTPA in early-stage diagnoses of HCC. We also analyse the guidelines for the diagnosis and management of HCC, the key concepts of HCC development, growth and spread and the imaging appearance of precursor nodules that eventually may transform into overt HCC.

Key words: Hepatobiliary contrast materials; Cirrhosis; Gadoxetic acid; Magnetic resonance imaging; Liver

Core tip: Hepatobiliary contrast agents improve detection and characterization of focal liver lesions in patients with cirrhotic liver. Gd-EOB-DTPA provides information not only on vascular changes but also on hepatocyte function. Based on the recent advances in liver magnetic resonance imaging (MRI) technology, in this review, we discuss the pivotal role of Gd-EOB-DTPA enhanced MRI for the future of hepatocellular carcinoma's management.

Inchingolo R, Faletti R, Grazioli L, Tricarico E, Gatti M, Pecorelli A, Ippolito D. MR with Gd-EOB-DTPA in assessment of liver nodules in cirrhotic patients. *World J Hepatol* 2018; 10(7): 462-473 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i7/462.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i7.462

INTRODUCTION

Liver cancer is the fifth most common cancer in men, the ninth in women and is the second most common cause of death from cancer worldwide[1]. Hepatocellular carcinoma (HCC) is a primary tumour of the liver and several risk factors for its development have been identified. These include hepatitis C viral (HCV) infection, hepatitis B virus (HBV) infection, hereditary hemochromatosis and cirrhosis of almost any cause[2].

The diagnosis of HCC can be difficult and often requires the use of one or more imaging modalities[3-6]. Surveillance for HCC aims to reduce disease-related mortality because an accurate and early detection and characterization of focal liver nodule is mandatory since the management of HCC patients differs to other malignant or benign nodules and the prognosis of HCC depends mostly on the stage at which the tumour is identified[7].

Liver cirrhosis is the underlying and common condition associated with hepatocarcinogenesis. Cirrhosis develops after a long period of chronic liver disease when the risk of HCC is still low. The nodules that could be potentially find in a cirrhotic liver comprise: regenerative nodule (RN), low-grade dysplastic nodule (LGDN), high-grade dysplastic nodule (HGDN), early HCC, well differentiated HCC and moderately-poorly differentiated HCC. Hepatocarcinogenesis is a multistep event during which cell density increase, Kuppfer cells decrease, nodules enlarge and hemodynamics changes occur. To date, the imaging diagnosis of HCC is based on the characterization of vascular features, which are typical for overt HCC[3,6,8]. In fact, in the final step of hepatocarcinogenesis, the tumor blood supply consists of nontriadal or unpaired arteries and sinusoidal capillarization, with reduced or absent portal blood supply[9]. However, an atypical vascular behaviour is quite common in small (< 2 cm) nodule and almost one-third of these are malignant ("the one-third rule")[10]. These features depends on intra-nodular perfusional changes during carcinogenesis, starting with arterial hypovascularity with portal supply still present, followed by a decrease of both arterial and portal blood flow and, subsequently, to an hypervascular pattern[5].

At the same time organic anionic transporting polypeptide (OATP), transporters of bile salts, simultaneously and gradually decrease. OATP is expressed in RNs and LGDNs and its levels are lower in many HGDNs, early HCCs and progressed HCCs. The hemodynamic changes are well depict during dynamic multi detector computed tomography (MDCT) and magnetic resonance imaging (MRI) and both European and American guidelines have endorsed this techniques for the diagnosis of HCC > 1 cm, based on the typical hallmarks of hypervascularity in arterial phase with wash-out in portal phase, avoiding liver biopsy[10,11]. Moreover, OATP8 expression level reduces prior to complete neoangiogenesis[12] and the use of hepatospecific contrast media in MRI is considered a "new" HCC diagnostic tools that allows either to increase sensitivities for detection of HCC of all sizes[13] or would make it possible to identify preneoplastic lesions, such as HGDNs[14]. Furthermore, MRI offers additional imaging sequences that can be helpful in nodule characterization, including T2-weighted imaging (T2-WI) and diffusion-weighted imaging (DWI), which provides information on cellularity and has shown additional value to gadolinium-enhanced MRI by increasing the detection rate of HCC[15]. However, precise differentiation of preneoplastic lesions remain uncertain to date. The recent introduction of hepatobiliary MRI contrast agent gadolinium-ethoxybenzyl-dieth-ylenetriamine pentaacetic acid (Gd-EOB-DTPA, Primovist®; Bayer Schering Pharma, Berlin, Germany) which gives information not only on vascular changes but also on hepatocyte function, raises the sensitivity for the detection of early HCC to 91%-93%[16]. Many authors[9,16-21] suggested that Gd-EOB-DTPA-enhanced MRI is itself the most relevant diagnostic tool for differentiating low-risk nodules (LGDN, RN) from high-risk nodules (HGDN, early HCC, overt HCC) and hence new diagnostic algorithms for HCC have been proposed[4,22].

Based on the recent advances in MRI imaging technology and since early-stage diagnoses of HCC have increased and opened the possibilities to curative therapy, the purpose of these review is to the analyze the guidelines for the diagnosis and management of HCC; the basic MRI protocol and the advanced techniques, the key concepts of HCC development, growth and spread and the imaging appearance of precursor nodules that eventually may transform into overt HCC in order to eluci-

date the pivotal role of Gd-EOB-DTPA-enhanced MRI for the future of HCC's management, through a review of the published literature.

INTERNATIONAL GUIDELINES

Many studies have examined the clinical management of HCC, detecting guidelines to define a standardized approach to surveillance, diagnosis and treatment, with the aim of improve timely diagnosis and early intervention.

Guidelines in fact could be a roadmap to develop decision making algorithms, improving quality of treatment and patients' outcomes according with support of regional or national resources. From 2001 to 2017 at least 20 guidelines have been published worldwide, with some differences in surveillance and diagnostic criteria[23].

The American Association for the Study of Liver Diseases (AASLD) was founded in 1950, providing recommendation for surveillances, diagnosis, staging and treatment of HCC, in a similar way of European Association for the Study of the Liver (EASL)[8], while the first edition of practice guidelines for HCC in Japan was published in 2005[24]. Most of the guidelines devises the form of surveillance depending on risk factors for HCC and on individuating patients with risk factor that have to be monitored. Risk factors are divided into those that are cirrhosis-related (HBV, HCV, alcoholic cirrhosis, genetic causes such as hemochromatosis, non-alcoholic steatohepatitis, stage IV primary biliary cirrhosis, alpha one antitrypsin deficiency) and those that are non-cirrhosis related, (being an HBV carrier with family history of HCC, being Asian and > 40 years old, being African/North American black infected with HBV). The distribution of risk factors is different among the world, being HBV the leading cause of HCC in Africa and East Asia, instead HCV is the main cause in Europe, Japan and North American. However, cirrhosis of any aetiology, is the strongest predictor of HCC[25,26].

The main differences in surveillance among guidelines is represented by the use of serologic markers [alpha-fetoprotein (AFP), agglutinin-reactive fraction of AFP, des-gamma-carboxy prothrombin (DCP)] and grouping the patients depending on risk to develop HCC[23,27]. The use of serologic markers in HCC surveillance is still recommended only by the Japan Society of Hepatology (JSH) Guideline and Japan-HCC (J-HCC) Guideline[4,24], while guidelines in many western countries, such as the AASLD Guideline, the National Comprehensive Cancer Network (NCCN) Guideline and the EASL guideline have excluded serologic markers from surveillance criteria[3,8,28].

Guidelines have some differences in definition of high-risk population: JSH Guideline and J-HCC Guideline divide patients in very-high risk population, including individuals with HBV and HCV cirrhosis, and high risk population, including individuals with cirrhosis with other causes or with chronic HBV or HCV infection. The two groups of patients have a different surveillance protocol: very high-risk patients undergo ultrasound (US) and measurement of serologic markers every 3-4 mo, or dynamic CT/MRI every 6-12 mo for patients that are not suitable for US examination, while high-risk patients undergo US and measurement of tumour markers every 6 mo[23].

The NCCN guideline and EASL guideline divide patients in cirrhosis group and non-cirrhosis group, including liver function for EASL guideline. NCCN Guideline considers only cirrhotic patients as candidates for surveillance, while EASL Guideline recommends surveilling the non-cirrhosis group for chronic HCV with advanced fibrosis[3].

In NCCN, EASL and AASLD guidelines surveillance is performed with only US scan every 6 mo. If a nodule $\leqslant$ 1 cm is found at US, EASL and AASLD guidelines recommend another ultrasound examination performed every 3 or 4 mo; if the lesion grows and exceed 1 cm, CT or MRI are performed, instead a stable lesion undergo US follow-up every 3 or 4 mo for 1 or 2 years, with regular checking every 6 mo thereafter. NCCN Guideline recommends CT, MRI or US examination with contrast enhancement (CEUS) at 3 to 6 mo if a nodule < 1 cm is found. MDCT or MRI examinations are mandatory if the nodule at first US examination exceeds 1 cm, and the non-invasive diagnosis of HCC is possible if the nodule shows arterial enhancement and venous equilibrium phase washout. A difference between EASL guideline and AASLD guidelines is that EASL guideline states that typical feature of HCC have to be identified in both CT and MRI for 1 to 2 cm nodules in other than centres of excellence; a single imaging modality is sufficient for 1 to 2 cm nodules for AASLD guideline, independently from the centre of examination. If the typical pattern of HCC is not observed the patient undergo the other imaging modality, and if this is not diagnostic too, biopsy is recommended and if it is inconclusive, US is performed after 4 mo.

All these pathways examined, extensively used in western countries are "size based", while J-HCC, JSH and APASL guidelines' algorithms are "non-size based", with all patients undergo dynamic imaging regardless of nodule size. If dynamic CT/MRI reveal typical HCC pattern a definitive diagnosis can be made. JSH Guideline includes Gd-EOB-DTPA MRI (gadoxetic acid disodium, a liver-specific contrast agent) as a tool for first-line surveillance and diagnosis of HCC. Otherwise, J-HCC guidelines recommend the use of Gd-EOB-DTPA MRI, together with SPIO-MRI, CEUS, CTA and biopsy for nodules larger than 1 cm revealing only hypervascularity with no wash-out, while only US follow-up at 3 mo is suggested for nodule < 1 cm[24].

The APASL Guideline recommend SPIO-enhanced MRI or Sonazoid CEUS for patients with atypical vascular pattern: diagnosis of HCC can be made if there is not uptake, otherwise follow-up is recommended[27].

On summary, the main differences among guidelines are represented by the use or serologic markers for surveillance, the distribution of patients in different risk categories and finally in the use of more detailed

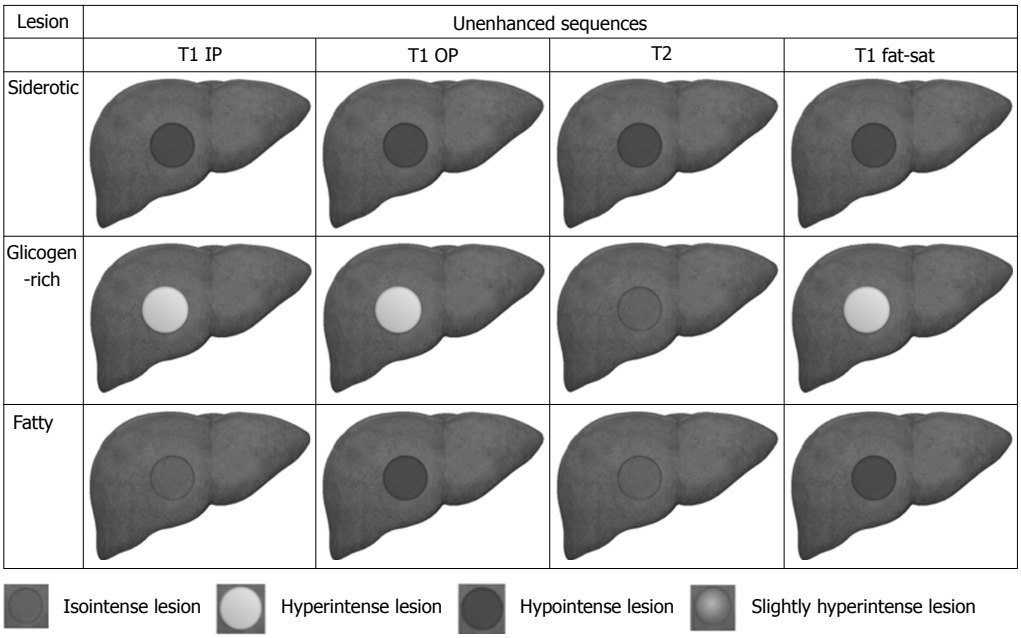

Figure 1 Schematic representation showing pre-contrast magnetic resonance imaging features of cirrhotic nodules such as siderotic (iron reach), glicogen rich and fatty nodule. T1 IP: T1-weighted in-phase image; T1 OP: T1-weighted out-of-phase image.

algorithm in J-HCC, JSH and APASL guidelines in case of hypervascular and hypovascular nodules, which maybe makes them more defined pathways.

RNS AND LGDNS

HCC is a complicated disease with a multi-step process from preneoplastic lesions, including cirrhosis, RN, LGDN, HGDN to HCC[29]. A RN is a well-defined area of liver parenchyma that has enlarged in response to necrosis, altered perfusion or other stimuli and consist of proliferating normal liver cells surrounded by a fibrous stroma[30].

Because of their histopathological nature, RNs are often not visible on T1 and T2 WI. However, they may appear hypointense, isointense or hyperintense related to the background liver on T1-WI or to the presence of paramagnetic materials a glycogen which contributes to T1-WI hyperintensity (Figure 1). On T2-WI, the signal intensity of the RNs is not hyper (unlike HCC) and they are often hypointense or isointense; low signal intensity may be due to iron deposition[9,31]. On DWI, RNs could be iso or less than a few times mild hyperintense compared to the surrounding parenchyma and the likely explanation for this mild hyperintensity was local areas of active fibrosis or infarction[32,33].

These nodules preserve hepatocellular function and lack neoangiogenesis. Thus, after extracellular contrast agent injection, enhancement is similar to, or slightly lower than that of the surrounding liver parenchyma while, after hepatobiliary MRI contrast agent injection, they usually appear iso- or hyper-intense on hepatobiliary phase images when compared to the surrounding liver[9,31].

The pre-malignant potentiality of RNs was controversial, but recently Sato *et al*[34] reported a rate of progression to malignancy of 13.6% at 50 mo and 32% at 100 mo for large RNs and these data stressed the outcome reported by Kobayashi *et al*[35] who reported an evolution rate into HCC of 12.4% in a 5-year period.

A DN is a focal area of hepatocytes $\geqslant$ 1 mm in diameter with dysplasia, without definite histologic malignant features[30]. They are classified into LGDNs and HGDNs based on cytological and architectural atypia as seen on microscopic evaluation.

In LGDNs, the hepatocytes rarely show a clonal population, there is minimal nuclear atypia and only an initial increase in the nuclear/cytoplasmic ratio. Large cell change is often present, but mitotic figures are absent. Without obvious clonal population, the distinction between LGDN and a large RN is difficult and does not comport any practical consequences as long as features of HGDN are absent[30].

The combination of iso- or hyper-intensity on T1-WI and iso- or hypo-intensity on T2-WI strongly suggests a DN. The reasons for the high intensity on T1 images include fatty change, intratumoral copper and increased zinc in the surrounding parenchyma. As RNs, LGDNs are iso or mild hyperintense compared to the surrounding parenchyma on DWI[32,33]. LGDNs display enhancement characteristics similar to that of the background liver parenchyma on all dynamic phases; because they remain mainly supplied by the portal circulation. LGDNs have been recently demonstrated as the tipping point (*i.e.*, pre-HCC state rather than HCC state) of hepatocarcinogenesis[36] and the evolution of dysplastic nodules into early HCC includes appearance of arterial blood supply and stromal invasion[37]. Channual

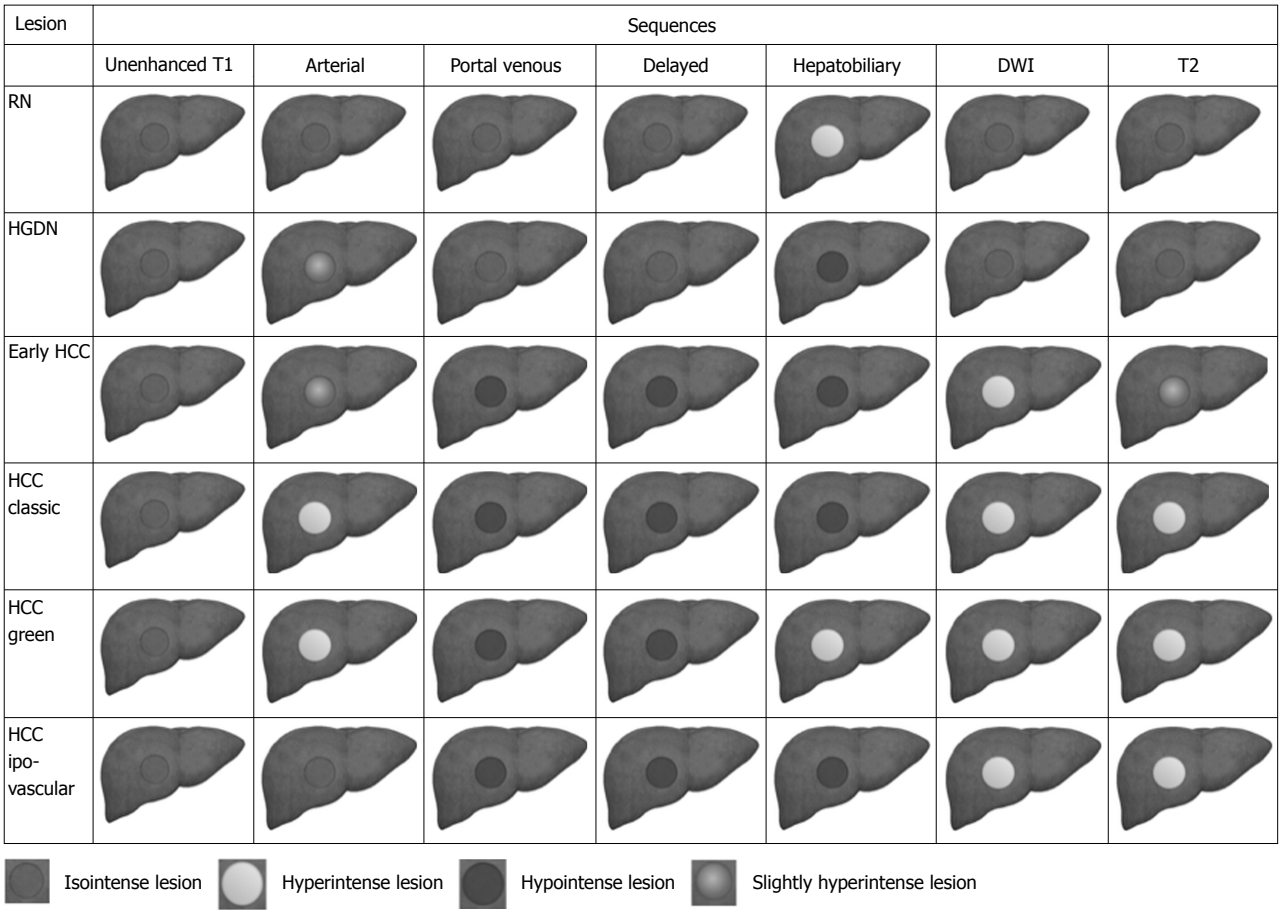

Figure 2 Schematic representation showing dynamic, diffusion weighted images and T2-weighted features, of regenerative nodules, high-grade dysplastic nodule, early hepatocellular carcinoma, classic hepatocellular carcinoma, green hepatocellular carcinoma and hypovascular hepatocellular carcinoma. DWI: Diffusion weighted images; HGDN: High-grade dysplastic nodule; RN: Regenerative nodule; HCC: Hepatocellular carcinoma.

et al[19] recently reported that LGDNs show lower relative intensity ratio on T2-WI and higher unenhanced to arterial signal intensities when compared with HGDNs and HCC.

All LGDNs demonstrated OATP1B3 expression similar to or higher than that of the surrounding liver and because of this OATP1B3 expression, commonly show iso/hyperintensity relative to surrounding liver in the hepatobiliary (HB) phase (Figure 2). According to the latest EASL and AASLD guidelines[3,5], DNs should not be treated or managed as cancers, nevertheless also LGDNs should be followed by regular imaging studies, since as firstly reported by Kobayashi *et al*[35] there is an annual transition rate of 10% for patients with LGDN and a 5-year cumulative transition rate of 30.2 and more recently Sato *et al*[34] studied founded a 50-mo transition rate of 40% in DNs.

Many authors demonstrated that reduction of signal intensity on both the late dynamic and hepatobiliary phase should then be considered an high feature of malignancy and could predict malignant transformation[16,38,39]. Therefore, a more frequent surveillance imaging is fundamental in these cases taking into account that the difference in the rates of malignant transformation between RNs and DNs is also important and highlighting the importance of classifying non-HCC lesions in cirrhotic liver into RNs and DNs. MRI features proposed by different authors for LGDN are summarized in Table 1.

HGDN

HGDNs usually have a vaguely nodular shape and lack of a capsule. Their cells are structured in an irregular trabecular pattern and are increased in density (2 times higher than the normal surrounding parenchyma). HGDNs often contain fat and sometimes copper and/or iron. Unpaired arteries are present even if in small number, and feeding portal veins are diminishing but still present. Finally organic anionic transporting polypeptide (OATP) expression progressively decrease[35]. The histopathological features of HGDNs are responsible for the lack of typical enhancement hallmark of HCC, that is arterial enhancement and delayed wash-out, and for their different appearance on the radiological imaging. MRI, better than other imaging techniques, is able to depict all this hepatocarcinogenesis changes[40].

Depending on iron or fat concentration, HDGNs can differently appear on pre-contrast sequences. Non-siderotic dysplastic nodules typically are hyperintense on T1-weighted sequence and iso-hypointense on T2-

Table 1 Magnetic resonance imaging features of low-grade dysplastic nodules according to various authors

	No. of nodules	T1w	T2w	Arterial phase	Delayed phase	DWI	HB
Golfieri *et al*[10]	38		=/-	=	=		=/-
Bartolozzi *et al*[17]	32	NA		=/+	=/+		+/=
Golfieri *et al*[16]	27	=/+	=/+	=	=		=/+
Chen *et al*[33]	10	+	=/-	=/+	=/-	=/+	
Di Martino *et al*[21]	29	+	-	=/-	=		=/+
Shin *et al*[44]	6	=/-	=/-	=	=	=	

+: Hyperintense; =: Isointense; -: Hypointense; NA: Not available; DWI: Diffusion-weighted imaging; HB: Hepatobiliary.

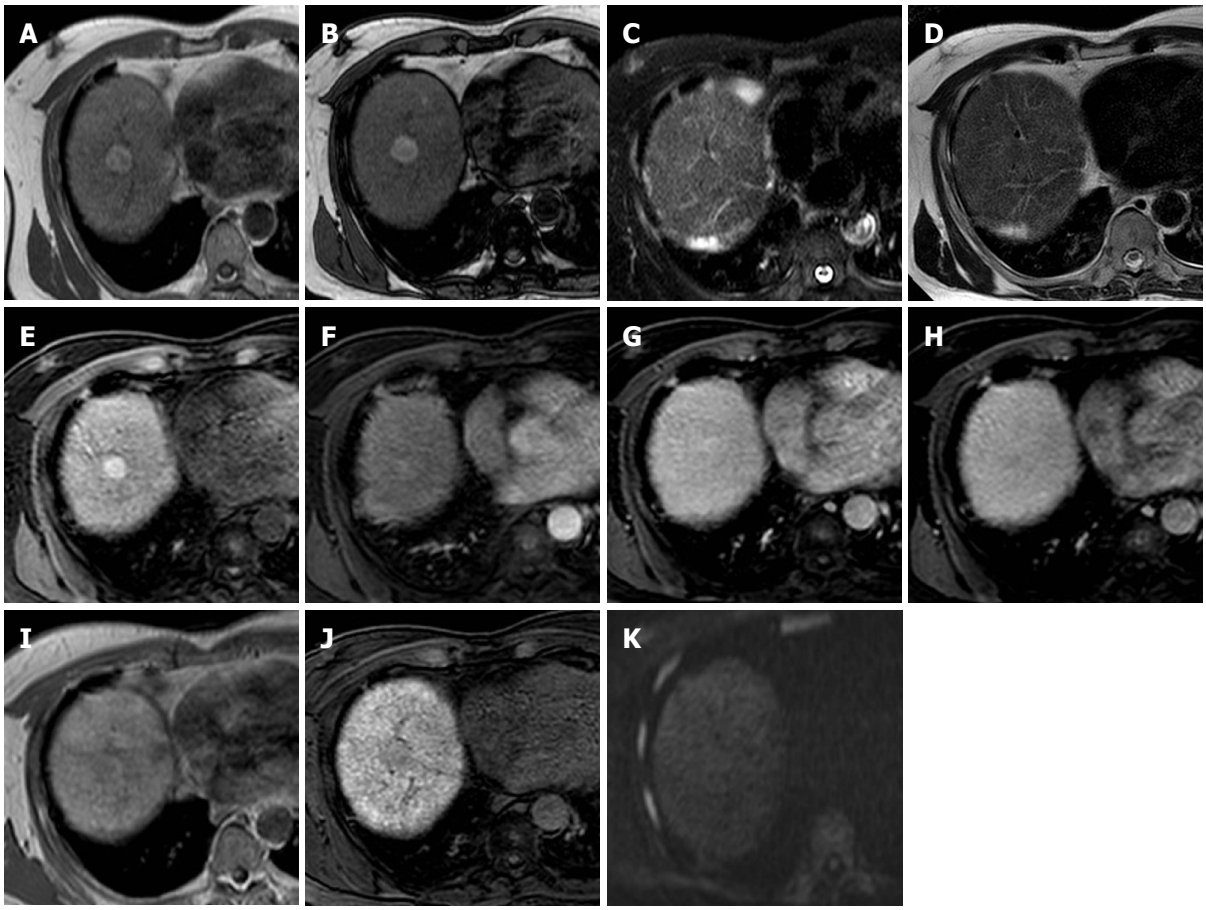

Figure 3 High-grade dysplastic nodule. Gd-EOB-DTPA enhanced MR images of a 57 years old cirrhotic patient with a liver nodule in the Ⅷ segment. A and B: Axial T1-weighted sequences both "in phase" and "out of phase" show a hyperintense nodule; C and D: On T2-weighted image with and without fat saturation the nodule appears as isointense; E-H: during the dynamic contrast-enhanced images the nodule shows a slight enhancement in the arterial phase, without wash-out in portal and delayed phases; I and J: Diffusion weighted image demonstrate no restriction to the diffusion; K: In hepatobiliary phase the nodule is hypointense in comparison to the surrounding liver parenchyma. The MRI features, suggestive of high grade dysplastic nodule, have been later confirmed by the histological examination. MRI: Magnetic resonance imaging.

weighted sequence[41]. With the increase of iron concentration HGDNs will appear as hypointense both on T1w and T2w images, although hyperintensity on T1w in siderotic nodules has been described which is related to low amount of iron[42] (Figure 3). Fat-rich HGDNs show hyperintensity on T1w in-phase images with a signal drop on out-of-phase images[43]. However intracellular fat is typical also in early HCC and can even found in overt HCC. In DWI, which provides information about cellular density, HGDNs have no restriction. For all this reason a correct characterization only based on pre-contrast images is not possible and the use of hepatospecific contrast agent to differentiate pre-neoplastic lesions and HCC is essential. A PubMed search using "high grade displastic nodules", "HGDN", "Gd-EOB-DTPA" and "Primovist" as keywords, identified 9 studies from 2011 to 2017, of whom only 4 focused on vascular pattern[1,11,16,17,44-48]. Typically, after hepatobiliary contrast agent injection, dysplastic nodules appear iso or hypointense on the arterial phase due to the uncomplete capillarization, and hypointense in delayed phase. Bartolozzi *et al*[17], in their study correlated dynamic MRI with histological findings on explanted cirrhotic lesions. They found 30 HGDNs of whom the majority (20/30) were iso-

hypointense on arterial phase and hypointense on late phase and the remaining 10 cases were iso-hyperintense on both arterial and late phases[17]. Kim *et al*[45] confirmed the prevalence of the iso-hypointense appearance of HGDNs in arterial phase. However vascular changes are a dynamic event and HGDNs may show hypervascular arterial enhancement[45]. Golfieri *et al*[10,16] in two different studies (2011 and 2012) reported a non-negligible amount of hypervascular enhancement in arterial phase, probably related to the fact that in both series HGDNs and early HCC were considered in the same group. The different radiological appearance clearly reflect the hemodynamic changes: Hypointense nodules in arterial phase are those lesions with arterial hypovascularity and a normal portal perfusion, isointense nodules are lesions with a perfect balance in the decrease of both arterial and portal blood supply and finally hyperintense nodules are lesions with an increase in arterial vascularity and the complete disappear of portal perfusion. In all studies the majority of HGDNs were hypointense on hepatobiliary phase (HBP). During hepatocarcinogenesis, OATP expression reduces prior to complete neoangiogenesis and increased arterial flow and so dysplastic nodules appear non-hypervascular and hypointense on HBP[9]. HB hypointensity is the most sensitive MRI feature to discriminate benign from malignant/pre-malignant lesions[49]. Moreover according to the literature non-hypervascular nodules with hypointensity on HBP have been shown to develop subsequent hypervascularization (range, 31%-35%) during the follow-up period of 1–3 years, being a risk factor for the development of HCC[50]. The probability to become hypervascular nodule increases with the nodule's dimension. According Kumada *et al*[51] a tumour diameter of 15 mm is the critical threshold for the vascularization of hypointense nodule since, at this size, nodules proliferate more actively and develop unpaired arteries. Akai *et al*[52] confirm these results. In fact, in their series hypointense nodules = 15 mm has a higher risk to progress to overt HCC in comparison to hypointense nodules > 15 mm (HR = 3.55; 95%CI: 0.79.12.3), although no significant difference was observed. As previously reported HGDNs have no restriction in the DWI. Shin *et al*[44] demonstrated that hyperintensity in DWI, combined with high signal intensity on T2, was the most specific feature to differentiate atypical HCCs from dysplastic nodules (sensitivity 80.0%, specificity 100%, positive predictive value 100%, negative predictive value 78.3%) due to the low cellularity of HGDNs when compared to HCC.

To conclude the combination of all MRI features (lesion size, intranodular fat, T2 and T1 intensity, DWI, HBP intensity), improves the characterization of lesions developed in a cirrhotic liver without the typical vascular pattern of HCC[53].

EARLY HCC

The multistep process of hepatocarcinogenesis, from RN to overt HCC, passes through an early stage of HCC. From a pathological point of view cancer cells of early HCC show unremarkable cellular atypia however nuclear-cytoplasmic ratio is increased and cellular density may be more than twice that of the surrounding non-cancerous liver tissue[54]. Early HCC proliferates by replacing adjacent hepatocytes in a trabecular pattern at the boundary with surrounding normal liver tissues, resulting in a poorly demarcated margin[55]. The pathologic features of early HCC closely resemble those of high grade dysplastic nodule (HGDN) and a distinct pathological differentiation between them is still lacking. According the International Consensus Group for Hepatocellular Neoplasia (ICGHN), stromal invasion should be considered as the most characteristics pathologic findings of early HCC[56]. The challenge for the radiologists is not only to differentiate between early HCC and HGDN but also with progressed HCC since early HCC shows a longer time to recurrence and a higher 5-year survival rate[57]. In this setting, magnetic resonance imaging using hepatospecific such as Gd-EOB-DTPA represents a major breakthrough in the proper characterization of liver nodules. In the last years some authors have described the MRI findings of histologically proven early HCC[10,58]. The largest series, published by Sano *et al*[59], described the MRI behaviour of 180 resected early HCC. In this study authors demonstrated that early HCC (n = 30) mostly appear as isointense on T1-WIand isointense or hyperintense on T2-WI. In some cases it may contain fat and appear as hypointense on T1-weighted sequences "out of phase" (Figure 4). Regarding contrast enhancement behaviour early-HCC is mainly hypovascular during arterial phase due to the lack of unpaired arteries. However some early HCCs may show hypervascular foci within them and this is the so called "nodule in nodule" appearance. During portal and delayed phases early HCC mostly appears as hypointense due to the progressive reduction of portal blood flow and in hepatobiliary phase is typically hypointense, because of the absence or the reduction of OATP1B3 carriers[59]. However, hypointensity in the hepatobiliary phase is not an peculiar finding of early or progressed HCC but can also be seen in dysplastic nodules, particularly in high grade[38]. In this setting, hyperintensity on DWI is the more accurate imaging feature to differentiate between early HCC/HCC and HGDN, with a sensitivity of 72.0%, higher than that of hyperintensity on T2 weighted images which is about 40.0% as reported by Hwang *et al*[60]. This finding has been recently confirmed by Renzulli *et al*[61]. In their series of 420 liver nodules, all the 24 histologically proven early-HCC were hypointense on hepatobiliary phase, hypovascular during arterial phase and hyperintense on DWI[61]. The explanation of the restriction to the diffusion, which is responsible for the hyperintensity on DWI, may rely on the increased cellular density during the histologic transition from dysplastic nodule to HCC. MRI features proposed by different authors for HGDN and early-HCC are summarized in Table 2.

HCC

HCC may arise from pre-existing DN or from an early

Table 2 Magnetic resonance imaging features of high-grade dysplastic nodules and early hepatocellular carcinoma according to various authors

	No. of nodules	T2w	Arterial phase	Delayed phase	DWI	HB
High grade dysplastic nodules						
Golfieri *et al*[10]	20	+	+/=	=		-
Golfieri *et al*[16]	41		+/=	=		-
Bartolozzi *et al*[17]	30		-/=	-		-
Choi *et al*[48]	17					-
Sugimori *et al*[46]	7					-
Kim *et al*[45]	17		-/=			-
Shin *et al*[44]	12	-/=			-	
Early HCC						
Sano *et al*[59]	180	=/+	+/=	-		-
Renzulli *et al*[61]	24	=/+	+	-	+	-

+: Hyperintense; =: Isointense; -: Hypointense; DWI: Diffusion-weighted imaging; HB: Hepatobiliary; HCC: Hepatocellular carcinoma.

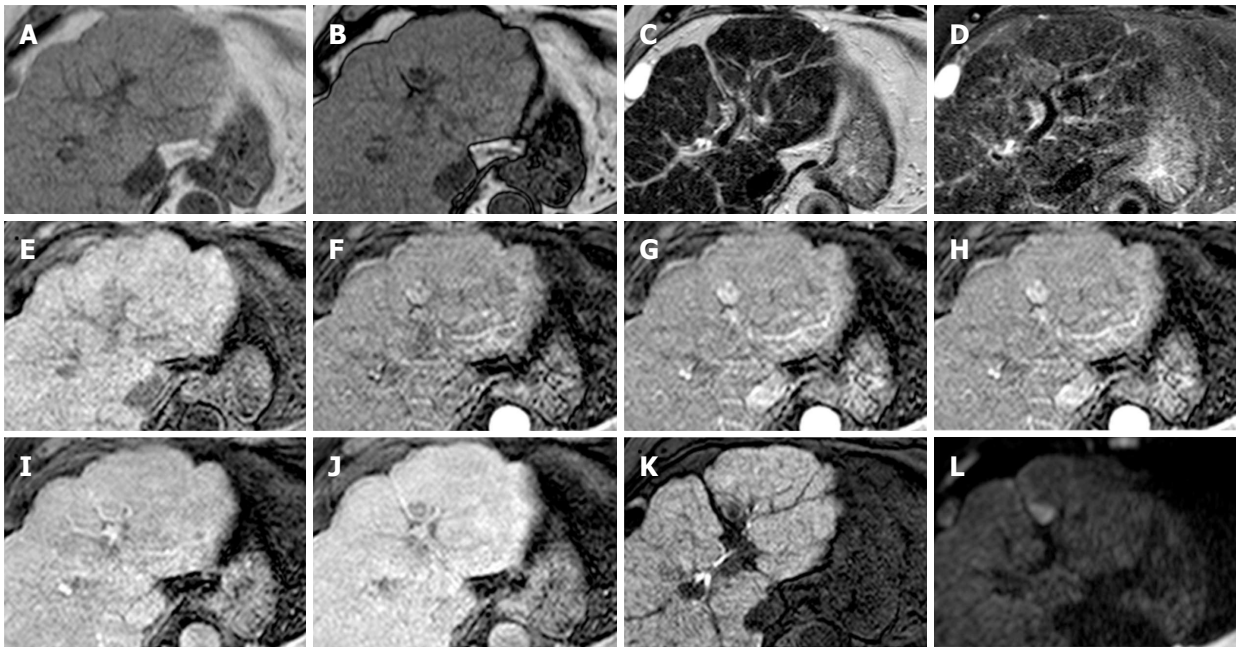

Figure 4 Typical case of lipid-rich hepatocellular carcinoma. A and B: The signal loss of nodule on opposed-phase compared to in-phase indicates intralesional fat; C and D: There is mild signal intensity on T2 and DWI restriction (L); E: The nodule appears hypointense in pre-contrast phase; G-J: Note the typical arterial phase hyperenhancement (G and H) followed by washout appearance in the portal venous (I) and/or delayed phase (J) that is the key diagnostic feature of HCC; K: The lesion also demonstrate hypointensity in the hepatobiliary phase; F-H: Note how the multi-arterial phase imaging (F and H) able improve the conspicuity of the HCC, especially in the late arterial phase (H). DWI: Diffusion weighted images; HCC: Hepatocellular carcinoma.

HCC. In both cases, it can take the macroscopic and radiological feature of the well-known "nodule in nodule". At the earliest stage, progressed HCC can be small, less than 2 cm and it is rarely a diagnostic dilemma either from a radiological and histological point of view. It is characterized by a destructive growth pattern with neoarterialization and microscopic vascular invasion in 1/4 of the cases. Portal tracts are no visible anymore and the borders of the tumour are usually rimmed by fibrosis that create a tumour capsule. Histologically, small but progressed HCC is usually well to moderately differentiated (G1/G2)[62].

According to the AASLD practice guidelines, typical enhancement of HCC during dynamic phases was defined as a combination of iso- to hypo-intensity on precontrast images, hyperintensity on arterial phase images, and hypointensity on PVP or delayed phase images, which represents washout[5]. This is based on previous studies that have demonstrated that HCCs contain solely arterial blood, whereas the normal liver parenchyma contains both arterial and portal blood[63]. However, many nodules in cirrhotic liver may have atypical enhancement patterns especially smaller ones (< 2 cm), and following "the one-third rule", approximately 30% of these are malignant[10].

In fact, HCC nodules smaller than 20 mm may be hypovascular, showing isointensity during the arterial and portal venous phases or may not have wash-out in portal phase[64]. Yoon *et al*[64] reported that the probability to have arterial phase enhancement was not only related to the size of the tumor, but also to the degree

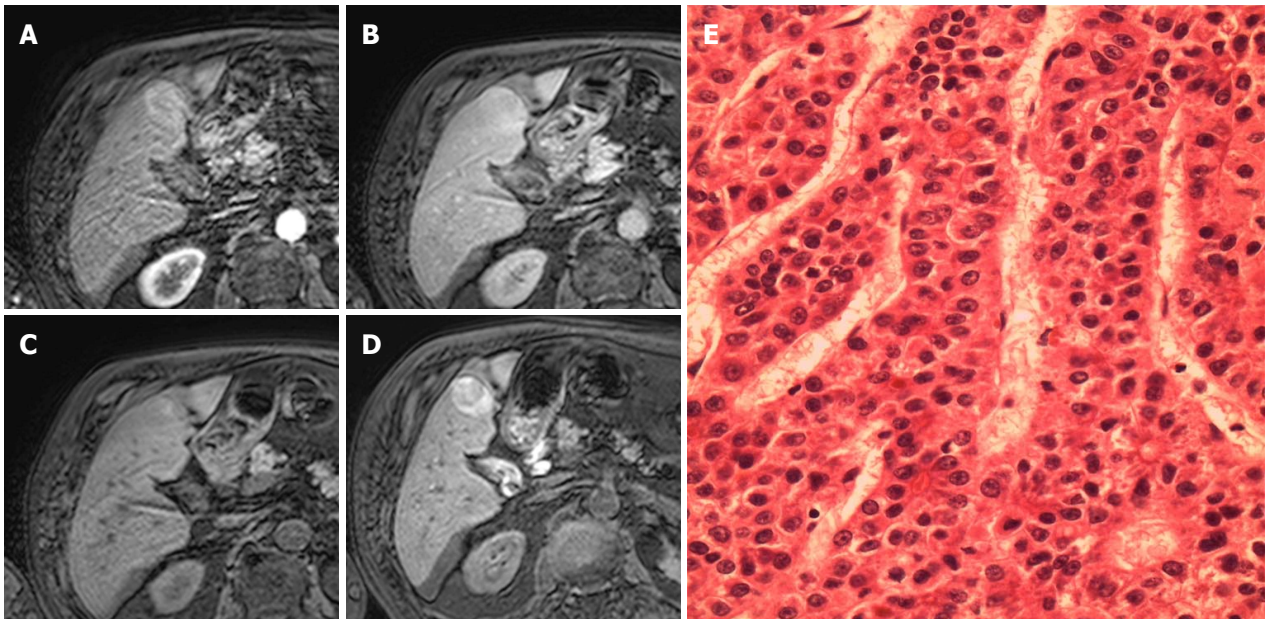

Figure 5 Atypical hepatocellular carcinoma. A and B: There is arterial phase (A) mild hyperenhancement and absence of washout appearance in the portal venous phase (B); C and D: In the delayed phase (C), there is a minimum hypointensity of the nodule, suspected for washout. The nodule resulted hyperintense in hepatobiliary phase (D); E: The lesion was biopsied and the specimen diagnosis was HCC solid-trabecular; G2, pT3a in non-cirrhotic liver with mild (< 5%) macrovescicular steatosis with portal fibrous enlargement and without septae formation.

of tumor differentiation, with poor differentiated HCC nodules having more probability to have arterial phase enhancement. In the same studies, the authors show that also the wash-out in portal phase was also related the degree of dedifferentiation with moderately and poorly differentiated HCCs have more probability to have wash-out than well-differentiated tumors. The atypical enhancement of small HCCs and well-differentiated HCCs during dynamic phase MR imaging may be explained by their immature arterialization during hepatocarcinogenesis[34,35,65,66].

Several previous studies[49,67] have demonstrated that HB phase imaging of hepatocyte-specific contrast agents such as Gd-EOB-DTPA can improve the detection and characterization of small nodules in the cirrhotic liver in comparison with dynamic phase imaging. However, Choi *et al*[68] have shown in their study that 10%-27% of HCCs remain iso- to hyper-intense on HBP images (Figure 5). The signal intensity on HBP may not depend on tumor differentiation, but rather on the degree of OATP8 expression and other potential genetic alterations, with possibility of poor differentiated HCC showing a high degree of OATP8 expression[12,69]. Furthermore, HCC can produce bile, thus appearing macroscopically green after fixing with formalin, the so called "green HCC"[55]. Asayama *et al*[70] in their study, significantly correlated the uptake of Gd-EOB-DTPA with green HCC, even if it was found that the location of uptake of Gd-EOB-DTPA in the tumor did not macroscopically correspond to the greenish areas.

Atypical enhancement pattern on dynamic phase images and iso- to hyperintensity on HBP images were more frequently detected in patients with worse Child Pugh class. The reason is that both the tumor and liver characteristics can affect the relative signal intensity of HCC and the gadoxetic acid uptake in the liver at HBP can particularly be diminished and delayed in the cirrhotic liver[67]. Therefore in cirrhotic patients the imaging interpretation of HCCs has to include a multiparametric assessment with other MR sequences.

However, Renzulli *et al*[61], in a recent study, included HBP hypointensity as an hallmark sufficient for diagnosis of HCC together with arterial phase hyperintensity and DWI restriction, purposing that only these three elements allow the diagnosis, excluding portal venous and delayed phase evaluation. Their diagnostic algorithm shows higher sensitivity and a specificity not very lower than that of the AASLD even for lesion smaller than 2 cm in cirrhotic patients evaluated with Gd-EOB-DTPA MRI, suggesting the increase value of the HBP signal intensity for diagnosis of HCC.

CONCLUSION

In summary, the use of MR with Gd-EOB-DTPA is increasing and progressively changing the standard of diagnosis of HCC and some international groups have boosted its role in new diagnostic algorithms for HCC. Indeed, to date the use of Gd-EOB-DTPA should be considered as an integral part and first line approach in diagnostic management of liver nodules in patients with liver cirrhosis, because it offers information not only related to the vascular pattern but also significant information on hepatocellular function. In most of cases the Gadoxetic acid improves detection of focal liver lesions and the categorization from LGDN form HGDN

and early HCCs lesions, avoiding the liver biopsy, an invasive procedure, that should be considered only in very few and specific cases. Moreover the identification of the borderline nodules from those with progression to HCC on the basis of the imaging appearance, related to hepatocellular function, is critical for determining the better management strategy of these patients and MR with Gd-EOB-DTPA can thus play a crucial role for the correct and non-invasive of HCC's management.

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MINIREVIEWS

Associations between nonalcoholic fatty liver disease and ischemic stroke

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the commonest chronic liver disease and affects a considerable proportion of the general population. NAFLD is independently associated with increased risk for cardiovascular events, particularly coronary heart disease. Importantly, even though NAFLD is more prevalent in patients with major cardiovascular risk factors (*e.g.*, type 2 diabetes mellitus, obesity and hypertension), the association between NAFLD and cardiovascular disease appears to be independent of these risk factors. However, NAFLD also appears to increase the risk for ischemic stroke, a leading cause of mortality and long-term disability worldwide. It also appears that nonalcoholic steatohepatitis is more strongly related to the risk of ischemic stroke than isolated hepatic steatosis. Moreover, emerging data suggest that patients with NAFLD experience more severe ischemic stroke and have more unfavorable prognosis after an acute ischemic stroke in terms of functional dependency and short- and long-term mortality. These associations have major public health implications, since ischemic stroke is the second leading cause of death worldwide and an important cause of long-term disability. The aim of the present review is to summarize the current knowledge regarding the relationship between NAFLD and ischemic stroke incidence, severity and outcome. Given these associations, it might be useful to evaluate patients with acute ischemic stroke for the presence of NAFLD and to manage those with NAFLD more aggressively.

Key words: Nonalcoholic fatty liver disease; Ischemic stroke; Risk; Incidence; Severity; Outcome; Functional dependency

Core tip: Accumulating data suggest that nonalcoholic

fatty liver disease (NAFLD) is independently associated with increased risk for ischemic stroke, a leading cause of mortality and long-term disability worldwide. Moreover, emerging evidence shows that patients with NAFLD experience more severe ischemic stroke and have more unfavorable prognosis after an acute ischemic stroke in terms of functional dependency and short- and long-term mortality.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disease, affects approximately 25% of the general population and is the leading cause of abnormal liver function tests[1,2]. Moreover, the prevalence of NAFLD is even higher in elderly subjects and in patients with type 2 diabetes mellitus (T2DM), obesity, hypertension and/or metabolic syndrome[2,3]. Nonalcoholic steatohepatitis (NASH), a more advanced form of NAFLD, is present in up to 10% of adults[4]. NAFLD can progress to cirrhosis, hepatocellular cancer or liver failure, and is a major cause of liver transplantation[3,5].

Several studies showed that NAFLD is a risk factor for cardiovascular disease (CVD)[6,7]. Importantly, even though NAFLD is more prevalent in patients with major cardiovascular risk factors (*e.g.*, T2DM, obesity and hypertension), the association between NAFLD and CVD appears to be independent of these risk factors (Figure 1)[6,7].

In this context, accumulating data suggest that NAFLD is also associated with increased incidence of ischemic stroke. Moreover, emerging evidence suggests that NAFLD might also be related with more severe stroke and with worse outcome of patients with acute ischemic stroke. These associations have major public health implications, since ischemic stroke is the second leading cause of death worldwide and an important cause of long-term disability[8,9]. The aim of the present review is to summarize the current knowledge regarding the relationship between NAFLD and ischemic stroke incidence, severity and outcome.

NAFLD AND INCIDENCE OF ISCHEMIC STROKE

Several studies reported that elevated aminotransferase and g-glutamyltransferase (gGT) levels, which are mostly due to NAFLD, are associated with increased incidence of ischemic stroke (Table 1). In a small case-control study in 103 patients with acute ischemic stroke and 200 controls, alanine or aspartate aminotransferase levels were independently associated with increased odds ratio for ischemic stroke[10]. In a larger prospective study in 6997 men without established CVD or T2DM, gGT levels, a more specific marker of NAFLD, was independently associated with higher risk of ischemic stroke, even in subjects at low- or moderate cardiovascular risk[11]. In EUROSTROKE, a nested-case control study performed in 3 European countries (Finland, Netherlands and United Kingdom), the association between gGT levels and ischemic stroke risk appeared to be stronger in patients without T2DM[12]. Importantly, gGT appears to play a role in atherogenesis[13]. Indeed, gGT has been isolated from atheromatic plaques, macrophages and foam cells[14] and appears to contribute to atherosclerosis by inducing oxidative stress[15]. It was shown than gGT promotes oxidization of low-density lipoprotein and that it plays a crucial role in the catabolism of glutathione and the release of reactive oxygen species[16,17]. In a recent cross-sectional study, NAFLD diagnosed with ultrasonography was associated with increased prevalence of lacunar infarcts in non-obese subjects but not in obese patients[18]. In contrast, in another recent case-cohort study in 572 patients with incident ischemic stroke and 1017 controls, NAFLD defined as fatty liver index > 60 was associated with lower risk in men but there was no association in women[19]. In a meta-analysis of 9 case-control and cohort studies, NAFLD was independently associated with 2.3 times higher risk for ischemic stroke (95%CI: 1.84-2.93)[20]. The strength of this association was comparable in Caucasian and Asian patients[20]. Importantly, this association was independent of traditional cardiovascular risk factors, including dyslipidemia, obesity and T2DM[20].

It appears that NASH is more strongly related to the risk of ischemic stroke than isolated hepatic steatosis. Indeed, in a case-control study in 295 patients with acute ischemic stroke and 1942 subjects who underwent a health check-up, the degree of liver fibrosis, evaluated with transient elastography, was independently associated with increased stroke risk[21]. In contrast, isolated steatosis was not related with the risk of stroke[21].

NAFLD AND SEVERITY AND OUTCOME OF ISCHEMIC STROKE

There are very few data regarding the impact of NAFLD on the severity and outcome of ischemic stroke (Table 1). In an early prospective study in 200 patients admitted with acute ischemic stroke, NAFLD (defined as elevated aminotransferase levels in the absence of other causes) was present in 42.5% of patients[22]. Patien-

Table 1 Major studies that evaluated the association between nonalcoholic fatty liver disease and ischemic stroke

Ref.	Design	Mean age (yr)	Follow-up	Outcome
[7]	Prospective observational study in 1637 healthy Japanese men/women	47.8	2 yr	Higher incidence of CVD including stroke in patients with NAFLD compared with controls
[22]	Prospective study in 242 patients admitted with acute stroke	66	2 yr	Increased risk of acute stroke, more severe stroke and worse outcome in patients with NAFLD
[11]	Prospective study in 6997 men with no history of CVD or diabetes mellitus	40-59	24 yr	Association between gGT and higher incidence of fatal, major stroke events and total CVD mortality
[10]	Cross sectional study in adults with suspected acute stroke	20-27	Not applicable	Elevated ALP and ALT levels independently associated with higher risk of acute stroke
[12]	Case-control study using data from 3 European cohort studies in 13177 subjects	40-60	3-8 yr	Elevated gGT levels associated with higher risk of stroke
[21]	Case-control study in 295 patients with acute stroke and 1942 healthy subjects	60	Not applicable	Liver fibrosis was associated with higher incidence of ischemic stroke
[20]	Meta analysis of 9 studies that examined relation of NAFLD and stroke	Not reported	Not applicable	Higher risk of ischemic stroke and hemorrhagic stroke in patients with NAFLD
[23]	Retrospective study in 306 patients with confirmed brainstem infractions	65	Not applicable	Higher incidence of brainstem infracts in patients with NAFLD
[24]	Study in 415 patients admitted with acute ischemic stroke	78.8	Duration of hospitalization	NAFLD was not associated with stroke severity at admission or outcome during hospitalization
[19]	Case-cohort study in 572 patients with stroke and 1017 controls	> 45	5.8 yr	Fatty liver index associated with increased risk for ischemic stroke in women and with lower risk in women
[20]	Cross-sectional study in 1277 subjects who underwent brain magnetic resonance imaging and abdominal ultrasound during check-up	> 40 yr	Not applicable	NAFLD diagnosed with ultrasonography was associated with increased prevalence of lacunar infarcts in non-obese subjects but not in obese patients

CVD: Cardiovascular disease; gGT: G-glutamyltransferase; ALP: Alkaline phosphatase; ALT; Alanine aminotransferase; NAFLD: Nonalcoholic fatty liver disease.

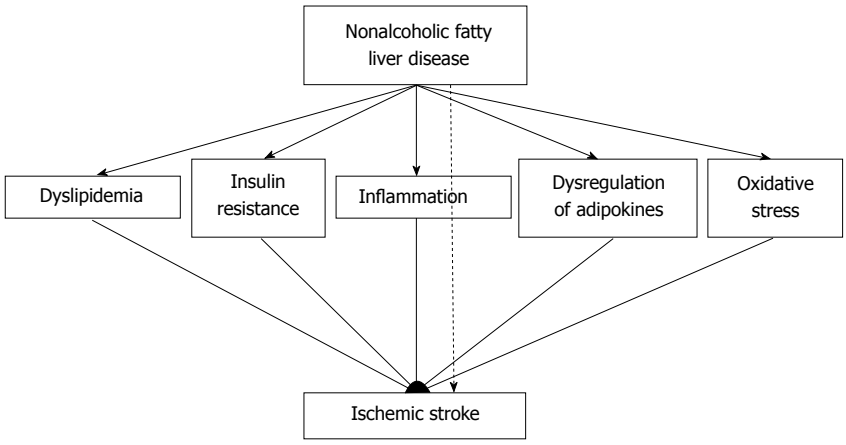

Figure 1 Mechanisms underpinning the association between nonalcoholic fatty liver disease and ischemic stroke (broken line suggests the independent association between nonalcoholic fatty liver disease and ischemic stroke).

ts with NAFLD had more severe stroke at admission and worse functional outcome at discharge[22]. However, patients with NAFLD were more obese, had higher prevalence of T2DM and had higher low-density lipoprotein cholesterol and triglyceride levels than patients without NAFLD; these differences were not adjusted for in the comparisons of stroke severity and outcome between the 2 groups[22]. In a more recent retrospective study in 306 patients with brainstem infarction, a similar prevalence of NAFLD (defined as elevated aminotransferase levels in the absence of other causes) was reported (42.5%)[23]. Patients with NAFLD had more severe stroke at admission and higher risk for neurological deterioration during hospitalization, independently of other risk factors[23]. In contrast, in a prospective study in 415 consecutive patients with acute ischemic stroke, stroke severity at admission, functional outcome at discharge and in-hospital mortality did not differ between patients with NAFLD (defined as elevated aminotransferase levels without another apparent cause) and those without NAFLD after adjustment for other cardiovascular risk factors[24]. However, only 32 patients (7.7% of the study population) had NAFLD[24]. Therefore, this study might have lacked the power to

identify an association between NAFLD and stroke severity and outcome[24].

CONCLUSION

Accumulating data suggest that NAFLD is independently associated with increased risk for ischemic stroke. Moreover, it appears that patients with NAFLD experience more severe stroke and have more adverse functional outcome than patients without NAFLD. Therefore, it might be useful to evaluate patients with acute ischemic stroke for the presence of NAFLD and to manage those with NAFLD more aggressively. It remains to be established whether management of NAFLD will also reduce the risk and improve the outcome of ischemic stroke.

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MINIREVIEWS

Status and perspective of laparoscopic repeat liver resection

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Abstract

Liver resection (LR) is now actively applied to intrahepatic recurrence of liver metastases and hepatocellular carcinoma. Although indications of laparoscopic LR (LLR) have been expanded, there are increased risks of intraoperative complications and conversion in repeat LLR. Controversy still exists for the indication. There are 16 reports of small series to date. These studies generally reported that repeat LLR has better short-term outcomes than open (reduced bleedings, less or similar morbidity and shorter hospital stay) without compromising the long-term outcomes. The fact that complete adhesiolysis can be avoided in repeat LLR is also reported. In the comparison of previous procedures, it is reported that the operation time for repeat LLR was shorter for the patients previously treated with LLR than open. Furthermore, it is speculated that LLR for minor repeat LR of cirrhotic liver can be minimized the deterioration of liver function by LR. However, further experience and evaluation of anatomical resection or resections exposing major vessels as repeat LLR, especially after previous anatomical resection, are needed. There should be a chance to prolong the overall survival of the patients by using LLR as a powerful local therapy which can be applied repeatedly with minimal deterioration of liver function.

Key words: Hepatocellular carcinoma; Laparoscopic liver resection; Repeat surgery; Metastasis

Core tip: There are 16 reports of repeat laparoscopic liver resection (LLR). They reported that it has better short-term outcomes than open (reduced bleedings, less or similar morbidity and shorter hospital stay). The fact that complete adhesiolysis can be avoided in repeat LLR is also reported. It is speculated that LLR for minor repeat LR of cirrhotic liver can be minimized the deterioration of liver function by LR. Repeated application of LLR as a powerful local therapy, which can be applied repeatedly with minimal deterioration of liver function, could improve the overall survival of the patients.

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INTRODUCTION

The neoplastic liver background of hepatocellular carcinoma (HCC) with chronic liver disease (CLD) develops multifocal and metachronous liver tumors repeatedly. Also, metastases of various tumors can occur repeatedly in the liver. Repeat treatments for HCC and metastases, especially of colorectal cancer, are often needed.

Nowadays, liver resection (LR) is often performed to such lesions, if they are resectable without other uncontrollable/distant disease, and the reports for repeat LR has increased[1-4]. Furthermore, indications of laparoscopic LR (LLR) are expanding with the accumulation of experiences and technical/instrumental developments[5-8]. In LLR, surgeons should overcome restricted manipulation, lack of tactile sensation and three-dimensional (3D) vision (which is recently partially resolved by 3D-laparoscope), and disorientation from the lack of an overview of operative field, during liver mobilization, pedicle control and parenchymal transection, which is a trade-off to magnified fine local view[9,10]. Postoperative adhesions with the need for adhesiolysis are known to increase the operation time of subsequent surgeries and the incidence of bowel injury[11,12]. Therefore, increased rates of complications and conversion from laparoscopic to open surgery had been reported in repeat laparoscopic surgery[13]. A previous history of surgery had been among the contraindications for laparoscopic surgery. However, many laparoscopic procedures with previous surgical history, such as cholecystectomy[12,13], appendectomy[14], colectomy[15], and gastrectomy[16], can be performed nowadays with technical and instrumental improvements. On the other, LLR itself remains a demanding procedure and the indications of repeat LLR are under discussion. Adequate dissection of adhesion and mobilization of the involved liver should be performed before repeat LR. Adhesion can disrupt the dissection of hilar area and hepatoduodenal ligament, which is often crucial in LR. The deformity of the liver and surrounding scars and adhesion makes the localization of tumors and the important structures (vessels) difficult. The fact that liver capsule bleeds easily during adhesiolysis and mobilization leads to increase the intraoperative bleeding and create a suboptimal operative field[17]. These changes after previous surgery can increase the risks of intraoperative injury to vascular or biliary structures.

STUDIES OF REPEAT LLR

Only 16 reports of small series were found out under Medline-search with the words "repeat" and "laparoscopic liver resection" and their re-quotations[18-33] (Table 1), although they are gradually increasing. Belli *et al*[20] reported that LLR with its magnifies view facilitates more meticulous dissection of adhesions strained by the pneumoperitoneum. An additional possible advantage of repeat LLR is reported that complete adhesiolysis can be avoided when the adhesion does not affect the current operative procedure[24,29]. Generally, these studies reported that repeat LLR has better short-term outcomes (similar or longer operation time, reduced bleedings, less blood transfusion, less or similar morbidity and shortened hospital stay) with the comparable long-term outcomes. Each study concluded that repeat LLR is feasible and safe for selected patients, although those studies are the mixtures of the patients with HCC and metastases. The settings of the patients with HCC and metastases are different in LR. The patients with metastases sometimes undergo major LR with the handling of Glissonian pedicles on the soft liver with congestion and/or steatosis. Minor LR on the fibrous hard liver with poor functional reserve and surrounding collateral vessels is often performed for HCC patients. Five studies of repeat LLR, which only include HCC patients[20,24,26,27,31], reported the outcomes for the series of 12, 6, 3, 20 and 8 patients. The conclusions of all studies are that repeat LLR for recurrent HCC in CLD backgrounds is a safe and feasible procedure. It is mentioned that the adhesiolysis was easier and the operation time was shorter in repeat LLR for the patients with previous LLR compared to open LR[20]. Belli *et al*[20] referred the advantages of LLR for the management during the long history with repeat oncogenesis in cirrhotic patients. Kanazawa *et al*[27] mentioned that the complication rate and the hospital stay had been decreased in their institute by the introduction of LLR for recurrent HCC patients.

LLR CHARACTERISTICS

It is previously reported that LLR is especially beneficial for severe CLD patients[34]. LLR with minimal laparotomy and mobilization can minimize the destruction of blood and lymphatic collaterals, as well as the parenchymal injury by compression. It reduces postoperative ascites and liver failure for CLD patients[35]. In LR, resection of the liver inside the subphrenic rib cage is performed. The cage is opened with a big subcostal incision and then the liver is picked up with mobilization in open LR. On the other, laparoscope and forceps intrude into the cage directly from the caudal direction ("Caudal approach"[36-38], Figure 1) and perform LR in the small targeted area without damages to the surrounding area in LLR. LLR also facilitates the usage of postural change and the gravity for handling organs/tumors, since the same surgical view under position changes can be established by the adjustments of laparoscope's positioning and rotation. That reduces compression on the liver during surgery. Our previous

Table 1 Summary of previous reports of repeat laparoscopic liver resection

n	Disease	Previous LR (open:lap)	Procedure	Bleeding (mL)	Operating time (min)	Conversion (*n*)	Postoperative hospital stay (d)	Morbidity	Mortality	Ref.
12	HCC	4:8	LLS (*n* = 5), Pt (*n* = 4), Seg (*n* = 3)	297 ± 134 272.2 ± 120	114.4 ± 11.0 63.9 ± 13.3	1	7.4 ± 2.5 6.2 ± 3.0	26.60%	0%	[20]
2	Met	ND	ND	ND	ND	ND	ND	ND	ND	[21]
6	HCC	3:3 (Lap RFA, *n* = 2)	LLS (*n* = 2), Pt (*n* = 4)	283.3 ± 256.3	140.8 ± 35.7	0	5.67 ± 1.63	16.7%	0%	[24]
76	Met (*n* = 63), HCC (*n* = 3), others (*n* = 10)	28:44	LLS (*n* = 4), Pt, seg (*n* = 53), above-seg (*n* = 19)	300 (0–5000)	180 (80–570)	8	6 (2–42)	26%	0%	[23]
4	HCC (*n* = 3), Met (*n* =1)	0:4	LLS (*n* = 1), Pt (*n* = 3)	481.7 ± 449.5	312.3 ± 158.4	1	10.6 ± 7.4	23.4%	0%	[22]
3	HCC	0:3	ND	281.3 (mean)	264.6 (mean)	0	8.6 (mean)		0%	[26]
17	ND	ND	ND	ND	ND	ND	ND	ND	ND	[25]
20	HCC	15:5	Pt	78 (1–1500)	239 (69–658)	2 (HALS)	9 (5–22)	5%	0%	[27]
20	HCC (*n* = 2), Met (*n* = 16), others (*n* = 2)	0:20	Minor (*n* = 14), major (*n* = 6)	400 (IQR 150-200 mL)	285 (IQR 195-360)	3	4 (1-57)	10%	0%	[30]
12	HCC (*n* = 8), Met (*n* = 2), others (*n* = 2)	8:4	Pt (*n* = 9), Subseg (*n* = 3)	50 (NC–840)	301 (104–570)	0	12 (9–30)	0%	0%	[29]
11	HCC	6:5	LLS = 2 Subseg = 9	100 (50-500)	200 (131-352)	0	6 (3-17)	18.2%	0%	[33]
27	Met	ND	Major = 25 Minor = 2	ND (4 patients received transfusion)	252.5 (180-300)	1	9 (IQR 8-18)	48.1%	0%	[32]
8	HCC	6:2	Sec = 2 Seg = 2 Subseg = 4	200 (30-5000)	343 (120-530)	1	3.5 (3-8)	12.5%	0%	[31]
20	HCC (*n* = 15) Met (*n* = 5)	12:8	Anatomical = 1 Non-anatomical = 19	159 +/- 256	225 +/- 85	1	14.2 +/- 5.4	0%	0%	[19]
33	HCC and combined (*n* = 18) Met (*n* = 15)	21:12	Anatomical = 11 Non-anatomical = 22	30 (NC-1012)	217 (43-356)	0	6.5 (3-47)	6.1%	3%	[18]

Data are expressed as median (range) or mean ± SD, unless stated otherwise. In the paper from Belli, operation time, bleeding and postoperative hospital stay are described separately for patients whose previous hepatectomy was open (upper) or laparoscopic (lower). LLR: Laparoscopic liver resection; LR: Liver resection; HCC: Hepatocellular carcinoma; LLS: Left lateral sectorectomy; Met: Metastasis; Minor: Resection of 2 segments or less; Major: Resection of 3 segments or more; ND: Not documented; Pt: Partial resection; Sec: Sectionectomy; Seg: Segmentectomy; Subseg: Subsegmentectomy; IQR: Interquartile range; NC: Not countable.

report of the caudal approach posterior sectionectomy in the left lateral position[36] posed the novel concept of "caudal approach" in LLR. Although the supine to semi-lateral positioning had been employed for the other resections, the transection plane of posterior sectionectomy was horizontal and gravity obstructs the exposure of the plane in the supine position. A clear view from the caudal direction and an easy access to postural changes is among the advantages of LLR (Figure 1). We perform parenchymal transection prior to mobilization in the left lateral position for laparoscopic posterior sectionectomy. It facilitates exposure of the cutting plane during the transection in caudal-to-cranial one direction. The transection plane is well-opened between the retroperitoneal-fixed posterior section and the remnant liver falling down to left by gravity. Moreover, the resection of segment(s) 7 should be performed in the deeper and smaller cranial subphrenic space and S6 is an obstacle under the laparoscopic caudal view even in the left lateral position. Semi-prone position with only partial dissection of the retroperitoneum is employed for those resections[39]. Our key aim in LLR is to carry out minimal dissection around the liver with the intrusion and manipulation of laparoscope and forceps to the small target area under postural changes. In the same context, repeat LLR requires smaller (than open) working space between adhesions. Direct approach to the tumor after

Table 2 The summary of present status and future perspectives of repeat laparoscopic liver resection

Present status
There are 16 reports of small series. Controversy still exists in the indication of repeat LLR
These studies generally reported that it has better short-term outcomes without compromising the long-term outcomes (similar or longer operation time, reduces bleedings, reduced blood transfusion rate, less or similar morbidity and shorter hospital stay)
It facilitates more meticulous dissection of adhesions strained by the pneumoperitoneum using magnified laparoscopic view
Complete adhesiolysis can be avoided when the adhesion does not affect the current operative procedure
Operation time was shorter and the adhesiolysis was easier for the patients previously treated with LLR than open LR
It requires smaller (than open) working space between adhesions (this fact allows for minimal adhesiolysis, and operation time and bleeding amount were similar in primary and repeat LLR, although those from open LR are longer and increased)
Future perspectives
Further evaluations of anatomical resection or resections exposing major vessels after previous anatomical resection are needed
One of the possible advantages for minor repeat LR of CLD liver is that the deterioration of liver function can be minimized
It could prolong the overall survival of the HCC patients with CLD as a powerful local therapy which can be applied repeatedly with minimal deterioration of liver function

LLR: Laparoscopic liver resection; LR: Liver resection; HCC: Hepatocellular carcinoma; CLD: Chronic liver disease.

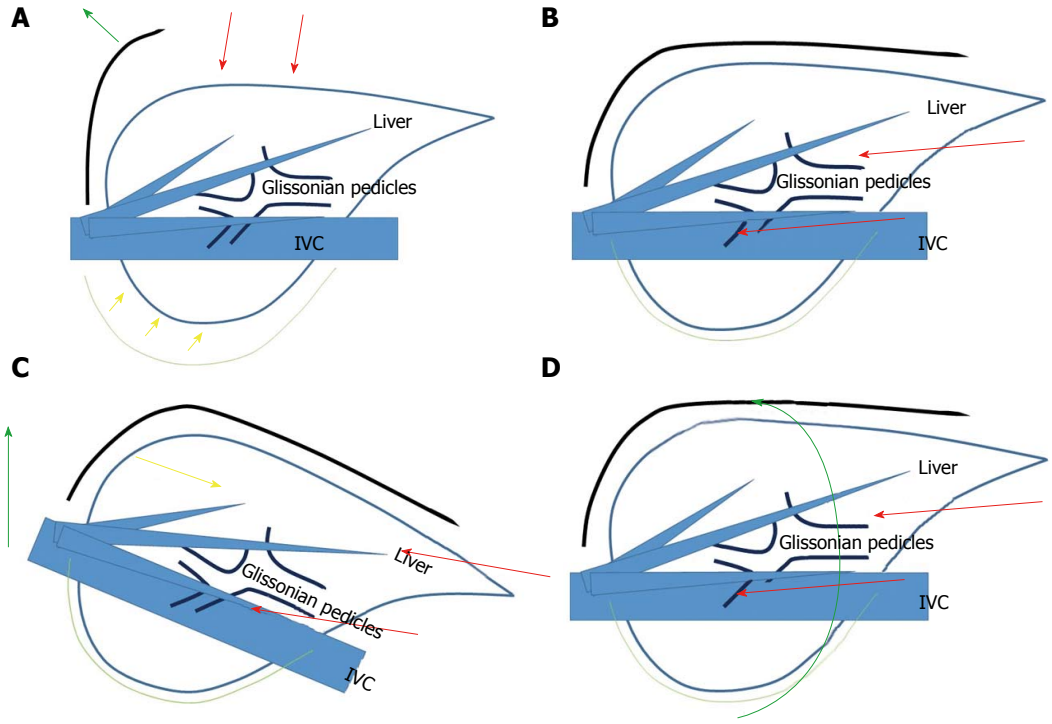

Figure 1 Schema of open liver resection (A), laparoscopic liver resection (B), position change in laparoscopic liver resection (tilting the bed for head-up position, C) and position change in laparoscopic liver resection (rotation from supine to semi-prone position, D). Red arrows indicate the directions of the view and manipulation in each approach. A: In the open approach, the subcostal cage containing the liver is opened with a large subcostal incision, and instruments are used to lift the costal arch up. The liver is dissected and mobilized (picked up) from the retroperitoneum; B: In the laparoscopic caudal approach, the laparoscope and forceps are placed into the subcostal cage from caudal direction, and surgery is performed with minimal alteration and destruction of the associated structures; C and D: In the laparoscopic approach, the same surgical view under position changes (tilting the bed and rotation of the patient's body), acquired by the adjustments of laparoscope's positioning and rotation, allows for handling large-volume liver/tumor by postural changes.

minimal adhesiolysis for the space where laparoscope and forceps can intrude and do manipulation can be allowed especially in repeat small LLR[23,24,29]. That is why some studies showed that operation time and bleeding amount were similar in primary and repeat LLR[18,29]. The operation time and blood loss are usually much longer and larger in open repeat than open primary LR. Operation time and bleeding amount of repeat partial resection could be reduced under laparoscopic approach.

OUR EXPERIENCES AND FUTURE PERSPECTIVES OF REPEAT LLR

Most reported cases of repeat LLR underwent minor resection of HCC with CLD, as mentioned above. The impact of alterations from the previous surgery on hepatic parenchyma and intrahepatic structure could be smaller in such cases. There were three repeat cases with anatomical resection or resections exposing major vessels (including S8 segmentectomy after

4-times LLR[40]) after previous anatomical resection who developed bile leakage and > 30 d hospital stay, among our 33 repeat and 12 three or more-time repeat LLR cases. Anatomical alterations surrounded by the scars and adhesions on major vessel structures could have big impacts on subsequent anatomical resection or resections exposing major vessels, experiences and evaluations of such setting of repeat LLR are required for the settlement (Table 2).

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MINIREVIEWS

Balloon-occluded transcatheter arterial chemoembolization for hepatocellular carcinoma

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Abstract

Transcatheter arterial chemoembolization (TACE) is widely accepted as a treatment for patients with hepatocellular carcinoma (HCC) in the intermediate stage according to the Barcelona Clinic Liver Cancer (BCLC) guidelines. Recently, balloon-occluded TACE (B-TACE) was developed in Japan. Despite the lack of a clear definition, B-TACE is generally defined as the infusion of emulsion of chemotherapeutic agents with lipiodol followed by gelatin particles under the occlusion of feeding arteries by a microballoon catheter, which leads to the dense lipiodol emulsion (LE) accumulation in HCC nodules. This phenomenon cannot be explained only by the prevention of proximal migration and leakage of embolization materials; it further involves causing local changes in the hemodynamics of the surrounding occlusion artery and targeted HCC nodules. Balloon-occluded arterial stump pressure plays an important role in the dense LE accumulation in targeted HCC nodules. Although randomized controlled trials comparing the therapeutic effect and the prognosis of B-TACE to those of the other TACE procedures, such as conventional-TACE and drug-eluting beads TACE, are still lacking, B-TACE is thought to be a promising treatment. The purpose of this review is to summarize the mechanism, therapeutic effect, indication, prognosis and complications of B-TACE.

Key words: Hepatocellular carcinoma; Treatment effect; Transcatheter arterial chemoembolization; Prognosis; Balloon-occluded arterial stump pressure; Dense lipiodol emulsion accumulation; Balloon-occluded transcatheter arterial chemoembolization; Microballoon catheter

Core tip: Balloon-occluded transcatheter arterial chemoembolization (B-TACE) was recently developed in Japan. Despite the lack of a clear definition, B-TACE is generally defined as the infusion of emulsion of chemotherapeutic agents with lipiodol followed by gelatin particles under the occlusion of feeding arteries by a microballoon catheter. Although randomized controlled trials comparing the therapeutic effect and the prognosis of B-TACE to those of the other TACE procedures are still lacking, B-TACE is thought to be a promising treatment. The purpose of this review is to summarize the mechanism, therapeutic effect, indication, prognosis and complications of B-TACE.

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INTRODUCTION

Liver cancer ranks sixth as the common malignant neoplasm and second as the cause of death worldwide[1], and hepatocellular carcinoma (HCC) is the most common type of malignant liver tumors[2]. According to the Barcelona Clinic Liver Cancer (BCLC) guidelines, transcatheter arterial chemoembolization (TACE) is an established treatment for patients with HCC in the intermediate stage[3]. The median survival of untreated patients at the intermediate stage, who present with multinodular without vascular invasion or extrahepatic metastasis, performance status 0 and Child-Pugh class A or B, is reported to be 16 mo[4,5], and TACE prolongs their overall survival compared to that of a control group[6,7].

TACE is performed through the injection of single or multiple chemotherapeutic agents after the catheterization of tumor-feeding arteries, followed by the embolization of the same vessels in order to gain a synergistic effect of cytotoxicity and ischemia[3,8]. Conventional-TACE (C-TACE) is defined as the injection of a mixture of anticancer agents with lipiodol followed by embolic materials, such as gelatin particles, calibrated microspheres or polyvinyl alcohol, and drug-eluting beads TACE (DEB-TACE) is defined as the infusion of microspheres onto which chemotherapeutic agents is loaded or adsorbed to achieve a sustained *in vivo* drug release[8,9].

Recently, balloon-occluded TACE (B-TACE), which was first reported by Irie *et al*[10], has been developed in Japan. Although it is not mentioned in several guidelines[3,11,12] and its clear definition has not been established, it is generally defined as the infusion of emulsion of chemotherapeutic agents with lipiodol followed by gelatin particles under the occlusion of feeding arteries by a microballoon catheter. The occlusion of the feeding arteries result in the dense lipiodol emulsion (LE) accumulation in targeted nodules[10]. The therapeutic effect of B-TACE was better than that of C-TACE according to several previous reports[13-15], although those were retrospective and small in scale. In addition, randomized controlled trials (RCTs) comparing the therapeutic effect and the prognosis of B-TACE to those of other TACE procedures are still lacking.

Table 1 depicts the key findings of the therapeutic effect and overall survival of B-TACE. With this potential limitation in mind, in the present review, we describe the current understanding of the mechanism, therapeutic effect, indication, overall survival and complications of B-TACE.

BALLOON-OCCLUDED TACE PROCEDURE

Microballoon catheters

A 3-Fr microballoon catheter was used in the study reported by Irie *et al*[10]. However, it is sometimes difficult to advance a microballoon catheter into the targeted tumor-feeding arteries selectively[16]. Moreover, a 5- or 6-Fr guiding catheter was required when we used a 3-Fr microballoon catheter for B-TACE, which is more invasive than C-TACE[16]. Microballoon catheters have improved in recent years, and a 1.8-Fr microballoon catheter (Logos, Piolax, Kanagawa, Japan; or Attendant, Terumo Clinical Supply, Tokyo, Japan) is now available, enabling us to insert the catheter more selectively and more peripherally[16]. We are able to coaxially advanced these microballoon catheters through a typical 4-Fr parent catheter[16]. The proximal side consists of two ports in these microballoon catheters: the microballoon lumen and the microguidewire lumen, the diameter of which is 0.017-0.018 inches[16].

B-TACE procedure

Although a clear definition of B-TACE has not been established and several details concerning the procedure vary markedly among institutions, we will attempt to briefly describe the main points of B-TACE.

The right femoral artery is punctured under local anesthesia. A 4- or 5-Fr parent catheter is then placed in the celiac artery (CeA) or superior mesenteric artery (SMA), and angiography is carried out to assess the anatomy of the hepatic artery, tumor stains, the feeding arteries and arteriovenous shunting. In some institutions, cone-beam computed tomography (CBCT) and angiography-assisted computed tomography (CT) is performed to diagnose the HCC and evaluate the tumor-feeding arteries. A microballoon catheter is inserted into the tumor-feeding arteries over the guidewire as selectively as possible. When the selective insertion of the microballoon catheter into the targeted arteries is difficult, such as in patients with tortuous anatomies

Table 1 Summary of retrospective studies of therapeutic effect and overall survival of balloon-occluded transcatheter arterial chemoembolization for hepatocellular carcinoma

Ref.	Year	No. of patients	Antitumor agent and embolic materials	Therapeutic effect	Overall survival	Commentary
Hatanaka *et al*[43]	2018	66	Miriplatin-lipiodol suspension + gelatin particle	CR 53.0%, PR 10.6%, SD 19.7%, PD 16.7%, RR 63.6%	The 1-, 2-, and 3-yr survival rates were 76.8%, 57.3%, and 46.7%, respectively	The number of tumors and α-fetoprotein level were predictive factors for the tumor response and serum albumin and overall response (CR + PR) were predictive factors for prognosis
Kawamura *et al*[40]	2017	30	Miriplatin-lipiodol suspension + gelatin particle	TE4 51.0%, TE3 8.5%, TE2 19.1%, TE1 21.3%, RR 59.6%	NA	The presence of portal vein visualization, tumor on the subcapsular portion, and successful subsegmental artery embolization were predictive factors for the tumor response
Maruyama *et al*[20]	2016	50	Epirubicin-lipiodol suspension + gelatin particle	There was no statistically significant difference between the B-TACE group and the C-TACE group	NA	
Irie *et al*[15]	2016	28	Doxorubicin, mytomysin-lipiodol suspension + gelatin particle	TE4 89.3%, TE3 10.7%, TE2 0%, TE1 0%, RR 100%. The local recurrence rates at 1, 3, and 5 yr were 92.4%, 69.9%, and 69.9%, respectively	The 1-, 3-, and 5-yr survival rates were 96.4%, 60.3% and 31.1%, respectively	B-TACE was an independent factor for improving both the control rate of the primary nodule and the overall survival rates
Asayama *et al*[32]	2016	29	Miriplatin-lipiodol suspension + gelatin particle	TE4 8.6%, TE3 48.6%, TE2 17.1%, TE1 25.7%, RR 57.1%	NA	
Ogawa *et al*[14]	2015	33	Miriplatin-lipiodol suspension + gelatin particle	TE4 49.2%	NA	The percentage of TE4 in B-TACE was significantly higher than that in the C-TACE
Minami *et al*[41]	2015	27	Miriplatin-lipiodol suspension + gelatin particle (epirubicin was used in 3 patients)	Countable HCC (n = 17): TE4 43.8%, TE3 12.5%, TE2 37.5%, TE1 6.3%, RR 56.3%, Uncountable HCC (n = 10): CR 0%, PR 0%, SD 10%, PD 90%	NA	
Arai *et al*[13]	2014	49	Miriplatin-lipiodol suspension + gelatin particle	TE4 55.1%, TE3 38.8%, TE2 4.1%, TE1 2.0%, RR 93.9%	NA	
Ishikawa *et al*[44]	2014	51	Miriplatin-lipiodol suspension + gelatin particle	The local recurrence rates at 6, 12 mo were 11.1 %, 26.2%, respectively. The medan recurrence time was 9 mo	NA	The CT value just after B-TACE was a predictive factor for the tumor response

According to Response Evaluation Criteria in Cancer of the Liver (RECICL), the treatment effect (TE) was defined as follows: TE4, tumor necrosis of 100% or 100% reduction; TE3, tumor necrosis of 50%-100% or 50%-100% reduction in tumor size; TE1, tumor enlargement of > 50% regardless of necrosis; TE2, effect other than TE3 or TE1. B-TACE: Balloon-occluded transcatheter arterial chemoembolization; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; RR: Response rate; NA: Not available; C-TACE: Conventional transcatheter arterial chemoembolization; CT: Computed tomography.

of the CeA or SMA, using a microballoon catheter as an anchor might be useful for achieving deep cannulation with the parent catheter[17]. The tumor-feeding artery is then occluded, and the emulsion of lipiodol and anticancer drugs is infused under radiographic guidance. The proportion of lipiodol to anticancer drugs was as follow: 70 mg of Miriplatin[18,19], which is most commonly used in B-TACE, was suspended with 3.5 mL of lipiodol (20 mg/mL); 10 mg of epirubicin[20] with 1-2 mL of lipiodol (5-10 mg/mL); 10 mg of doxorubicin hydrochloride and 2 mg of mitomycin C with 5-10 ml of lipiodol (5-10 mg/mL and 1-2 mg/mL, respectively)[10,15]. Digital angiography is sometimes useful for checking for the flow of LE droplets outside the target areas. LE is infused until sufficient filling of the targeted nodule, overflow into the intrahepatic collateral arteries or the presence of portal vein visualization is observed. The fragmented gelatin sponge slurry is then injected into

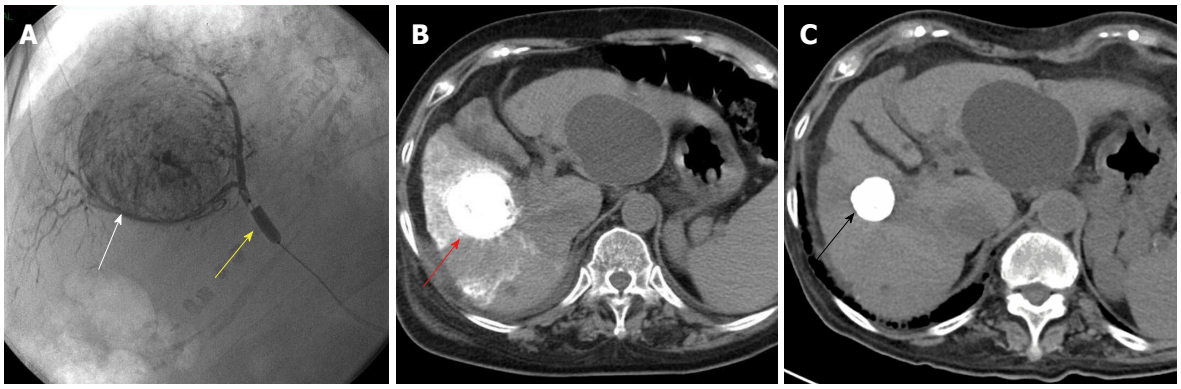

Figure 1 A 71-year-old female patient with hepatitis-C related hepatocellular carcinoma 45 mm in diameter underwent balloon-occluded transcatheter arterial chemoembolization. A: A 3-Fr microballoon catheter was inserted into a tumor-feeding artery. After achieving occlusion with the microballoon catheter (yellow arrowhead), emulsion of lipiodol and miriplatin was infused until the cancer nodule (white arrowhead) was sufficiently filled. Fragmented gelatin sponge slurry was then injected; B: Just after B-TACE, computed tomography (CT) showed a dense lipiodol emulsion (LE) accumulation in HCC nodules (red arrowhead); C: Four years after B-TACE, CT showed that the volume of the LE accumulation was reduced (black arrowhead) with no local recurrence. HCC: Hepatocellular carcinoma; B-TACE: Balloon-occluded transcatheter arterial chemoembolization.

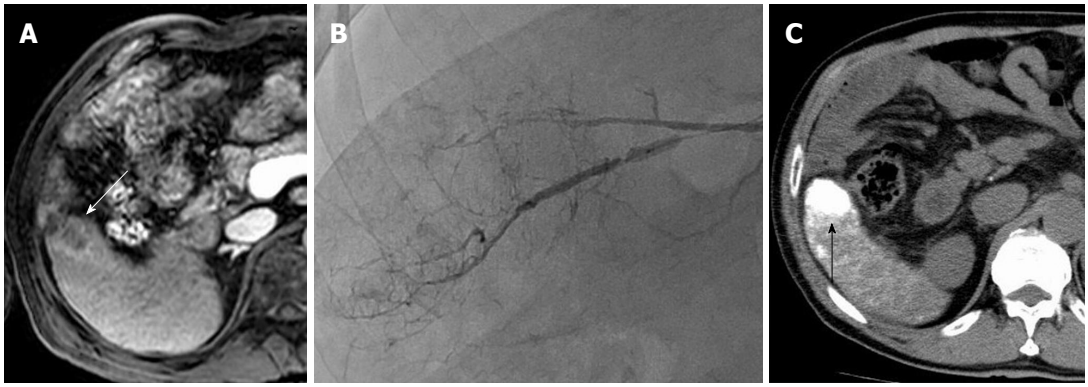

Figure 2 A 75-year-old man with hepatitis-B related hepatocellular carcinoma. A: Gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging (EOB-MRI) showed that HCC was slightly enhanced in the arterial phase (white arrowhead); B: While angiography did not show the tumor stain obviously, we injected the lipiodol emulsion (LE) and fragmented gelatin sponge slurry from tumor-feeding artery under occlusion with microballoon inflation; C: A favorable LE accumulation (black arrowhead) was seen on computed tomography one day after balloon-occluded transcatheter arterial chemoembolization. HCC: Hepatocellular carcinoma.

the blood vessels until the distal vessel is embolized. We describe a typical B-TACE procedure in Figures 1-3.

MECHANISM OF B-TACE

Irie *et al*[10] reported that they found dense lipiodol emulsion (LE) accumulation in HCC nodules when they used a 3-Fr microballoon catheter in selective TACE with occlusion of feeding arteries. Denser LE accumulation in targeted HCC nodules means more accumulation of anticancer drugs, leading to more therapeutic effect[21,22]. Therefore, revealing this mechanism is important for hepatologists and interventional radiologists in daily clinical practice.

Relationship between the balloon-occluded arterial stump pressure and the dense LE accumulation

The dense LE accumulation caused by the occlusion of feeding arteries cannot be explained only by the prevention of proximal migration and leakage of embolization materials; it also involves causing local changes in the hemodynamics of the surrounding occlusion artery and targeted tumors. Irie *et al*[10] reported that the pulsatile movement of LE droplets was observed after the inflation of the tip of a balloon catheter, with the peripheral arterial blood flow unable to be stopped. Because stasis of an LE droplet was observed even at the tip of the microballoon catheter, the authors speculated that the distal arterial blood flow did not come from leakage though the space between the balloon and the occluded artery. The intrahepatic collateral arteries, such as the peribiliary plexus[23], interlobar communicating arcade[24] and isolated artery[25], were considered to help maintain the distal arterial blood flow, based on the observation of anastomotic vessels with collateral arteries on digital subtraction angiography (DSA) under occlusion[10]. Consequently, the BOASP is reduced compared to the mean arterial pressure without balloon occlusion and more decreased BOASP allows the injection of LE under higher pressure. He

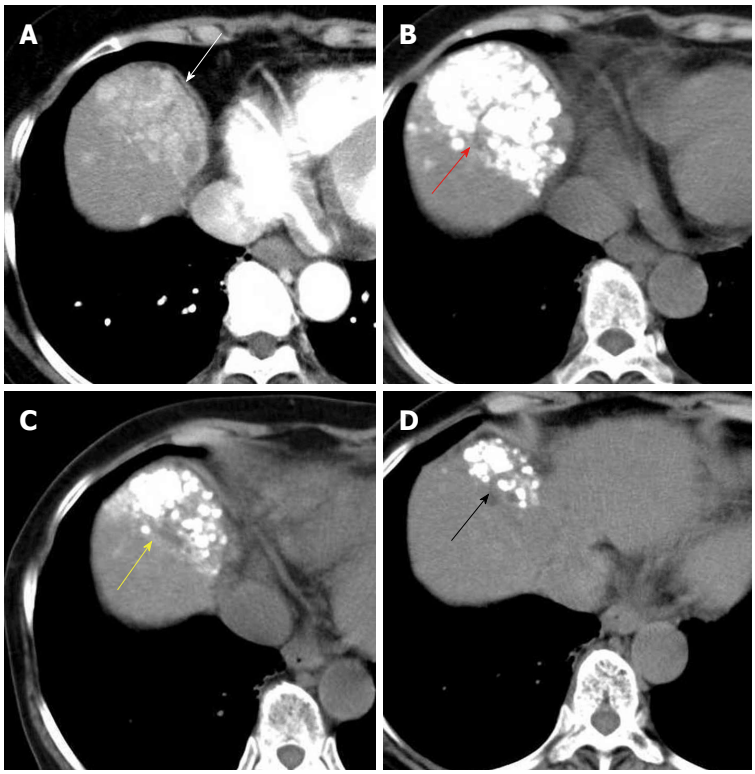

Figure 3 A 74-year-old woman with hepatitis-C related multiple hepatocellular carcinoma. A: Contrast-enhanced computed tomography (CT) showed that multiple HCC nodules were observed, aggregated at segment 8 (white arrowhead); B: Computed tomography showed that the dense lipiodol emulsion (LE) accumulations (red arrowhead) were observed just after balloon-occluded transcatheter arterial chemoembolization (B-TACE) for multiple HCC; C: We carried out additional B-TACE because local recurrence was detected on follow-up CT. Favorable LE accumulations were observed just after additional B-TACE (yellow arrowhead); D: Three month after additional B-TACE, local recurrence was not observed, and the LE accumulations were shrinking (black arrowhead). HCC: Hepatocellular carcinoma.

also demonstrated that BOASP is responsible for the dense LE accumulation and the BOASP ⩾ 64 mmHg is predictive for the presence of thick collateral arteries and less dense LE accumulation[10].

Matsumoto *et al*[26] reported that they used a 1.8-Fr microballoon catheter and measured the BOASP at each lobar, segmental and subsegmental arteries before B-TACE treatment. He showed that the BOASP ⩾ 64 mmHg was founded at A1, A4, A8, anterior segment artery, right hepatic artery (RHA) and left hepatic artery (LHA) whereas the BOASP ⩾ 64 mmHg was never seen at the other segmental and subsegmental arteries and the BOASP was independent of the size and number of tumors[26]. He presumed that this result was affected by the presence of intrahepatic collateral arteries, especially the communicating arcade in the hilum which is a relatively thick anastomosis between the right and left hepatic arteries and originates mainly from A4 and from anterior segment artery or the right hepatic artery[24,26]. BOASP ⩾ 64 mmHg at A1 artery could be explained by the studies reported by Miyayama *et al*[27,28] in which the right branch of the communicating artery in the hilum might have been A1[26]. So, B-TACE treatment in A1, A4, A8, anterior segment artery, RHA and LHA possibly caused less dense LE accumulation in targeted HCC nodules[26]. However, this study is small sample size in measured hepatic arteries and lack of the statistical analysis. A large number of validation studies are needed.

Kakuta *et al*[29] examined the changes in the stump pressure with and without balloon occlusion as well as after B-TACE treatment. The mean blood pressure at the targeted occluded artery was 97 mmHg before balloon occlusion and decreased to 49.1 mmHg immediately after balloon occlusion, with a statistically significance[29]. Five minutes after balloon occlusion, the mean blood pressure was 50.4 mmHg, and the maintenance of a decreased blood pressure was observed[29]. After the injection of LE and gelatin sponge particles, the mean blood pressure increased to 70.6 mmHg, which was significantly higher than that immediately after balloon occlusion ($P < 0.001$)[29]. The mean arterial blood pressure without balloon occlusion after B-TACE was not significantly different from that without balloon occlusion before B-TACE ($P = 0.9107$)[29]. Although no significant differences were observed among the stump pressures at the first-, second- and third-order branches, the proportion of stump pressure < 64 mmHg at third-order branches tended to be higher than that at first- or second-order branches[29]. Advancing a microballoon catheter to a more peripheral artery appeared to cause the stump pressure to decrease more[29]. A further investigation is needed to determine whether or not the occlusion of a more distal artery results in a more de-

creased BOASP.

Although these previous studies[10,26,29] have provided only preliminary and limited data, the findings indicate that the BOASP plays an important role in the dense LE accumulation in targeted HCC nodules. Further studies regarding whether or not the BOSAP can predict the long-term therapeutic effect should be conducted.

Hemodynamic changes assessed by CBCT and angio-assisted CT

Three reports[30-32] have explored the hemodynamic changes assessed by balloon occlusion using CBCT and angiography-assisted CT. Ishikawa *et al*[30] reported that the CBCT pixel values of tumor enhancement increased after balloon occlusion in 37 of the 52 nodules, whereas it decreased in the remaining 15 nodules. Yoshimatsu *et al*[31] showed that the degree of tumor enhancement on selective CT during hepatic angiography (CTHA) increased after balloon occlusion in 3 of the 27 nodules, while it decreased in 11 nodules. Asayama *et al*[32] also reported that 15 of the 35 nodules showed decreased enhancement or perfusion defects on CTHA with balloon occlusion. While the percentage of decreased tumor enhancement varied markedly among those studies, it remained a negative predictive factor for a dense LE accumulation[30,31] and short-term therapeutic effect[32]. It can be presumed that the blood flow from the occluded artery reduced and the blood flow from the collateral arteries relatively increased[31,32]. In relation to this, a discrepancy was noted between the tumor enhancement on selective CTHA with balloon occlusion and a dense LE accumulation after B-TACE, which was higher than the tumor enhancement grade[31]. Although CBCT[33-35] and CTHA[36,37] are well known to be useful tools for identifying the tumor-feeding arteries, evaluating the embolized area and avoiding nontargeted embolization, the same findings were reported in selective C-TACE[38]. A validation study will be needed to confirm whether or not a decreased tumor enhancement after balloon occlusion can predict the short- and long-term therapeutic effects.

With regard to the analysis of CT findings during arterial portography (CTAP), the reduction in size of the tumorous portal perfusion defects on CTAP with balloon occlusion was observed in 90% (18/20) of nodules. Its mean size significantly decreased from 21.9 to 19.1 mm in diameter (P = 0.0001). No decrease in the tumorous portal perfusion defect area significantly influenced the poor dense LE accumulation in the tumor[31]. The authors speculated that the decrease in the BOASP enabled the pressure gradient from the hepatic artery to the portal vein to also decrease, thus resulting in a relative increase in the blood flow from the portal vein in the surrounding tumor[31]. In other words, no change in the size of the tumorous portal perfusion defect may indicate no decrease in the BOASP[31]. To facilitate our understanding of the mechanism of B-TACE, we summarize the main findings mentioned above and present them in a schematic illustration in Figure 4.

THERAPEUTIC EFFECT OF B-TACE

Short-term therapeutic effect and local recurrence rate

In many previous studies, the short-term therapeutic effect of B-TACE was evaluated mainly by the Response Evaluation Criteria in Cancer of the Liver (RECICL) proposed by the Liver Cancer Study Group of Japan[39]. The treatment effect (TE) was determined by the radiological findings 1-3 mo after B-TACE treatment and defined as follows: TE4, tumor necrosis of 100% or 100% reduction; TE3, tumor necrosis of 50%-100% or 50%-100% reduction in tumor size; TE1, tumor enlargement of > 50% regardless of necrosis; TE2, effect other than TE3 or TE1[39]. According to previous reports[13,15,32,40,41], the rate of TE4 of targeted HCC nodules treated with B-TACE was 8.6%-89.3%, and that of TE3 was 8.5%-48.6%, with a response rate of 56.3%-100%.

Kawamura *et al*[40] showed that the presence of portal vein visualization during treatment was associated with an objective response, which is consistent with findings of the study reported by Miyayama *et al*[42]. However, the evaluation of the therapeutic effect of B-TACE in these studies[13,15,32,40,41] was limited to only targeted lesions and did not include the appearance of new lesions. According to a study[43] evaluated based on the overall response, including target lesions, non-target lesions and the appearance of new lesion, 35 patients (53.0%) experienced complete response (CR), 7 patients (10.6%) partial response (PR), 13 patients (19.7%) stable disease, and 11 patients (16.7%) progressive disease. The rate of a response rate and a disease control rate were 63.6% and 83.3%, respectively. Furthermore, a multivariate analysis showed that a solitary tumor and serum α-fetoprotein level were significantly relevant to the tumor response[43].

Regarding the analysis of the local recurrence rate, Ishikawa *et al*[44] showed that the median recurrence time and the local recurrence rate at 6 mo and 12 mo were 9 mo and 11.1% and 26.2%. The authors further revealed that only the CT value just after B-TACE was significantly relevant to local recurrence in a multivariate analysis[44]. CBCT was also deemed useful as a substitute for conventional CT to evaluate the degree of dense LE accumulation quantitatively in targeted HCC nodules[45]. Irie *et al*[15] also showed that the control rates of primary nodule treated by B-TACE at 1, 3 and 5 years were 92.4%, 69.9% and 69.9%, respectively. However, these were retrospective studies, and the number of nodules per patient was small (many patients had solitary nodules). The results should therefore be interpreted with caution when extrapolating to HCC patients with more nodules. A summary of the previous studies is shown in Table 1. A prospective validation study is warranted.

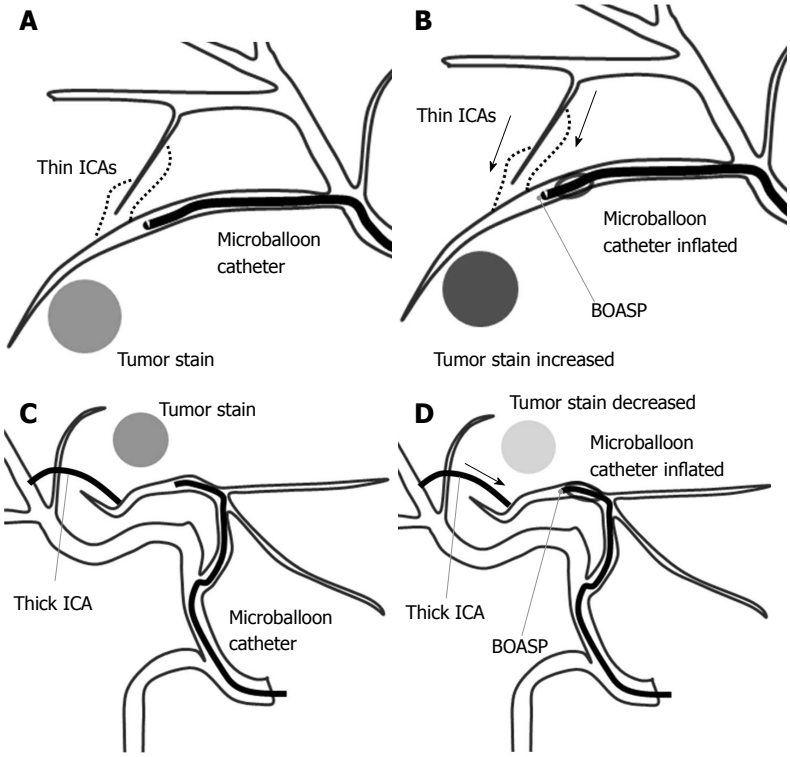

Figure 4 A schematic illustration of the balloon-occluded transcatheter arterial chemoembolization procedure. A: A microballoon catheter is inserted into the tumor-feeding artery and inflated to occlude it. If the distal arterial blood flow cannot be stopped, the intrahepatic collateral arteries (ICAs) may be maintaining the distal arterial blood flow; B: When the ICAs are thin, the balloon-occluded arterial stump pressure (BOASP) is remarkably reduced compared to the arterial pressure without balloon occlusion. In this situation, we might observe increased tumor enhancement on selective computed tomography during hepatic angiography (CTHA) after balloon occlusion. A remarkable reduction in the BOASP is thought to allow us to inject the lipiodol emulsion (LE) under higher pressure, leading to the denser LE accumulation in targeted tumors. The BOASP after the injection of LE and gelatin particles is significantly higher than that before treatment; C and D: When the ICAs are thick, the BOASP is expected to be mildly reduced or unchanged because of the adequate blood flow from a thick ICA. In this situation, we might observe decreased tumor enhancement on CTHA after balloon occlusion. We would probably inject the LE under lower pressure in such a situation, thereby achieving a less-dense LE accumulation.

Suitable anticancer agents

The anticancer agents that have been used in B-TACE are as follows: Miriplatin[13,14,31,32,40,41,44], which was the most commonly used; cisplatin[29,31]; epirubicin[20] and a mixture of doxorubicin hydrochloride and mitomycin[10,15]. Miriplatin, which is a lipophilic platinum complex[18,19], has been used in C-TACE[46]. A retrospective study[47] showed that the local recurrence rate was significantly higher after superselective TACE with miriplatin than with epirubucin and mitomycin. This may be attributed to its high viscosity, and warmed miriplatin may reduce its viscosity, possibly overcoming its disadvantages and increasing its therapeutic effect[48,49]. Therefore, B-TACE with warmed miriplatin may exert a better therapeutic effect than that with miriplatin at room temperature. In this connection, arterial vascular damage is less severe in patients treated with miriplatin than in those treated with epirubicin[50]. Although no RCT has compared antitumor agents, miriplatin might be suitable as an anticancer agent in B-TACE. Further studies should be conducted.

Comparing the effectiveness of B-TACE and C-TACE

Reliable data on the comparative efficacy between B-TACE and C-TACE are still lacking, with only four retrospective studies reported. Arai *et al*[13] reported that the targeted therapeutic effect of B-TACE with miriplatin was better than that of C-TACE, and the mean total dose of miriplatin in B-TACE was higher than that of C-TACE. They presumed that inflating the microballoon catheter decreased the arterial pressure proximal of the tip of the catheter and blocked the backflow of miriplatin, resulting in a higher total dose of miriplatin and a better therapeutic effect on targeted nodules than with C-TACE. Ogawa *et al*[14] also reported that TE4 was observed in 29 patients (49.2%) treated with B-TACE and in 10 patients (27%) treated with C-TACE, indicating that local efficacy of B-TACE was significantly better than that of C-TACE. Irie *et al*[15] reported that the short-term treatment effect based on RECICL criteria was significantly better in B-TACE group than that in the C-TACE group, and the tumor control rates of the targeted nodule were significantly improved in the B-TACE group compared to C-TACE group. However, Maruyama *et al*[20] reported that the local control rate in B-TACE group were not significantly better than in the C-TACE group. The major cause of this was the lack of a significant difference between the two groups

in the analysis of the LE ratio. The LE ratio, which was calculated as the ratio of the LE concentration in the targeted tumor to that in the surrounding embolized non-cancerous area, was considered to be an indicator of the selectivity of the LE accumulation in HCC[10]. Alternatively, this finding may have been observed because most patients received B-TACE treatment at the lobar or segmental level, resulting in a less-marked decrease in the BOASP and therefore a less-marked therapeutic effect[26]. More large-scale prospective studies are warranted.

INDICATIONS OF B-TACE

Since no definitive indications of B-TACE have been established to date, we explored potential factors and conditions supporting the performance of B-TACE. Summarizing the findings of the previous studies mentioned above, the following factors and conditions are known to be related to a dense LE accumulation in targeted HCC nodules and the therapeutic effect of B-TACE: a BOASP $\geqslant$ 64 mmHg[10]; microballoon occlusion at A1, A4, A8, anterior segment artery, RHA or LHA[26]; decreased tumor enhancement on CBCT and CTHA[30-32]; no decrease in the tumorous portal perfusion defect on CTAP[31]; tumor location on the subcapsular portion[40]; successful subsegmental feeding artery embolization[40]; the presence of portal vein visualization[40]; a high CT value just after B-TACE[44]; solitary tumor[43] and a low serum α-fetoprotein level[43]. Based on studies[13-15] showing that patients with fewer tumor nodules experienced a better therapeutic effect with B-TACE than with C-TACE, B-TACE might be a better indication than C-TACE in patients with fewer tumor nodules. Prospective validation studies are needed to clarify the indications of B-TACE.

The contraindications of B-TACE were thought to be almost the same as those for C-TACE treatment: Child-Pugh class C, no tumor thrombosis in the first branch or main portal vein, extrahepatic metastasis, refractory ascites, hepatic encephalopathy, presence of high-flow arterioportal or arteriovenous shunts, and allergy to contrast medium.

OVERALL SURVIVAL OF B-TACE

Hatanaka *et al*[43] reported that the survival rates at 1, 3 and 5 years in patients after B-TACE were 76.8%, 57.3% and 46.7%, respectively, and the median survival time was 902 d. They also revealed that a serum albumin level $\geqslant$ 3.4 g/dL and an overall response of CR+PR were favorable prognostic factors in a multivariate analysis[43]. Serum albumin is one of the most well-known prognostic factors for patients with HCC and has been adopted and integrated into several staging systems, including the BCLC staging system[3], Cancer of the Liver Italian Program (CLIP) score[51] and Japan Integrated Staging score (JIS score)[52]. According to previous studies of TACE[53,54], the overall response has been reported to be an independent factor affecting for the survival of HCC patients. Although the findings reported by Hatanaka *et al*[43] were consistent with those of previous studies[53,54], the results were based on a single-arm and single-institution study. Additional validation studies are therefore warranted.

One problem that remains to be solved is whether or not B-TACE improves the overall survival compared to other TACE treatments. Unfortunately, no RCTs have compared B-TACE with other TACE treatments, such as C-TACE and DEB-TACE, and only one historical control study[15] with patients who had solitary or two nodules has been reported. However, while it was small in scale, that study found that B-TACE was a significant factor predicting for the overall survival in a multivariate analysis[15]. A large-scale prospective study is therefore warranted.

COMPLICATIONS OF B-TACE

According to previous reports[13,20,43,44] evaluated by the Common Terminology Criteria for Adverse Effects (CTCAE), all grades of fever were reported as complications in 44.2%-68% of cases, nausea in 16.3%-28%, abdominal pain in 14%-36.7%, ascites in 12.2%-15.2%, elevated total bilirubin in 34%-62.2%, elevated alanine aminotransferase (ALT) in 78.8%-96%, elevated serum creatine in 8.1%-9.1%, leukocytopenia in 53.1%-59%, thrombocytopenia in 71.3%-87.7%, anorexia in 31.3% and vagovagal reflex in 12%, while a grade 3/4 fever was reported as a complication in 0%-6% of cases, elevated total bilirubin in 0%-6.1%, elevated ALT in 9.1%-18%, leukocytopenia in 0%-12.1% and thrombocytopenia in 7.6%-12.2%. In an analysis comparing the adverse events of B-TACE with those of C-TACE, the rate of increased levels of ALT was significantly higher in the B-TACE group, with no significant differences in the clinical symptoms or other laboratory data between the two groups[13,20].

Hatanaka *et al*[43] reported no mortalities, but the development of biloma requiring percutaneous transhepatic biliary drainage after B-TACE was observed in 1 patient (1.5%) who had intrahepatic dilation on preoperative CT. Maruyama *et al*[20] reported that liver abscess was observed in 3 patients (6%), and liver infarction was observed in 1 patient (2%), with bile duct dilation indicated as a significant predictive factor in a multivariate analysis. Careful consideration must be given to perform the B-TACE for the patients with bile duct dilation[43]. Large-scale validation studies are therefore needed to confirm these findings.

CONCLUSION

B-TACE represents a feasible and promising therapy for the treatment of HCC patients, especially those with few nodules. Although no definitive indication of B-TACE in the common clinical practice has yet been established, leaving the decision to perform this procedure up to

gastroenterologist or radiologist based on their expertise with or the availability of microballoon catheters, B-TACE may represent an established treatment in HCC patients.

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ORIGINAL ARTICLE

Basic Study

Amelioration of hepatotoxicity by biocleavable aminothiol chimeras of isoniazid: Design, synthesis, kinetics and pharmacological evaluation

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Abstract

AIM

To overcome the hazardous effects on liver caused by long-term use of antitubercular agent isoniazid (INH) by developing a novel hepatoprotective prodrug strategy by conjugating INH with aminothiols as antioxidant promoieties for probable synergistic effect.

METHODS

INH was conjugated with N-acetyl cysteine (NAC) and N-(2)-mercaptopropionyl glycine using the Schotten-Baumann reaction and with L-methionine using Boc-anhydride through a biocleavable amide linkage. Synthesized prodrugs were characterized by spectral analysis, and *in vitro* and *in vivo* release studies were carried out using HPLC. Their hepatoprotective potential was evaluated in male Wistar rats by performing liver function tests, mea-

suring markers of oxidative stress and carrying out histopathology studies.

RESULTS

Prodrugs were found to be stable in acidic (pH 1.2) and basic (pH 7.4) buffers and in rat stomach homogenates, whereas they were hydrolysed significantly (59.43%-94.93%) in intestinal homogenates over a period of 6 h. Upon oral administration of prodrug NI to rats, 52.4%-61.3% INH and 47.4%-56.8% of NAC were recovered in blood in 8-10 h. Urine and faeces samples pooled over a period of 24 h exhibited 1.3%-2.5% and 0.94%-0.9% of NAC, respectively, without any presence of intact NI or INH. Prodrugs were biologically evaluated for hepatoprotective activity. All the prodrugs were effective in abating oxidative stress and re-establishing the normal hepatic physiology. The effect of prodrug of INH with NAC in restoring the levels of the enzymes superoxide dismutase and glutathione peroxidase and abrogating liver damage was noteworthy especially.

CONCLUSION

The findings of this investigation demonstrated that the reported prodrugs can add safety and efficacy to future clinical protocols of tuberculosis treatment.

Key words: Aminothiols; Antioxidants; N-acetyl cysteine; N-(2-mercaptopropionyl) glycine; Isoniazid; L-methionine; Liver injury; Tuberculosis

Core tip: To overcome the deleterious effects caused by long-term use of isoniazid (INH), a novel hepatoprotective prodrug strategy was developed by integrating the antioxidant property of aminothiols with INH moiety to improve its therapeutic safety. The anticipated prodrugs were synthesized using facile method of synthesis, wherein aminothiols were tethered to the INH molecule *via* biocleavable amide linkage. Upon hepatoprotective potential assessment, the prodrug NI displayed noteworthy effects, whereas prodrugs MGI and MI exhibited moderate effects. The findings of this study strongly suggest that concept-based design of hepatoprotective prodrugs can be applied effectively in reversing toxic effects of INH and its metabolites on liver.

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INTRODUCTION

Tuberculosis (TB) is a worldwide pandemic and has existed for millennia. According to the Global Tuberculosis Report 2016 published by the World Health Organization, 10.4 million new TB cases (including 1.2 million among human immunodeficiency virus-positive people) occurred in 2015 and an astounding 1.4 million perished from TB that year. Overall, 10% of cases were children, whereas 90% were adults. TB disproportionately afflicts developing nations; India, Indonesia, China, Nigeria, Pakistan and South Africa were the countries that accounted for 60% of the global burden[1]. TB was one of the top 10 causes of death worldwide in 2015, despite the existence of curative chemotherapy.

Standard treatment recommended for adult respiratory TB is a 2-mo regime of isoniazid (INH), rifampicin, pyrazinamide and ethambutol, followed by INH and rifampicin for 4 mo[2]. Unfortunately, INH, the drug for the intensive phase of conventional anti-TB treatment, is highly hepatotoxic, with incidence varying between 4%-11%[3]. INH and/or its metabolites trigger systemic lupus erythematosus, peripheral neuropathy, steatosis, neurological disorders and hepatic necrosis[4]. N-arylaminoacetyl transferases (NATs) are the enzymes that metabolize INH in humans through acetylation at N2-center in the hydrazinic chain and release free radicals, which are implicated in the hepatotoxicity[4-7].

A well-established drug therapy to prevent or cure this hepatotoxicity does not exist, except discontinuing the treatment for a while and restoring it steadily later on when liver enzymes return to normal level. Such discontinuation of treatment may result in morbidity or prolonged disability. Therefore, clinical safety in long term treatment with INH is questionable and continues to be a subject of grave concern in the field of medicine[3]. In the past, considerable focus has been put on the development of INH conjugates with lower hepatotoxicity, improved hydrolytic stability, enhanced bioavailability and antitubercular activity. These include conjugation with polymers like gelatine[8], peptide[9], polyethyleneglycol[10], β-cyclodextrin conjugate[11] and antioxidants like cinnamic acids[12], curcumin[13], Schiff bases[14] and phenolic acids[15].

Resurgence of INH as a clinically safe drug appears to rely on its appropriate modification that can block acetylation by NATs and overcome toxicity. To engender these anticipated drug properties in INH, it can be structurally modified at N2-center in the hydrazinic chain[2,14]. The molecular mechanism of cytotoxicity is attributed to CYP2E1-induced oxidative stress as protein carbonyl formation and rise in reactive oxygen species occur before the beginning of hepatocyte toxicity[16,17].

Since nitrogen-centred free radicals play a critical role in instigating oxidative stress, by augmenting the hepatocellular antioxidant defence system, especially glutathione (GSH), cells can be protected against the INH-induced oxidative injury, as evidenced in previous reports[18]. Thiol-containing antioxidants have earned great interest of researchers because of the profound part played by intracellular GSH in redox regulation and antioxidant

cell defence machinery. In the present study, GSH depletion is the rationale for the selection of protective amino thiol antioxidants as carriers for conjugation with INH. As precursors of GSH, sulfhydryl group-containing compounds either increase hepatic stores of reduced GSH or substitute for it in the liver[19]. Among the sulphur-containing antioxidants, N-acetyl cysteine (NAC), N-(2-mercaptopropionyl) glycine (MPG) and L-methionine (Met) are known to be nontoxic and have been used in the treatment of various disorders[20-24].

Met plays a vital role in detoxification by acting as an amino acid precursor for GSH synthesis, which protects cells from oxidative damage[25]. NAC helps in maintaining intracellular GSH levels and scavenging reactive oxygen species and its potential in attenuating INH and rifampicin-induced hepatotoxicity has already been reported[20]. Protective effects of MPG have been reported in paracetamol-induced hepatic necrosis[26].

On these grounds, a novel series of prodrugs was designed with the aim of combining antioxidant properties of aminothiols (NAC, MPG and Met) with INH to yield codrugs with lower hepatotoxicity. The hypothesis behind this concept was based on transient masking of the hydrazinic chain of INH at the N2 position to block oxidation by CYP2E1 and acetylation by NAT. Release kinetics were studied to confirm the biocleavable nature of this newly formed amide bond, and hepatoprotective efficacy was assessed.

MATERIALS AND METHODS

INH was received as gift sample from Lupin Research Park (Lupin Ltd., Aurangabad, India). NAC was generously provided by Zim Laboratories Ltd (Nagpur, India). MPG and Met were purchased from Sigma Chemical Co. (St Louis, MO, United States). Precoated silica gel plates (60 F254; Merck, Kenilworth, NJ, United States) with fluorescent indicator were used for thin-layer chromatography, and ultraviolet (UV) light (254 nm) was used for spot detection. Programmable melting point apparatus (Veego, India) was used to record melting points and are uncorrected. The V530, UV-visible double-beam spectrophotometer (JASCO, Oklahoma City, OK, United States) was used to determine the λ_{max} of the synthesized compounds.

Spectroscopic methods were used for confirmation of structures. The infrared (IR) spectrum was recorded on a JASCO V-530 FTIR in potassium bromide (anhydrous IR grade). Proton- and ^{13}C-NMR were recorded in $CDCl_3$ and DMSO referring chemical shifts to TMS as the internal standard using an Avance Ⅱ 400 instrument (Bruker, Billerica, MA, United States) at 400 MHz, with super conducting magnet, at VIT University (Vellore, Tamilnadu, India). The mass spectra were recorded by employing an Agilent 1260 Infinity HPLC-MASS Analyzer 6460 Triple Quad LC/MS at the Poona College of Pharmacy, Food Testing Laboratory (Pune, India). The elemental analysis of synthesized prodrugs was performed on an elemental analyser (Vario Micro Cube; Elementar, Frankfurt, Germany) at the Poona College of Pharmacy.

In vitro and *in vivo* release studies were carried out on a JASCO PU HPLC model 2080, with UV detector UV-Vis 2070, using a Thermo C18 column (Hypersil gold, 250 mm × 4.36 mm, 5 μm). For pharmacological screening of synthesized prodrugs (CPCSEA/PCH/02/2016-17), Institutional Animal Ethical Committee-approved experimental protocols were followed and all procedures were carried out at the CPCSEA-approved animal facilities of Poona College of Pharmacy (Reg. No.100/1999/CPCSEA). The animals were bought from the National Institute of Biosciences (Pune, India).

Synthesis of aminothiol prodrugs of INH

Synthesis of prodrugs of INH with NAC (NI) and MPG (MGI): To the solution of amino acid (1) (0.005 mol/L) in THF (10 mL) thionyl chloride (2) (0.005 mol/L) was added drop-wise and stirred for 2 h at 55 ℃. The reaction was monitored by thin-layer chromatography using ethyl acetate:methanol:glacial acetic acid (GAA) (0.5:1.5:0.02 v/v/v). At the end of 2 h, the reaction mixture was evaporated using a rotary evaporator, and the residue of acid chloride (3) was dried under vacuum. It was then dissolved in THF and poured into a round-bottom flask which was placed in an ice bath and kept for stirring. To an ice-cold mixture of INH (4) in THF, TEA (0.5 mL) was added and the resultant solution was added drop-wise to the round-bottom flask containing acid chloride, and the temperature of the ice bath was maintained at 0 ℃. At the end of 4 h, the precipitated amide (5) was separated from the mixture by filtration and was recrystallized using mixture of ethanol:water (2:1 v/v). It was further purified by preparative thin-layer chromatography using the same mobile phase mentioned above. The reaction scheme is shown in Figure 1.

Synthesis of prodrug of INH with Met (MI): To a stirred solution of Met (6) (2 mmol) in THF (20 mL), a saturated solution of potassium carbonate (5 mL) was added. Di-*tert*-butyl dicarbonate (Boc_2O; 2 mmoL) was then added to this solution and the resultant reaction mixture was refluxed for 4 h (7) and monitored by thin-layer chromatography using ethyl acetate:hexane:GAA (0.7:0.3:0.01 v/v/v). INH (4) (2 mmol) was added to the reaction flask and the resulting mixture was refluxed for a further 6 h. Removal of solvent yielded a pale orange paste, which was further extracted using hexane: ethyl acetate (3:2 v/v) to remove excess Boc_2O and with water:DCM (1:5 v/v) to obtain a product which was dried over anhydrous magnesium sulphate.

Deprotection at the N-terminal (8) was done by addition of a solution of HCl in MeOH (10 mL) to the pale orange product obtained from the previous step. The resulting reaction mixture was stirred at room-temperature for 12 h, after which its pH was adjusted to 9 with 2 N NaOH[27]. The mixture was extracted with DCM (2 × 10 mL). The combined organics were washed with saturated aqueous brine (2 × 15 mL), dried over sodium sulphate

Table 1 Doses of test and standard drugs

S.N.	Compound	Dose[1], mg/kg/per day
1	H	0.9% saline (1 mL)
2	INH	150
3	NI	309
4	MGI	311
5	MI	293
6	INH + NAC	150 + 159
7	INH + MPG	150 + 161
8	INH + Met	150 + 143

[1]All doses were calculated on an equimolar basis to the dose of INH. H: Healthy control; INH: Isoniazid; NI: Prodrug of INH and N-acetyl cysteine; MGI: Prodrug of INH and N-(2-mercaptopropionyl) glycine; MI: Prodrug of INH and L-methionine; INH + NAC: Physical mixture of INH+N-acetyl cysteine; INH + MPG: Physical mixture of INH + N-(2-mercaptopropionyl) glycine; INH + Met: Physical mixture of INH and L-methionine.

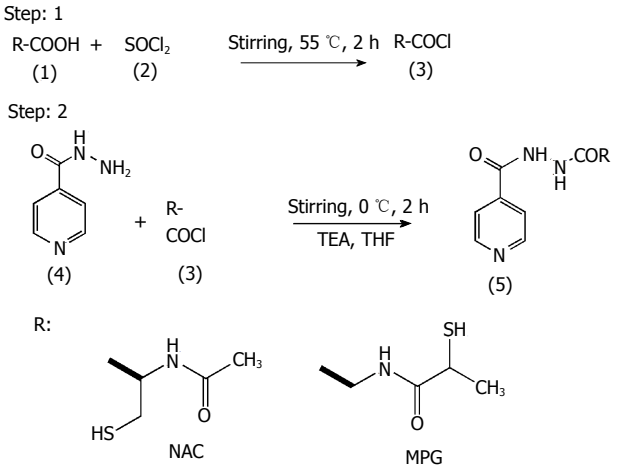

Figure 1 Scheme of synthesis for NI and MGI.

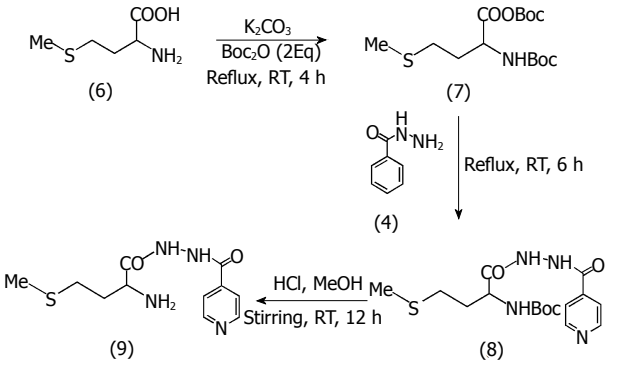

Figure 2 Scheme of synthesis for MI.

and concentrated *in vacuo*, to yield the final product (9) as a pale yellow solid. Further purification was carried out using preparative thin-layer chromatography with mobile phase ethyl acetate:hexane:GAA (0.7:0.3:0.01 v/v/v). The reaction scheme is shown in Figure 2.

In vitro stability and release studies

In vitro stability studies were performed in aqueous buffers of varied pH range and tissue homogenates of stomach and intestine of rats to ensure bioreversibility of the synthesized prodrugs. Triplicate samples were analysed and methods were validated as per ICH guidelines. Novel HPLC methods were developed for simultaneous estimation of prodrugs in the presence of their released active metabolite, INH. Percentage of hydrolysed prodrugs and release of INH was calculated using equations generated from calibration curves. Prodrugs in the presence of their hydrolysed products were estimated by simultaneous estimation method developed on reverse-phase HPLC. The method developed for estimation of NI and its hydrolysed products was comprised of water: methanol (80:20 v/v adjusted to pH 3.0 with OPA at flow rate 1 mL/min on a Thermo C_{18} column at 204 nm). For MGI, the mobile phase was comprised of acetonitrile: water (65:35; v/v adjusted to pH 2.5 with OPA at flow rate 1 mL/min on a Thermo C18 column at 365 nm). For the MI mobile phase, water:acetonitrile:methanol (20:20:60 v/v/v at flow rate 1mL/min on a Thermo C_{18} column at 205 nm) was used.

Calibration curves of prodrugs and INH were constructed in HCl buffer (pH 1.2) and phosphate buffer (pH 7.4) in the range of 10-100 μg/mL. Release study was performed in HCl buffer (pH 1.2) and stomach homogenates till 3 h and in phosphate buffer (pH 7.4) and intestinal homogenates till 6 h respectively. Prodrug (10 mg) was introduced in 100 mL of 0.05 mol/L HCl (pH 1.2) or 0.05 mol/L phosphate buffer (pH 7.4) in a beaker kept in a constant-temperature bath at 37 ± 1 ℃ with occasional stirring. Aliquots (5 mL) were withdrawn and replaced with fresh aqueous buffer at regular intervals of 0, 15, 30, 45, 60 min and every 30 min thereafter till 3 h for HCl buffer and 6 h for phosphate buffer, and 50 μL sample reconstituted with the mobile phase was injected in the column and analysed by HPLC.

In vivo release studies

In vivo behaviour of the orally-administered prodrug NI was investigated in Wistar rats (200-250 g; n = 3) housed in metabolic cages individually under normal conditions (at 27 ± 0.5 ℃ and a relative humidity of 70% ± 0.5% under natural light/dark conditions). To study the *in vivo* behaviour, an HPLC method was developed for simultaneous estimation of INH, NI and NAC. The same HPLC system, column and mobile phase were used for this purpose, as mentioned in the *in vitro* release study section above. All the kinetic studies were carried out in triplicate.

Equimolar dose of NI (Table 1) was orally administered to the 6 male Wistar rats kept in individual metabolic cages. Male Wistar rats were fasted for 24 h prior to use and were administered water *ad libitum*. Blood (0.5 mL) was withdrawn by retro-orbital puncture and the reading was considered as the 0-min reading. A suspension of NI in 1.0 mL of physiological saline was administered to the animals (Table 1). Blood samples were collected in EDTA-coated tubes at an interval of 15 min for the first 1 h. Then, subsequent blood collection

was made on a bihourly basis till the 10th h and finally at the 24th h. These EDTA-coated tubes were centrifuged for 10 min at 5000 rpm with temperature set at 0-5 ℃. The supernatant solution (0.1 mL) of centrifuged blood was added to an Eppendorf tube (1 mL capacity) and methanol (0.9 mL) was added to it for instant plasma protein precipitation. After vortexing all the solutions for 2 min, they were again centrifuged at 5000 rpm for 10 min at 0-5 ℃ in order to precipitate any solid matter or impurities present in the biological samples. These samples were then analysed by HPLC, using the procedure described above.

Urine/faeces samples were also collected at various time intervals and pooled together over a period of 24 h and analysed similarly by HPLC.

Biological evaluation

Animals: For screening of prodrugs for hepatoprotective potential, Wistar rats (male, 180-200 g) were used. Solid-bottom polypropylene cages were used to house the animals and were maintained at 24 ± 1 ℃, with a relative humidity of 45%-55% and 12:12 h dark/light cycle. They were acclimatized to laboratory conditions for 1 wk. The animals had free access to food (standard chow pellets; Chakan Oil Mills, Sangli, India) and water. The doses of prodrugs were calculated on an equimolar basis to INH and are presented in Table 1. The standard and the test compounds were administered orally as a suspension in 1% sodium CMC.

Evaluation of hepatoprotective potential: Rats were randomly divided into eight groups, after an acclimatization period of 1 wk, with each group consisting of 6 animals. Doses as mentioned in the Table 1 were administered for 21 d to the respective groups. Body weights and relative liver weights of animals from prodrug-treated groups and physical mixture-treated groups were calculated at the end of study. After completion of 21 d, on the 22nd d, the animals were fasted overnight and sacrificed immediately after withdrawal of blood from the retrobulbar venous plexus. Afterwards, centrifugation of whole blood was carried out to obtain serum samples. Liver samples were dissected out and washed immediately with ice-cold saline to remove as much blood as possible. One fraction of the liver samples was excised, fixed in a 10% formalin solution and sent for histopathological analysis; another fraction was immediately stored at -80 ℃ for future analysis. The histopathologist was unaware of the protocols to ensure unbiased analysis.

Assessment of antioxidant parameters: Liver tissues were washed with normal saline to remove any blood or clots, and homogenized on ice in Tris-HCl (5 mmol/L containing 2 mmol/L EDTA, pH 7.4). Homogenates were centrifuged at 1000 × *g* for 15 min at 4 ℃. Aliquoted samples of the supernatants were used immediately for the assays of superoxide dismutase (SOD) and glutathione peroxidase (GSHPx). SOD activity was estimated by the method described by Marklund and Marklund (1974). GSHPx content was determined by the method of Moron *et al*[28-30] (1979).

Assay of lipid peroxidation products: The extent of lipid peroxidation was assessed by estimating the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) as described by Ohkawa *et al*[31] (1979). MDA concentrations were measured using 1,1,3,3-tetraethoxypropane as standard and expressed as micromoles per gram of tissue[31].

Estimation of alanine aminotransferase and aspartate aminotransferase: Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated with help of commercially available kits (Coral Clinical Systems, Mumbai, Maharashtra, India) using Reitman and Frankel's colorimetric method[32].

Biochemical parameters

Commercially available spectrophotometric kits (Coral Clinical Systems) were used to measure triglycerides and cholesterol levels. For determination of triglycerides in plasma, glycerol-3-phosphate-oxidase enzymatic colorimetric (GPO/PAP) method was used. CHOD/PAP method based on estimation of D4 cholestenone after enzymatic cleavage of cholesterol ester by cholesterol esterase was used for the determination of cholesterol in plasma[33].

Histopathological analysis

Histopathology of rat liver was carried out at SAI Research Laboratories (Pune, India). The sections were stained by haematoxylin and eosin. Coloured photomicrographs of the sections were taken on Nikon Optical Microscope, Eclipse E-200 (resolution of 10 × 10 X), with an attached trinocular camera.

Statistical analysis

An average of six readings was calculated and data were expressed as mean ± SEM; *n* referred to number of animals in each group. Statistical differences between the groups were calculated by one-way ANOVA followed by Dunnett's post-hoc test. Differences were considered at *P* values of < 0.001-0.05 when compared with the INH group.

RESULTS

Partition coefficient and aqueous solubility

The log P_{oct} of INH was found to be -0.71 and aqueous solubility was 135 mg/mL. The partition coefficients of NI, MGI and MI were found to be 0.11, 0.27 and 0.06 respectively and their aqueous solubilities were 104 mg/mL, 94 mg/mL and 114 mg/mL respectively.

Spectral analysis

For confirming the structures of synthesized prodrugs, they were subjected to spectral analysis by IR, NMR,

Table 2 *In vitro* release kinetics data

Prodrug	Incubation medium						
	HCl buffer, pH 1.2	Phosphate buffer, pH 7.4	Stomach homogenates	Intestinal homogenates			
				K ± SD, min-1[1]	$t_{1/2}$, min	% Prodrug hydrolysed	% INH released
NI	Stable	Stable	Negligible	3.4×10^{-3}	233.42	94.93	46.10
MGI	Stable	Stable	Negligible	3.3×10^{-3}	217.34	70.11	33.83
MI	Stable	Stable	Negligible	5.1×10^{-3}	181.36	86.55	44.26

[1]Average of three readings; follows first-order kinetics.

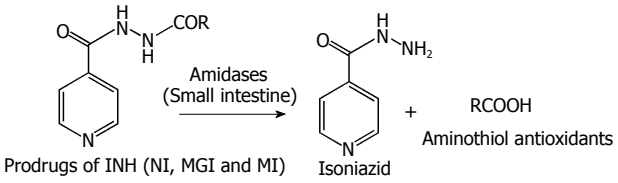

Figure 3 Proposed activation mechanism of prodrugs.

mass spectroscopy and elemental analysis.

Amide NI: N-{1-mercaptomethyl-2-oxo-2-[N'-(pyridine-4-carbonyl)-hydrazino]-ethyl}-acetamide, M.P.: 175-178 ℃ (uncorrected), R_f: 0.58 (ethyl acetate: methanol:GAA; 0.5:1.5:0.02 v/v/v), Aq. Sol. : 104 mg/mL, Log P_{oct}: 0.11, IR (anhydrous KBr; cm-1): 3300 (NH, sec amide, str), 2545 (SH, str), 1704 (C=O, CHCONH, str), 1663 (C=O, aromatic CONH, str.), 1532 (C-N, pyridine ring, str), 1406 (CH_2, bend), 751 (N-N, hydrazine, sym wag) ^{1}HNMR ($CDCl_3$; 400 MHz): δ 2.02 [s, 3H]-CH_3O, 3.05-3.06 [d, 2H]-CH_2S, 4.68-4.71 [t, 1H]-CH_2-CH_2-SH, 7.82-7.83 [d, 2H]-pyridine ring, 8.78-8.79 [d, 2H]-pyridine ring, 9.84 [s, 3H]-NH ^{13}C-NMR ($CDCl_3$; 400 MHz): 19.07, 28.85, 63.75, 121.31, 134.41, 140.51, 150.22, 163.75, 164.22, 170.22 MS: m/z ratio: 282.09 ($C_{11}H_{14}N_4O_3S$, predicted: 282.32). Elemental analysis: Calculated for $C_{11}H_{14}N_4O_3S$: C, 46.80; H, 5.00; N, 19.85; S, 11.36; Found: C, 46.77; H, 5.03; N, 19.87; S, 11.32.

Amide MGI: 2-Mercapto-N-{2-oxo-2-[N'-(pyridine-4-carbonyl)-hydrazino]-ethyl}-propionamide, M.P.: 189-191 ℃ (uncorrected), R_f: 0.66 (ethyl acetate:methanol:GAA; 0.5:1.5:0.02 v/v/v), Aq. Sol. : 94 mg/mL, Log P_{oct}: 0.27, IR (anhydrous KBr; cm-1): 3583 (NH, sec amide, str), 2560 (SH, str), 1692 (C=O, CHCONH, str), 1679 (C=O, aromatic CONH, str.), 1533 (C-N, pyridine ring, str), 1401 (CH_2, bend), 760 (N-N, hydrazine, sym wag) ^{1}HNMR ($CDCl_3$; 400MHz) δ 1.20-1.21[d, 3H]-CH_3, 3.04-3.07 [1H, m]-CH-CH_3, 4.04 [2H, s]-CH_2CO, 7.94-7.96 [d, 2H]-pyridine ring, 8.861-8.863 [d, 2H]-pyridine ring, 10.21 [s, 3H]-NH. ^{13}C-NMR ($CDCl_3$; 400 MHz): 18.03, 45.81, 59.18, 122.43, 139.18, 150.03, 164.38, 170.03, 175.97 MS: m/z ratio: 283.89 ($C_{11}H_{14}N_4O_3S$, predicted: 282.32). Elemental analysis: Calculated for $C_{11}H_{14}N_4O_3S$: C, 46.80; H, 5.00; N, 19.55; S, 11.36; Found: C, 46.79; H, 5.01; N, 19.88; S, 11.34

Amide MI: Isonicotinic acid N'-(2-amino-4-methylsulfanyl-butyryl)-hydrazide, M.P.: 199-201 ℃ (uncorrected), R_f: 0.66 (ethyl acetate:hexane:GAA; 0.7:0.3:0.01 v/v/v), Aq. Sol.: 114 mg/mL, Log P_{oct}: 0.06, IR (anhydrous KBr; cm^{-1}): 3461 (NH, sec amide, str), 2830, 2911 (CH, str), 1701 (C=O, CHCONH, str), 1692 (C=O, aromatic CONH, str.), 1538 (C-N, pyridine ring, str), 1325 (S-CH_3, str), 754 (N-N, hydrazine, sym wag), 661 (C-S-C, str) ^{1}HNMR ($CDCl_3$; 400MHz) δ 1.88-2.12[m, 2H]-CH_2-CH_2-S, 2.46[s, 3H]-CH_3S, 2.76-2.96 [2H, t]-CH_2-S, 3.85-3.88 [1H, t]-CH-NH_2, 7.88-7.89 [d, 2H]-pyridine ring, 8.82-8.83 [d, 2H]-pyridine ring, 10.06 [s, 4H]-NH. ^{13}C-NMR ($CDCl_3$; 400MHz): 18.36, 28.77, 29.98, 58.36, 122.36, 122.56, 140.40, 150.53, 150.63, 164.57, 170.17 MS: m/z ratio: 268.19 ($C_{11}H_{16}N_4O_2S$, predicted: 268.34). Elemental analysis: Calculated for $C_{11}H_{16}N_4O_2S$: C, 49.21; H, 6.07; N, 20.85; S, 11.89; Found: C, 49.25; H, 6.04; N, 20.89; S, 11.96.

In vitro release kinetics in aqueous buffers and tissue homogenates of rats

Kinetic parameters like rate constants, half-lives and order of kinetics of hydrolysis were also calculated (Table 2). No change in the peak of prodrugs was noted for the 0.05 M HCl buffer (pH 1.2) and phosphate buffer (pH 7.4). All the three prodrugs showed negligible release of INH in stomach homogenates of rats. They were readily hydrolysed in intestinal homogenates by first-order kinetics. There was gradual increase in the percentage of prodrugs hydrolysed in intestinal homogenates (59.43%-94.93%; Table 2); thus, confirming their activation by intestinal amidases (Figure 3).

In vivo release kinetics

Prodrug NI was selected as a representative of the three synthesized prodrugs to investigate the *in vivo* behaviour. NI appeared in blood between 2-2.5 h after oral administration, indicating absorption of intact prodrug through stomach (Figure 4). There was consistent rise in the concentration of NI in blood till 4.5-5 h, indicating absorption of intact prodrug from the small intestine also. INH and NAC appeared in the blood from 4 h onwards, indicating hydrolytic activation of NI in the small intestine. The concentration of intact NI in the blood started declining from 5.5-6.5 h and completely disappeared at 7.5-9 h. The concentration of INH and

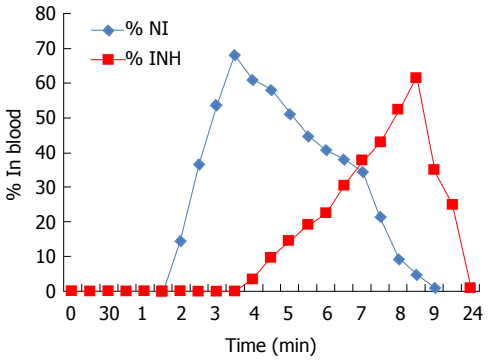

Figure 4 ***In vivo*** **pharmacokinetics after oral administration of the prodrug NI (blood).** NI: Prodrug of INH and N-acetyl cysteine.

NAC reached a maximum of 52.4%-61.3% at 9.5-10 h and 47.4%-56.8% at 8-9 h respectively. Intact NI was not observed in urine and faeces samples, whereas 1.3%-2.5% and 0.94%-0.9% of NAC was detected in 24 h pooled samples of urine and faeces respectively.

Biological evaluation

The body weights and relative liver weights of animals from prodrug-treated groups and physical mixture-treated groups calculated at the end of study had no statistical significance when compared to those of the INH-treated group.

Antioxidant markers (SOD, GSHPx and MDA): The SODs are a group of closely associated enzymes that catalyse the breakdown of the superoxide anion into hydrogen peroxide and oxygen. The function of GSHPx is to reduce free hydrogen peroxide to water and reduce lipid hydroperoxides to their corresponding alcohols[34]. Elevating GSH levels aids the productivity of the GSHPx and *vice versa*. The process of oxidative degradation of lipids is brought about by the enzyme lipid peroxidase. In this course, free radicals steal electrons from the lipids that are present in cell membranes, causing cell damage. This process progresses through a free radical chain reaction mechanism[35,36]. Polyunsaturated fatty acids are most often affected by this reaction, as they possess multiple double bonds within which are methylene groups that contain specifically reactive hydrogen. It is a free radical-mediated destructive autocatalytic process, whereby polyunsaturated fatty acids undergo degradation to form MDA. Raised MDA levels in liver signify enhanced lipid peroxidation causing hepatic tissue damage and breakdown of antioxidant defence mechanisms to obviate the excessive free radical formation. In the group treated with INH, the level of SOD and GSHPx was significantly lower and the MDA level was significantly higher. This finding confirmed the induction of oxidative stress due to administration of INH[37,38].

Treatment with NI, MGI and MI significantly re-established the levels of SOD to 93.39%, 71.98% and 88.69% respectively and GSHPx to 100%, 91.83% and 93.87% respectively (Figure 5A and B). Physical mixture INH + NAC had significant effect, whereas the effect of INH + MPG and INH + MI on both the enzymes was moderate. MDA levels were significantly decreased by prodrugs compared to the INH-treated group (Figure 5C). The effect of NI and MGI in reducing the MDA level was better compared to that achieved with MI. Physical mixtures moderately decreased the MDA level. Thus, prodrugs were found to restore the levels of antioxidant enzymes, demonstrating their hepatoprotective potential.

ALT and AST: Raised levels of ALT and AST, bilirubinuria, bilirubinaemia, jaundice and rarely severe and occasionally fatal hepatitis often occur with normal dosing procedures of antitubercular drugs. ALT and AST levels are often used to assess hepatic damage. Hepatic injury causes necrosis or membrane damage, which permits intracellular enzymes to circulate and, hence, to be detected in serum. Elevated concentration of these enzymes in the serum is an indication of loss of functional integrity of the hepatic membrane[39,40]. Elevated ALT level, especially, is a signal of hepatocellular necrosis and has been used as a surrogate marker of liver injury[41].

In the present study, drastic elevation in ALT and AST levels (144 ± 7.70 U/L and 148 ± 7.60 U/L respectively) occurred in the INH-treated group, indicating pathological conditions in liver (Figure 5D and E). Physical mixtures as well as prodrug-treated groups displayed decline in ALT and AST levels, signifying restoration of these enzymes. The effect of prodrugs compared to physical mixtures was significantly superior; the effect of prodrug NI was especially noteworthy as it restored levels of ALT and AST to 48 ± 4.50 and 46 ± 7.30 U/L respectively. This is in agreement with the generally established observation that levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes.

Triglycerides and cholesterol: Previous studies have documented a strong association between hypercholesterolemia and increased free radical production[42]. Increase in cholesterol levels in the liver might be due to increased uptake of low-density lipoprotein from the blood by the tissues[43]. Earlier studies state that treatment with INH resulted in accumulation of triglycerides (TGs) in the liver of INH-treated animals. This was found to be a consequence of decreased lipoprotein synthesis, causing impaired mobilization of lipids[44]. Moreover, the levels of total cholesterol also increased, indicating that INH induces the hypercholesterolemic condition. Increased uptake of low-density lipoprotein from the blood by the tissues and inhibition of bile secretion might be the reason behind this increase in cholesterol levels[45]. Hepatotoxic agents like INH have also been reported to disrupt the membrane fluidity and thus affect the normal hepatocellular functions[45].

In groups administered with prodrugs, TG and cholesterol level decreased remarkably in comparison to the group administered with INH (Figure 5F and G). TG and cholesterol level decreased, to a lesser extent, in

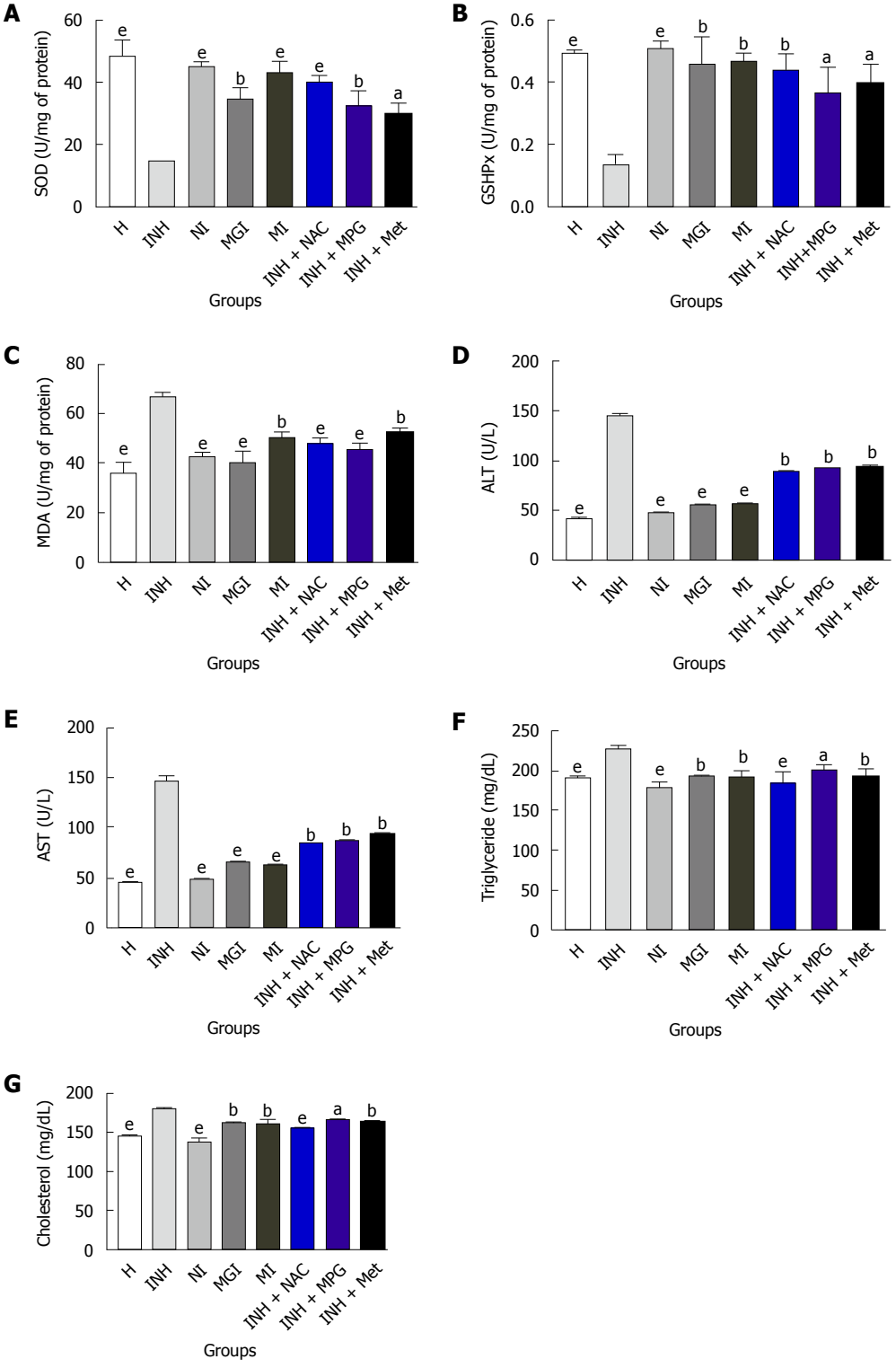

Figure 5 Effect of prodrugs on markers of liver function and oxidative stress and biochemical parameters. Average of six readings; One-way ANOVA followed by Dunnett's multiple comparison test, statistical significance considered at $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ and ns: non-significant, when *vs* INH-treated group. H: Healthy control; INH: Isoniazid; NI: Prodrug of INH and N-acetyl cysteine; MGI: Prodrug of INH and N-(2-mercaptopropionyl) glycine; MI: Prodrug of INH and L-methionine; INH + NAC: Physical mixture of INH + N-acetyl cysteine; INH + MPG: Physical mixture of INH + N-(2-mercaptopropionyl) glycine; INH + Met: Physical mixture of INH and L-methionine.

the groups treated with physical mixtures INH + MPG and INH + Met, whereas the effect of INH + NAC was more significant. Prodrugs provided remarkable defence against INH-induced aberrations in liver.

Histopathological analysis

Microscopic examination of liver sections of healthy rats showed intact parenchymal cells and normal lobular architecture (Figure 6). Polygonal hepatocytes (single headed black arrow) arranged in hexagonal lobules and portal triads at vertices were also visible. Inside each lobule, adjacent blood sinusoids (white triangle) separated the hepatocytes and Kupffer cells (two headed black arrow), along with abundant eosinophilic cytoplasm

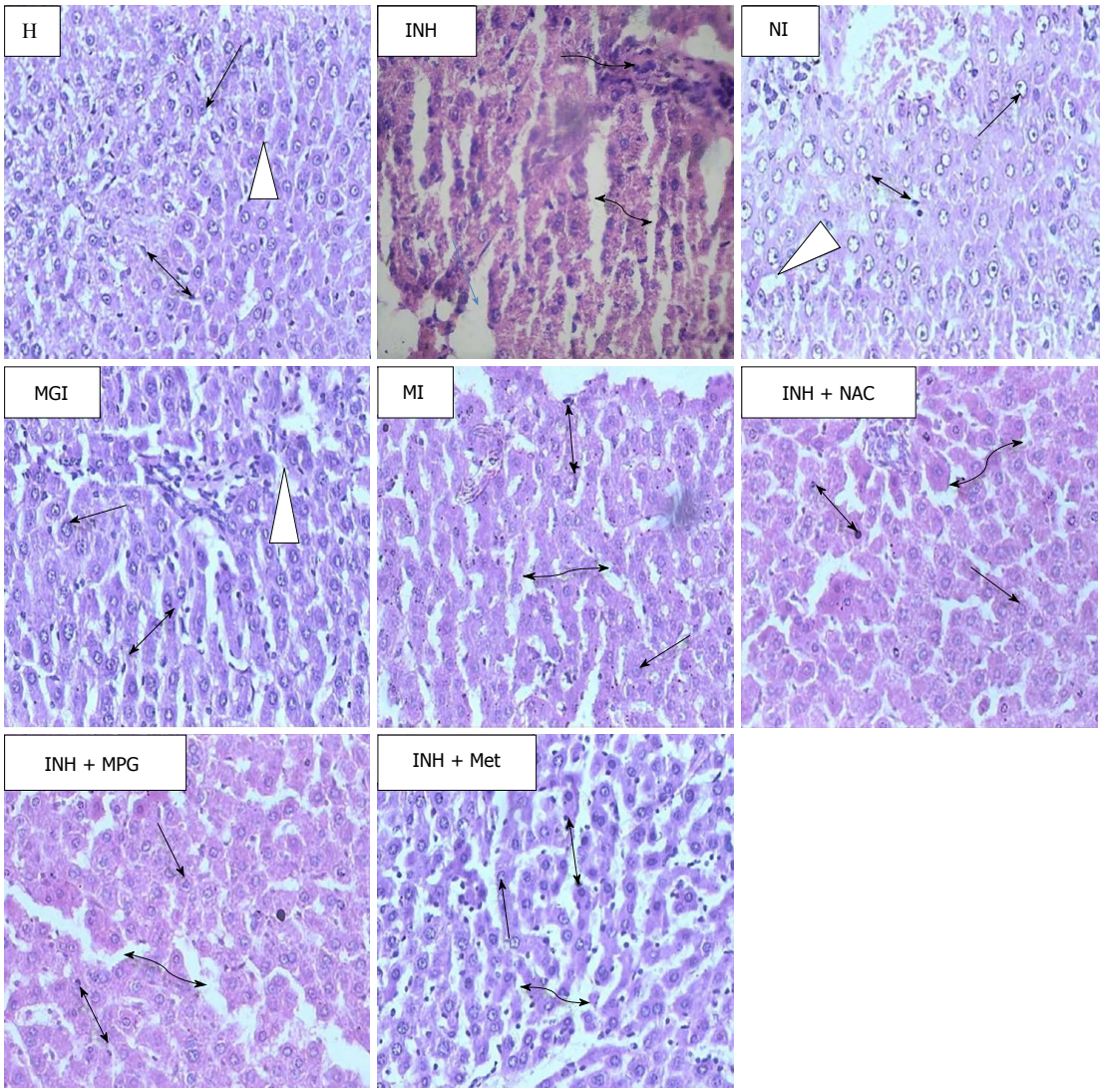

Figure 6 Photomicrographs of haematoxylin and eosin-stained histological sections of normal, isoniazid-intoxicated, prodrugs- and physical mixtures-treated rat livers.

being observable.

Membrane disintegration, degenerated nuclei, intense vacuolation accompanied by cytoplasmic rarefication leading to loss of the polyhedral structure is generally seen in liver sections of rats treated with INH. Blood sinusoidal dilatation (double headed curved black arrow), portal triaditis, multifocal area of necrosis (single headed blue arrow) and inflammatory cells (single headed curved black arrow) with granular swelling are also the prominent features in the histopathology of INH-damaged liver[46]. In the present study, all these pathological changes were noticed in liver sections of INH-treated rats (Figure 6).

The liver of rats treated with NI showed reversal of such pathology, with no evidence of necrosis, inflammation or fibrosis. Normal lobular structural design of the liver with well-preserved cytoplasm and blood sinusoids running in between normal hepatocytes were seen. Liver sections of groups treated with prodrug MGI and MI showed minimal inflammation, with minimal portal triaditis and mild dilation of blood sinusoids. The lobular architecture and hepatocytes were normal, and overall normal hepatic morphology with no histopathological derangements was observed.

Histological observations of rats administered with physical mixtures INH+NAC and INH+MPG displayed slight degenerative alterations in the parenchymal lining. Moderate sinusoidal dilatation was also observed. However, liver sections of the INH+ Met-treated group exhibited hepatocytic vacuolation, necrotic areas, moderate inflammation and severe blood sinusoidal dilatation.

DISCUSSION

Synthesis and characterization

Amide prodrugs of INH and three different sulphur containing amino acids (NI, MGI and NI) were synthesized. NI and MGI were synthesized using Schotten-Baumann reaction as described in the above section of synthesis of NI, and MGI and MI were synthesized by using the Boc-

anhydride as a protecting as well as an activating group in amide formation, as described in section of synthesis of MI. Reactions were monitored by thin-layer chromatography (ethyl acetate:methanol:GAA; 0.5:1.5:0.02 v/v/v for NI and MGI and ethyl acetate:hexane:GAA; 0.7:0.3:0.01 v/v/v for MI). Partition coefficients and aqueous solubilities of standard drugs and synthesized conjugates were determined experimentally. Log P_{oct} of INH was found to be -0.71 and aqueous solubility was 135 mg/mL. Partition coefficients of prodrugs were in the range of 0.06-0.27. The results of partition coefficients of prodrugs were in accordance with their determined aqueous solubilities (94-114 mg/mL). Increased partition coefficients might enhance the penetration and bioavailability of prodrugs.

The synthesized prodrugs were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, elemental analysis and mass spectroscopy. Formation of amide prodrugs was confirmed through IR by carbonyl stretching between 1663-1692 cm^{-1} and NH stretching of secondary amide between 3300-3583 cm^{-1}. Chemical shifts of protons of INH and amino acids backbones were confirmed by ^{1}H-NMR, in which number of protons matched with the anticipated structures. ^{13}C-NMR confirmed formation of amide linkage between one molecule of INH and one molecule of amino acid. Mass spectroscopy also exhibited molecular ion peaks in accordance with the molecular weights (268-283) of respective prodrugs. Elemental analysis results were within the permissible range that confirmed molecular weights of prodrugs.

In vitro release kinetics

All prodrugs when incubated with HCl buffer (pH 1.2) and stomach homogenates resisted hydrolysis for 3 h and when incubated with phosphate buffer (pH 7.4) resisted hydrolysis for 6 h, as HPLC analysis did not show any peaks of INH, aminothiols or unknown hydrolysis products except for the peaks of intact prodrugs. Prodrugs were readily hydrolysed in intestinal homogenates by first-order kinetics, confirming their activation by intestinal amidases. The rate constant values increased while half-lives of the prodrugs decreased, as shown in Table 2. The extent of hydrolysis was highest for NI (94.93%; Table 2).

In vivo release kinetics

In vivo pharmacokinetic properties and factors influencing it are the guiding principles for effective prodrug design and hence need to be accurately estimated. Appearance of INH and NAC in blood from 4 h onwards indicated enzymatic activation of NI in the small intestine. From these results, we hypothesize that NI must have been activated in the small intestine by enzymatic (amidase) hydrolysis. Interestingly, many unknown metabolites were detected in urine and faeces, which might be the consequence of metabolism of INH and NAC.

Biological evaluation

Results of estimations of antioxidant markers, including SOD, GSHPx and MDA, of liver function markers, including ALT and AST, and biochemical parameters, including cholesterol and TG, clearly indicated the abrogating effect of synthesized prodrugs. Histopathological observations correlated with the biochemical findings, thus confirming that prodrugs were more effective than physical mixtures in re-establishing the normal hepatic cytoarchitecture[47,48].

The ameliorative effect of prodrugs observed in this study can be accredited to the thiol-containing amino acids which were used as carriers. They can cause hepatotropic detoxification by augmenting synthesis of GSH, which is an endogenous antioxidant and is usually depleted as a consequence of increased oxidative stress[49]. They also act either by maintaining GSH in the reduced state or providing an alternative nucleophilic target and, thus, protect protein-SH groups[18].

In summary, to address the issue of INH-induced hepatotoxicity, prodrugs of INH with aminothiol antioxidants were successfully synthesized employing simple methods for amide synthesis. *In vitro* and *in vivo* release studies confirmed the bioactivation of these prodrugs. Observations obtained from hepatoprotective potential assessment demonstrated that the prodrugs had remarkable restorative effect on levels of enzymes and biochemical parameters involved in liver injury, to normal. Further confirmation was obtained from histological micrographs of liver that displayed complete reparation of hepatic tissue. The underlying mechanism of this protective action of prodrugs involved the suppression of oxidative stress, cell membrane stabilization and reinforcement of cellular antioxidant defences, as evidenced from interpretations of hepatoprotective activity. Findings of this study proved the success of concept-based prodrug design in abating the deleterious effects of INH and its metabolites on liver.

We believe that these novel prodrugs may offer a new therapeutic strategy in TB treatment by preventing complications associated with long-term use of INH. However, further studies to explore their *in vivo* antimycobacterial efficacy are required and are under progress in our laboratory, the results of which will be communicated in our future publications.

ARTICLE HIGHLIGHTS

Research background

Tuberculosis (TB) is widely viewed as a disease of the developing world and death rates are often attributed to failure of overburdened public health systems to deliver appropriate care to infected individuals. The logistics of delivering the complex regimen and ensuring patient compliance with the full course of chemotherapy challenge the resources of the public health sector, even in the most developed countries. TB drug development has made substantial progress in the past decade. There are currently at least 10 drugs being evaluated in clinical trials. Some belong to chemical classes already employed in first- or second-line treatment regimens and are being explored for more optimized use at higher doses or in new drug combinations (oxazolidinones, rifamycins and fluoroquinolones), while others represent potential novel members of the TB drug arsenal, killing *Mycobacterium tuberculosis* through previously untried mechanisms of action (diarylquinolines, nitroimidazoles, pyrroles and ethylene diamines). The typical challenges of drug development are augmented in TB by the complexity of the disease, the requirement for multi-drug regimens,

the relative lack of TB drug development for the past several decades, and inadequate resources being brought to bear despite the urgency of the global medical need. Yet, in the face of these challenges, none of the drugs have succeeded to reach the patients. The urgent need for innovation and persistent efforts to tap novel resources cannot be denied. A fine balance needs to be achieved between protecting novel drugs or modified derivatives of existing drugs so that resistance, side effects and toxicity could be minimized, ensuring that regimens are low-cost, safe, readily available, and adopted by healthcare systems and providers. The current treatment regimen has several drawbacks, including prolonged treatment time to completely eradicate the bacteria (sterilization). This increases the risk of toxicity associated with long-term use of antitubercular drug. Isoniazid (INH) is a major first-line drug used for the treatment of TB, although the metabolic and morphological aberrations that it causes and emergence of its resistance on wide scale have been a matter of great concern for future treatment schedules of TB. Therefore, a suitable molecular modification of INH in the form of codrugs was performed in order to resolve these issues. Antioxidant aminothiols were selected as carriers to minimize hepatotoxic effects of INH. The hepatoprotective potential of these prodrugs was investigated in Wistar rats to prove effectiveness in abrogating liver damage caused by INH.

Research motivation

The root cause of INH toxicity is believed to be in the metabolism of INH at the N2 centre in hydrazinic chain by the enzymes N-acetyltransferases, which are responsible for acetylation of INH. This acetylation by N-acetyltransferases in humans is under genetic control and can be divided into two categories, *viz*. "fast acetylators" and "slow acetylators". The fast acetylators, in long-term treatment of INH, lead to significant lowering of drug bioavailability and consequent generation of INH resistance. Whereas in the case of slow acetylators, high levels of INH lead to serious hepatotoxicity. In both the cases, optimization of dose is a major issue. INH after metabolism in the liver produces hydrazine metabolites (nitrogen-centred free radicals). These radicals generate highly reactive oxygen species, which act as stimulators of lipid peroxidation, resulting in cell death and hepatic necrosis. Acute poisoning leads to lactic acidosis and renal failure, development of agranulocytosis, INH-induced tenosynovitis, INH-induced liver injury, and fatal INH-induced acute liver failure, to name only a few of the toxic consequences of INH. Furthermore, INH and/or its metabolites (*e.g.*, hydrazine) are associated with causing mitochondrial injury that may lead to oxidant stress in mitochondria and destruction of energy homeostasis. This specific observation inspired us to design prodrugs of INH; transiently masking the N2 centre in the hydrazinic chain of the INH moiety by using aminothiols which could serve by protecting the liver against toxic effects of INH. Previous studies have shown that aminothiols have antioxidant potential and they attenuate liver injury induced by INH, acetaminophen, fluoride, cisplatin, carbon tetrachloride and lead overdose. But, none of the studies have been based on introducing these antioxidant promoeities [N-acetyl cysteine (NAC), N-(2-mercaptopropionyl) glycine (MPG) and L-methionine (Met)] in the INH molecule *via* amide linkage at the hydrazinic centre and exploring their therapeutic potential. This study is the first to explore the hepatoprotective potential of aminothiol-INH conjugates for restoration of normal hepatic physiology in INH-intoxicated rats and their possible healing mechanism. It was foreseen that these prodrugs may find utility in safer treatment of TB.

Research objectives

The chief objective of this work was to minimize hepatotoxic effects of the antitubercular drug INH and, thereby, improve its safety profile in the management of TB. As metabolism at the hydrazinic chain of INH is responsible for possible side effects, we thought of masking the N2 centre in the hydrazinic chain transiently. Hepatoprotective action was achieved by using aminothiols such as NAC, MPG and Met as antioxidant carriers, which act by scavenging free radicals. Signs of liver injury did not manifest in the prodrug-treated groups, which was one of the important objectives of the present study. Future research could be directed at investigating the *in vivo* antimycobacterial potential of these prodrugs.

Research methods

Facile synthesis of target mutual prodrugs was accomplished through optimization of the Schotten-Baumann reaction and Boc-anhydride to avoid complex purification procedures. Spectral analysis was used for extensive characterization of synthesized prodrugs. Novel HPLC methods were developed and validated for simultaneous estimation of INH and aminothiols in the presence of intact prodrugs in order to study their release profiles in buffers of varied pH, rat homogenates of the gastrointestinal tract, blood, urine and faeces. For *in vivo* study, male Wistar rats weighing 180-220 g were fasted for 24 h. The animals were given drug solution in stipulated dose, quantity depending on the body weight of each animal. At 0 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 hr and 24 h of treatment, 3 mL of blood was withdrawn by retro-orbital puncture into EDTA-coated tubes and centrifuged at 5000 rpm at 0-5 ℃ for 10 min. A 0.1 mL aliquot of the supernatant solution of centrifuged blood was added to an Eppendorf tube and 0.9 mL of methanol added to it for immediate plasma protein precipitation. The solution was vortexed for 2 min and then centrifuged at 5000 rpm for 10 min at 0-5 ℃ in order to precipitate solid matter present in the biological sample and other impurities. Then, 20 μL of the supernatant was injected into the HPLC instrument. Hepatoprotective potential was evaluated in male Wistar rats in a 21-d study. Doses calculated on equimolar basis were administered for 21 d to respective groups. All the animals were examined for liver function markers, antioxidant markers, biochemical parameters and liver histology. Livers of the sacrificed animals were removed and fixed in 10% buffered formalin and samples were sent for microscopic examination. Various markers like aminotransferases, superoxide dismutase, glutathione peroxidase and malondialdehyde, cholesterol and triglycerides were estimated at the end of study. Relevant statistical tests were used for analysing the data.

Research results

Activation of prodrugs by amidases in the small intestine, restoration of enzyme levels, re-establishment of the antioxidant defence system to avert the formation of excessive free radicals, regeneration of hepatocytes, maintenance of structural integrity of liver by released aminothiols and significantly notable reparation of INH-induced hepatotoxicity in Wistar rats were the promising outcomes of this study. This is the pioneer study to identify and describe the therapeutic potential of hepatoprotective prodrugs of INH in Wistar rats. These prodrugs could be explored further as an alternative to INH for treatment of TB.

Research conclusions

In the present work, the N2 centre of the hydrazinic chain in INH was transiently masked with aminothiols, as literature review revealed that antioxidant aminothiols play a vital role in restoring the antioxidant defence system of a host after a free radical-mediated destructive autocatalytic process which occurs in INH-induced hepatotoxicity. The novel hepatoprotective prodrug strategy proved to be advantageous in terms of lowering the biochemical parameters and liver function markers, and remarkably improving the levels of enzymes involved in the antioxidant defence system. This study emphasized the effectiveness of aminothiols to maintain integrity of the liver. These prodrugs have the potential to be screened further for their effectiveness in patients who are on long-term treatment with INH.

Research perspectives

This study proved that concept-based design of hepatoprotective prodrugs can be applied successfully in overcoming toxic effects of a drug. This work proved that an amide conjugation strategy can provide a potential synergistic effect in abrogation of INH-induced hepatotoxicity. Future research should be directed towards investigation of *in vivo* antimycobacterial potential and pharmacokinetic profiles in order to elucidate the exact molecular and biochemical mechanisms of these synthesized prodrugs.

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CASE REPORT

Hepatitis B virus subgenotype F3 reactivation with vaccine escape mutations: A case report and review of the literature

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Author contributions: Schlabe S and van Bremen K wrote the manuscript; Marsen T wrote the nephrological medical history, including transplantation and HBV management before transplantation; Mellin W discussed pathological findings of HBV reactivation; Eis-Hübinger AM, Aldabbagh S, Bremer CM, and Glebe D characterized the resistant virus and performed deep sequencing; Di Cristanziano V, Schlabe S, van Bremen K, Marsen T and Spengler U took care of the patient; escape mechanism and impact of second and third-generation vaccines were discussed and revised by Schlabe S, Spengler U, Eis-Hübinger AM, and Glebe D; the phylogenetic analysis and alignment were done by Eis-Hübinger AM and Aldabbagh S; all authors read and approved the final manuscript.

Informed consent statement: The patient gave informed consent for the publication of his medical history. Identifying details were omitted or anonymized.

Conflict-of-interest statement: Spengler U has consulted for AbbVie and has received a royalty from UpToDate for another study. Marsen T has received speakers honoraria from GHD Gesundheits GmbH and Fresenius Medical Care Nephrology for another study. Schlabe S has received sponsoring for educational events from Janssen for another study. All other authors state no conflict of interest.

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Abstract

Hepatitis B represents a global health threat because its chronic course and sequelae contribute to a high morbidity and mortality. Hepatitis B virus (HBV) infection can be controlled by vaccines, antiviral treatment, and by interrupting transmission. Rare vaccine escape mutants are serious because they eliminate vaccine protection. Here, we present a 74-year-old vaccinated patient with HBV reactivation 11 years after kidney transplantation. The patient was HBV-positive but HBsAg-negative prior to vaccination 6 years before transplantation. The reactivated virus was HBV genotype F3 with vaccine escape mutations G145R, P120Q, and Q129P. The patient was successfully treated with entecavir. The epidemiological reasons for this subgenotype, which is extremely rare in Western Europe, were unclear. This case illustrates that second-generation vaccines are not always effective in a specific group of patients.

Key words: Entecavir; Hepatitis B virus; Subgenotype F3; Kidney transplantation; Vaccine escape mutant G145R

Core tip: We report the first documented case of hepatitis B virus (HBV) subgenotype F3 reactivation with vaccine escape mutations in a patient after kidney transplantation. We successfully treated this patient with entecavir. This case illustrates a specific clinical situation in which the current World Health Organization HBV vaccine may be unsuccessful, and third generation vaccines should be considered.

Schlabe S, van Bremen K, Aldabbagh S, Glebe D, Bremer CM, Marsen T, Mellin W, Di Cristanziano V, Eis-Hübinger AM, Spengler U. Hepatitis B virus subgenotype F3 reactivation with vaccine escape mutations: A case report and review of the literature. *World J Hepatol* 2018; 10(7): 509-516 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i7/509.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i7.509

INTRODUCTION

Hepatitis B virus (HBV) is an enveloped virus of the *Hepadnaviridae* family. HBV is highly hepatotropic and is transmitted parenterally. There are 111.2 million cases of hepatitis[1] and 686000 hepatitis-related deaths reported worldwide each year. Long-term sequelae of hepatitis include liver cirrhosis and liver cell carcinoma, and these are responsible for the majority of hepatitis related deaths[2]. HBV has at least ten different genotypes, each with a specific geographic pattern[3].

There is no cure for chronic HBV infection. Antiviral agents are able to suppress viral replication but have to be taken permanently to prevent reactivation. A plasma-derived vaccine (first generation) was developed in 1982 and was replaced in 1986 by a recombinant monovalent yeast-derived vaccine (second generation). Global prevention strategies include vaccination at birth and during early childhood. However, 5%-10%of immunocompetent vaccines do not induce protective neutralizing antibody production (more so in immunodeficient patients)[4].

Immunization with second-generation vaccines relies on neutralizing antibody production against the "a" determinant, a hydrophilic antigenic domain (residues 100-170) of the HBV surface (S) protein. Specific single mutations, such as G145R, disrupt the structure of this epitope domain so that neutralizing antibodies (anti-HBs) cannot recognize it, thereby eliminating protection[5].

In this study, we report a case of HBV subgenotype F3 reactivation after kidney transplantation. Our findings highlight that current vaccines are not always effective against mutated HBV. Furthermore, current vaccines do not confer protection against HBV subgenotype F3, which is distantly related to those subgenotypes that current vaccines are based upon.

CASE REPORT

A 74-year-old patient was referred to the University Hospital of Bonn with acute hepatitis in March 2015. He initially presented with loss of appetite and acute watery diarrhea for 7 d. He was a frail patient, requiring long-term level Ⅲ care. Six weeks prior to admission he had slightly elevated alanine aminotransferase (ALT) levels (78 U/L) due to a urinary tract infection.

His medical history included chronic kidney failure due to tuberculous empyema, and the left kidney was resected in 1965. In May 1996, end-stage renal disease was diagnosed and hemodialysis was started. Prior to dialysis, chronic HBV infection had been diagnosed with partial seroconversion (anti-HBc and anti-HBe-positive and anti-HBs-negative). In November 1995, he tested positive for the HBV virus surface antigen (HBsAg). HBsAg declined to borderline levels in July 1996; and in May 1996, only low levels of HBV DNA viremia (9 pg/mL) were detected. Tests for HBs-Ag and HBV DNA have been repeatedly negative since July 1996. Two years later, seroconversion to anti-HBs had still not occurred, and the patient was repeatedly vaccinated with GenHBVax (January 1998, May 1998, and July 2002), which stabi-

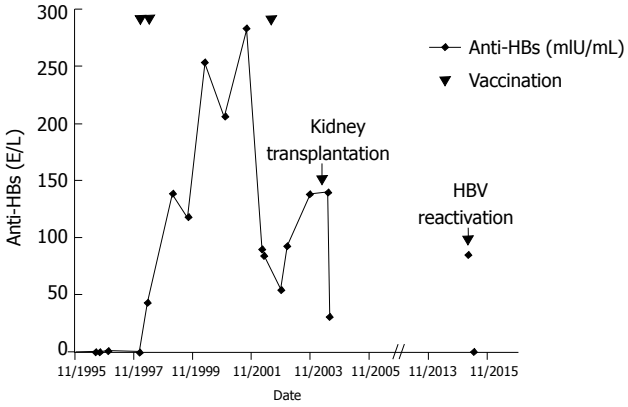

Figure 1 Course of anti-HBs levels since diagnosis of hepatitis B virus infection in 1995. Vaccine was administered in January 1998, May 1998, and July 2002. Kidney transplantation and hepatitis B virus reactivation are indicated.

lized anti-HBs levels (Figure 1).

In June 2004, he underwent cadaveric kidney transplantation and received three immunosuppressive treatments: sirolimus, tacrolimus, and methylprednisolone. This treatment was discontinued several months later because of severe side effects and was converted to 25 mg azathioprine every other day in combination with daily administration of 300 mg allopurinol plus 4 mg prednisolone with close lymphocyte monitoring. Permanent antiviral treatment was not administered. After the immunosuppressant regimen was changed, graft function remained stable with an eGFR (MDRD) grade of IIIb A1.

At admission (March 2015), his liver enzyme levels were massively elevated: ALT, 1462 U/L; aspartate aminotransferase (AST), 1459 U/L; gamma glutamyltransferase 407 U/L; lactate dehydrogenase (LDH), 616 U/L; and bilirubin, 5 mg/dL. Creatinine was elevated to 2.9 mg/dL (eGFR (MDRD) 25 mL/min). Ultrasound and CT scans excluded a tumor, thrombosis, or abscess. Serologic hepatotropic virus evaluation revealed highly replicative HBV infection with 22.5 million IU/mL HBV DNA and positive results for HBsAg (79.11 IU/mL) and anti-HBs (85.2 mIU/mL). Anti-HBe tests were also positive, while anti-HBc IgM tests were negative. Infection with hepatitis C/D viruses, CMV, and HIV was excluded. Markers of past hepatitis A/E virus, HSV, and Epstein Barr virus (EBV) infections were detected. A liver biopsy showed acute hepatitis with multiple disseminated acidophilic single cell necroses, pericentral lipofuscinosis, vacuolar lipid droplets, and minimal periportal fibrosis. The latter was interpreted as chronic toxic damage (Figure 2).

Aminotransaminase levels decreased slightly, but bilirubin peaked at 24 mg/mL 11 d after presentation. Because hepatitis B had reactivated despite high levels of anti-HBs antibodies, we initiated further analyses. Full genome analysis revealed the virus to be subgenotype F3 of genotype F (Figure 3). HBV genotype F is endemic in South America but rare in Europe. There were no reported contacts to South America, Spain, or any other epidemiological links to regions endemic for genotype F. The patient had received blood transfusions following a traffic accident with bilateral hip fracture requiring surgical procedures back in the 1960s, which may represent potential transmission routes. After kidney transplantation and revisions to the hip prosthesis, only minor surgical wound care interventions had been performed before presentation.

We found eight mutations and amino acid substitutions in the S protein, including the most common vaccine escape mutation G145R (Figure 4). Analysis of the HBV polymerase reverse transcription domain showed no known primary resistance mutations against the nucleos(t)ide analogues lamivudine, telbivudine, entecavir, tenofovir, or adefovir. Sequence analysis showed that wild-type and mutated viruses were present at a ratio of approximately 40:60 for five out of the eight identified mutations, indicating a mixed HBV population. The CD4 cell count was low (121/μL), the B cell count was strongly reduced (2/μL), and immunoglobulin levels were slightly reduced. Therefore, immunosuppressive treatment was reduced to 3 mg prednisolone, 100 mg allopurinol, and 25 mg azathioprine.

Daily treatment with 0.5 mg entecavir was initiated because of reduced kidney function. Viral replication decreased significantly and was negative 5 mo after the diagnosis (Figure 5). The entecavir dosage was later adjusted according to kidney function every fourth day. No complications with liver function were reported following treatment. Immunosuppressive treatment was changed to tacrolimus.

DISCUSSION

Reactivation of chronic hepatitis B in immunosuppressed patients is serious. In this patient, reactivation occurred approximately 11 years after immunosuppressive therapy was initiated, and no recent causes for reactivation, such as a change in immunosuppressive medication, were reported. HBV reactivation is a well described risk in renal transplant recipients. However, only one report of reactivation due to a vaccine escape mutant (HBV genotype E) in a successfully vaccinated post-transplant patient 4 years after kidney transplantation has been published[6]. Post-renal transplant patients are at risk of *de novo* HBV infections due to immunosuppression. At least two reported cases of HBV infection have been described in vaccinated kidney transplant patients carrying vaccine escape mutations[7,8]. A *de novo* infection, albeit unlikely, cannot be excluded completely in our patient.

We identified the immune escape mutation G145R in our patient. This mutation was discovered in 1988[9] and has been described in HBV genotypes A-D worldwide[10]. Reduced antibody binding is sufficient for immunologic breakthrough of HBV escape mutants[5]. We also identified a P120Q mutation in the upstream region of the HBV core gene and two different mutations at positions

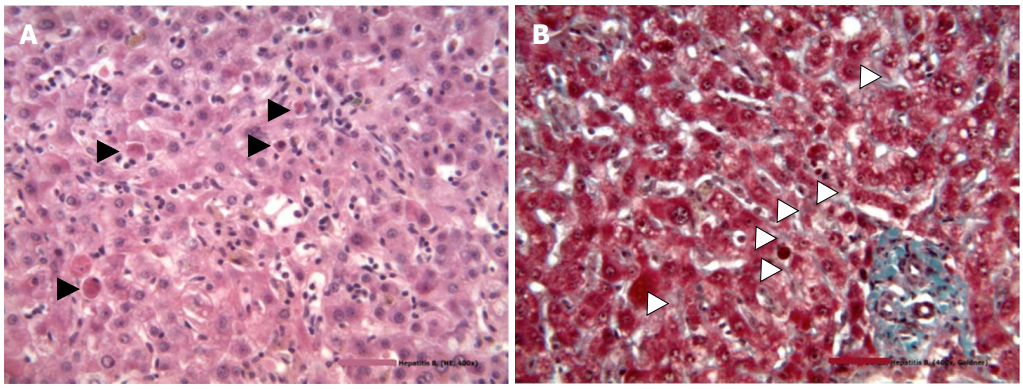

Figure 2 Hematoxylin and eosin stain of a liver specimen from the patient described in this study showing necrosis of hepatocytes (black triangles) without infiltrations and trichrome stain for fibrosis. A: Necrosis of hepatocytes; B: Trichromestain for fibrosis. Periportal fibrosis is indicated by white triangles.

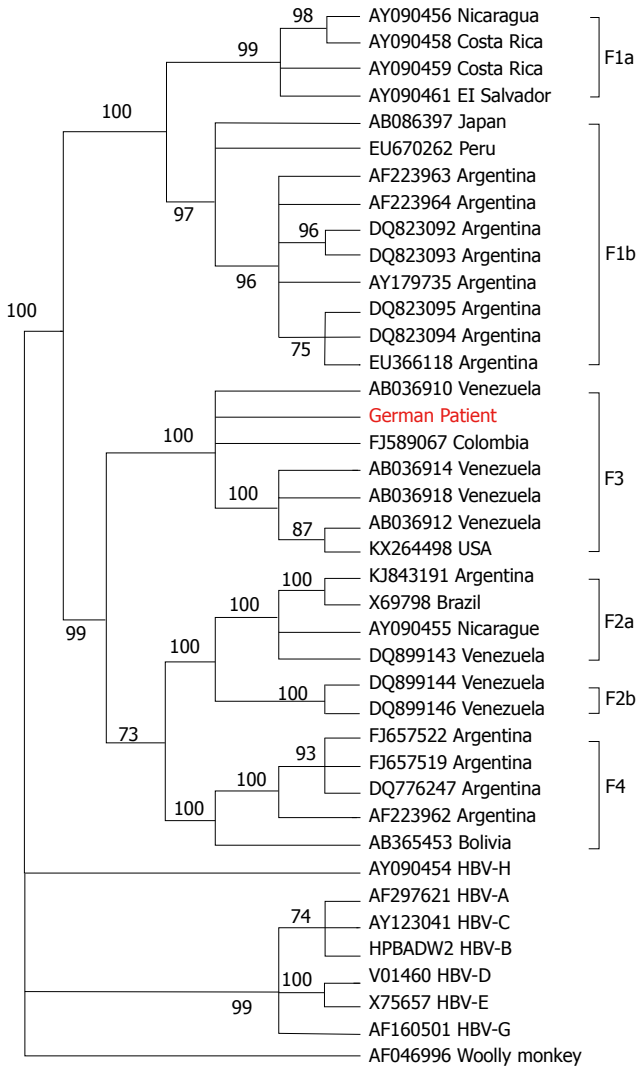

Figure 3 Phylogenetic tree of hepatitis B virus based on complete hepatitis B virus genomes. The tree was constructed using the MEGAv6.0 software package[29], (http//:www.megasoftware.net), with the neighbor-joining method with p-distance and 1000 bootstrap replicates. Bootstrap values of at least 70 are shown at the nodes. Phylogenetic analysis was performed using reference sequences from GenBank, indicated in the tree by their GenBank accession numbers. Country of virus origin is included for all genotype F sequences. Woolly monkey HBV was used as the outgroup. The sequence from the patient described in this study is presented in red. HBV: Hepatitis B virus.

Q129, Q129P, and H in our patient. Mutations at these positions have been described in immune escape variants or occult infection[10]. The combination of different mutations alters HBV antigenicity even further[11]. All eight mutations found in the case presented here and available previous reports on these specific mutations

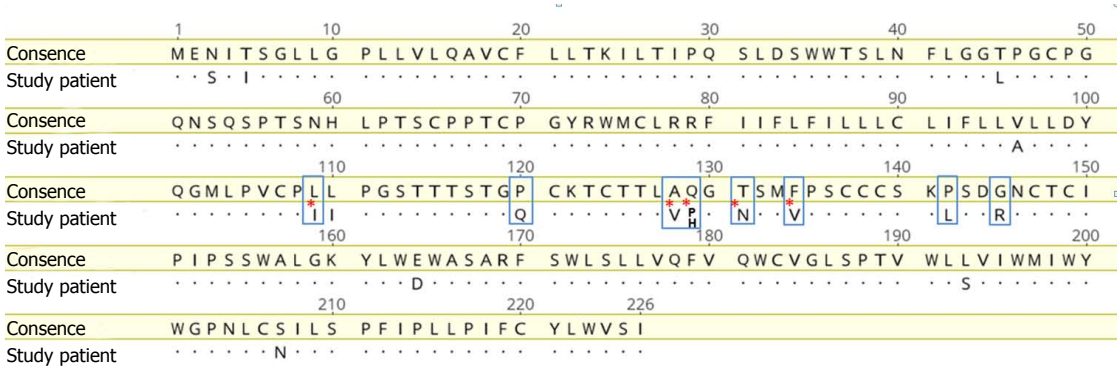

Figure 4 Vaccine escape mutations in the patient's hepatitis B virus strain. Alignment of the HBs antigen amino acid sequence (SHBs) of the patient´s strain with a HBV genotype F consensus sequence derived from the HIV-grade database (www.hiv-grade.de). Amino acids are named according to the one-letter code. Dots in the patient´s sequence represent amino acids identical to those in the consensus sequence. Positions relevant for drug resistance are indicated by blue boxes. Red asterisks denote positions in the patient´s strain (*i.e.*, 109, 128, 129, 131, and 134) where both the amino acid of the consensus strain and the vaccine escape mutation were observed. HBV: Hepatitis B virus.

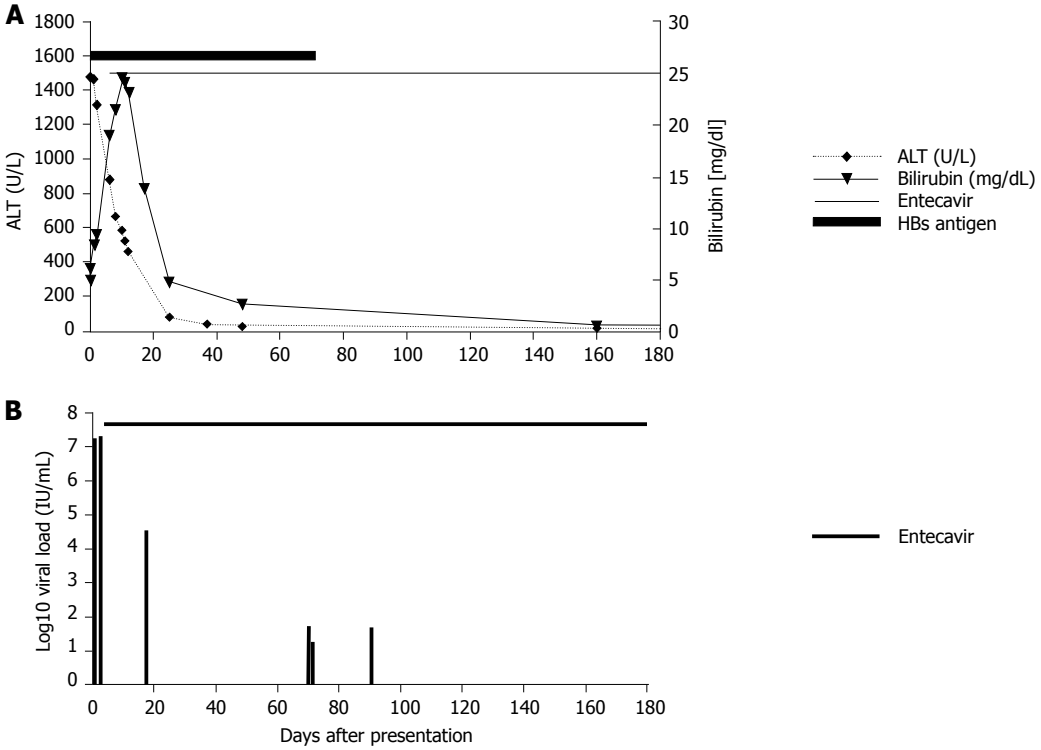

Figure 5 Course of amino transferases and bilirubin levels during acute hepatitis in March 2015 and HBV viremia levels after diagnosis of hepatitis B virus reactivation. A: Course of amino transferases and bilirubin levels during acute hepatitis in March 2015; B: Course of HBV viremia levels after diagnosis of HBV reactivation. Start of antiviral treatment is indicated. ALT: Alanine aminotransferase.

are provided in the supplemental file. Escape mutants are rare, but they can be selected by vaccination in highly endemic regions[12] and during reactivation of occult HBV.

HBV genotype F is endemic in Central and South America and has four subgenotypes, F1–F4[13]. Prevalence outside South America is very low (up to 7% in Spain[14]and 1.4% in the whole of Europe[15]). Surprisingly, the HBV genotype in our patient was subgenotype F3, subtype adw4q. The phylogenetic tree of this virus was similar to virus strains in Venezuela (Figure 3).

HBV genotype F is the most genetically divergent of the HBV genotypes[16]. It shares a close genetic and geographical association with related orthohepadnaviruses endemic in mammals of Middle and South America, like the woolly monkey, a nonhuman primate, and the Tent-making bat. This suggests an evolutionary link between these viruses in different host species[17]. The distant relationship of HBV genotype F strains with European HBV isolates has specific clinical implications. For example, the current World Health Organization (WHO) vaccine is based on the "a" determinant domain of subgenotype A2 (subtype adw2), which has a different antigen to genotype F (subtype adw4). Therefore, vaccine escape is more likely in genotype F. Moreover, HBV genotype F infections have high viral

loads and are typically HBeAg-positive in acute cases. This may facilitate breakthrough when anti-HBs titers are low[18,19]. However, only two cases of vaccine failure in patients infected with HBV subgenotype F have been reported. They were infected with genotype F1b (subtype adw4) after traveling to Northern Argentina[20] and with subgenotype F1 after traveling to Spain[21], respectively. Escape mutants were not detected in either patient, suggesting that the HBV genotype F breakthrough without the help of escape mutations. The patients described in these previous reports were vaccinated before infection. In contrast, our patient was vaccinated after HBV infection. In the 1990s, several clinical pilot trials tested vaccination in HBsAg-positive patients to induce virus-specific cellular immunity and HBsAg loss ("therapeutic vaccination") using vaccines containing HBsAg and later containing preS2/S proteins[22]. Initial results suggested a small effect on replication, seroconversion, and HBsAg loss, but the concept was later proven unsuccessful, at least with current vaccines[23]. In our case, vaccine escape was an additional negative effect of this vaccination therapy. Escape mutation selection in our patient after second-generation vaccination could be explained by: (1) incomplete neutralization of HBV genotype F by the generated antibodies; and (2) selection pressure from vaccination. To the best of our knowledge, this is the first case describing breakthrough of HBV genotype F due to G145R, P120Q, and Q129P escape mutations in a transplant setting.

The high viral load in our patient presented a substantial risk for virus transmission during blood sampling or laboratory blood work. The risk was aggravated because the virus was HBeAg-negative, which favors the development of severe and fulminant acute hepatitis due to abnormal immunopathology. Fatal outbreaks of HBeAg-negative HBV strains, even with common genotypes, have recently been reported in healthcare settings and nursing homes[24].

This case illustrates the limitations of the conventional HBV vaccine in individuals infected with escape mutant forms of the virus. These limitations include impaired immunogenicity and inability of neutralizing antibodies to recognize the virus. All available second-generation, yeast-derived vaccines are based on the non-glycosylated "a" determinant of the small surface HBV protein (SHBs). Several third-generation vaccines (Hepagene™, Bio-Hep B™, Sci-V-vac™) were developed in the 1990s and contain all three HBV envelope proteins: The SHBs, the medium (MHBs) (preS2-SHBs), and the large (LHBs) (preS1/preS2-SHBs) within subviral particles derived from mammalian cells[25]. The amino terminal of the preS1 domain contains the binding domain for sodium-dependent taurocholate co-transporting peptide (NTCP), which is the high-affinity receptor for HBV cell binding and entry[26]. PreS1-specific antibodies are interesting because the receptor-binding domain of preS1 is relatively conserved across all HBV genotypes. To date, a third-generation vaccine is not globally available; one has been on the market in Israel and in several countries in East Asia and Africa since 2001. The response rate in healthy non-responders is superior to second-generation vaccines[27] as well as in immunosuppressed post-liver transplantation patients who do not respond to second-generation vaccines[28].

In summary, we present a rare case of vaccine escape in an immunosuppressed patient infected with the rare HBV subgenotype F3 in Western Europe. This vaccine escape mutant replicated despite high levels of anti-HBs. Fortunately, antiviral agents effectively lowered the viral load and ameliorated liver inflammation. Despite this successful treatment, third-generation vaccines should be introduced as a protective measure to enhance the immunological protection of low-responders and risk groups (*e.g.*, immunosuppressed patients, patients with chronic kidney failure) and possibly induce seroconversion of HBs-Ag to anti-HBs in chronic HBeAg-negative patients with low viral loads. The HBV status of immunosuppressed patients should be carefully monitored in transplant settings to prevent possible reactivation.

ARTICLE HIGHLIGHTS

Case characteristics

A 74-year-old patient with previous hepatitis B infection presented 11 years after kidney transplantation with diarrhea, loss of appetite, and icterus.

Clinical diagnosis

The main clinical finding was acute liver dysfunction.

Differential diagnosis

The differentials of acute liver dysfunction in this patient were acute hepatitis caused by hepatitis B virus (HBV) reactivation or hepatitis D virus (HDV) superinfection or infection with another hepatotropic virus, sepsis, obstruction of bile ducts, cholangitis, tumor, abscess, or thrombosis.

Laboratory diagnosis

Laboratory results revealed acute hepatitis caused by highly replicative HBV subgenotype F3 with immune escape mutations.

Imaging diagnosis

A computed tomography (CT) scan excluded bile duct obstruction, tumor, abscess, and thrombosis.

Pathological diagnosis

Histologic examinations showed acute hepatitis with multiple disseminated acidophilic single cell necroses and pericentral lipofuscinosis, vacuolar lipid droplets, and minimal periportal fibrosis suggesting additional chronic toxic damage.

Treatment

Acute hepatitis B was treated with an antiviral medication, the nucleoside reverse transcriptase inhibitor (NRTI) entecavir (ETV).

Related reports

HBV reactivation has been reported in immunosuppressed patients, patients

with *de novo* infection, and in successfully vaccinated persons (caused by vaccine escape mutations or subgenotype F).

Term explanation

Vaccine or immune escape describes the ability of the HBV to reinfect or reactivate in the presence of neutralizing antibodies that cannot neutralize the virus.

Experiences and lessons

Acute hepatitis caused by hepatitis B infection may occur in a successfully vaccinated patient if the virus escapes antibody neutralization. Escape may be caused by escape mutations that change surface antigens or by a virus genotype that is distantly related to the virus genotype that the second-generation HBV vaccine is based on.

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CASE REPORT

Large primary hepatic gastrinoma in young patient treated with trisegmentectomy: A case report and review of the literature

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Author contributions: Pipek LZ, Jardim YJ, Mesquita GHA, and Martines DR wrote the article; Nii F, Medeiros KAA, and Carvalho BJ performed the literature search; Waisberg DR and D'Albuquerque LAC identified the case and revised the manuscript for important intellectual content; Iuamoto LR, Andraus W, and Meyer A reviewed the final version of the article.

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Abstract

Primary hepatic gastrinoma is a rare disease, with fewer than 40 cases reported in the medical literature. Because it is located in an organ in which metastases are common, its diagnosis is difficult. We report a case of a 19 years old male patient with a history of gastric

ulcers since the age of nine. Following gastric surgery, an antrectomy and a vagotomy, there was some alleviation of symptoms. Subsequently, the patient reported various intermittent episodes of diarrhea, diffuse abdominal pain, and vomiting. The patient underwent tomography, which revealed the presence of a hepatic mass measuring 19.5 cm × 12.5 cm × 17 cm. Primary hepatic gastrinoma was diagnosed based on laboratory examinations that indicated hypergastrinemia and a positron emission tomography/magnetic resonance study with somatostatin analogue that confirmed the liver as the primary site. After hepatic trisegmentectomy (Ⅱ, Ⅲ, Ⅳ, Ⅴ, Ⅷ), the patient's symptoms improved. The case is notable for the presence of a rare tumor with uncommon dimensions.

Key words: Gastrinoma; Primary hepatic gastrinoma; Zollinger-Ellison syndrome; Hepatic trisegmentectomy; Gastric surgery

Core tip: Primary hepatic gastrinoma is a very rare disease. Due to its location in an organ in which metastases are common, its diagnosis is difficult. We report a case of a 19 years old male patient with a history of gastric ulcers since the age of nine. Hypergastrinemia and a PET/MR study with somatostatin analogue confirmed that the liver was the primary site. After hepatic trisegmentectomy, the patient's symptoms improved. The case is notable for the presence of a rare tumor with uncommon dimensions.

Pipek LZ, Jardim YJ, Mesquita GHA, Nii F, Medeiros KAA, Carvalho BJ, Martines DR, Iuamoto LR, Waisberg DR, D'Albuquerque LAC, Meyer A, Andraus W. Large primary hepatic gastrinoma in young patient treated with trisegmentectomy: A case report and review of the literature. *World J Hepatol* 2018; 10(7): 517-522 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i7/517.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i7.517

INTRODUCTION

Zollinger-Ellison syndrome[1] is characterized by gastric hypersecretion, resulting in peptic disease and diarrhea[2]. Diagnosis is confirmed by gastrin levels of over 1000 pg/mg. This syndrome was first described by Zollinger and Ellison[2] in 1955. With an incidence of around 0.5 to 2 per million, the majority of patients are diagnosed between 20 and 50 years of age, with a higher prevalence among men. The majority of gastrinomas are sporadic, and 20%-30% are associated with type 1 endocrine neoplasias, NEM-1[3].

The classical clinical presentation of the syndrome is the presence of abdominal pain (75%) and chronic diarrhea (73%). Half of patients present with burning as a result of gastroesophageal reflux. There is still a significant population (17%-25%) that presents with weight loss and gastrointestinal bleeding[4]. Over 90% of cases of gastrinomas are present in the so-called "gastrinoma triangle", delimited by the cystic duct, the junction between the second and third portions of the duodenum, and the junction between the colon and the pancreatic body. Occasionally, gastrinomas have also been described in other areas, including lymph nodes, biliary tree, ovaries, kidneys, heart, gallbladder, omentum, and liver[5-7]. This article describes a case of primary hepatic gastrinoma in a 19 years old hypergastremic patient who underwent hepatic trisegmentectomy to remove the tumor.

CASE REPORT

A male patient, 19 years of age, was referred as a result of intermittent abdominal pain over the previous 10 years. He reported an attack of diffuse abdominal pain for 1 month at the age of nine. At that time, a gastric ulcer was diagnosed, and due to the failure of clinical treatment, he underwent antrectomy with vagotomy in another service. In the years following the surgery, the patient presented sporadic episodes of non-specific diffuse abdominal pain associated with nausea and vomiting. Diarrhea was also present, but dyspeptic characteristics were not. During that time, emergency treatment was sought with regularity, with improvement following clinical treatment. A loss of around 16 kg of body weight was reported over the year prior to admission to our service (from 68 to 52 kg).

Upper digestive endoscopy was carried out. In the third distal, close to the esophageal transition, the mucosa was pearlescent and thickened, which was associated with confluent erosions larger than 5 mm. This covered 100% of the circumference of the organ, with ulcerations and fibrin (intense erosive distal esophagitis-Los Angeles Class D). Furthermore, an image compatible with extrinsic compression of the stomach was revealed (Figure 1).

Computed tomography (CT) and magnetic resonance (Figure 2) were carried out, showing increased dimensions of the liver, as the result of a voluminous expansive hypervascularized lesion with necrotic central areas. Involvement in the portal vein was identified, and the mass mainly occupied the central portion of the liver and hilum, affecting segments Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅷ and measuring 19.5 cm × 12.5 cm × 17.0 cm.

A biopsy was carried out with the following immunohistochemical characteristics: cytokeratin 7 negative, cytokeratin 20 negative, chromogranin positive, synaptophysin positive, CD99 positive, Hep par 1 negative, polyclonal CEA positive (standard cytoplasma and membrane), and Ki 67 positive (around 5%). It was concluded that the profile was compatible with a well-defined neuroendocrine tumor (NET Grade 2). The diagnosis was confirmed by the discovery of gastrin hypersecretion, *via* chemiluminescence, with a resulting

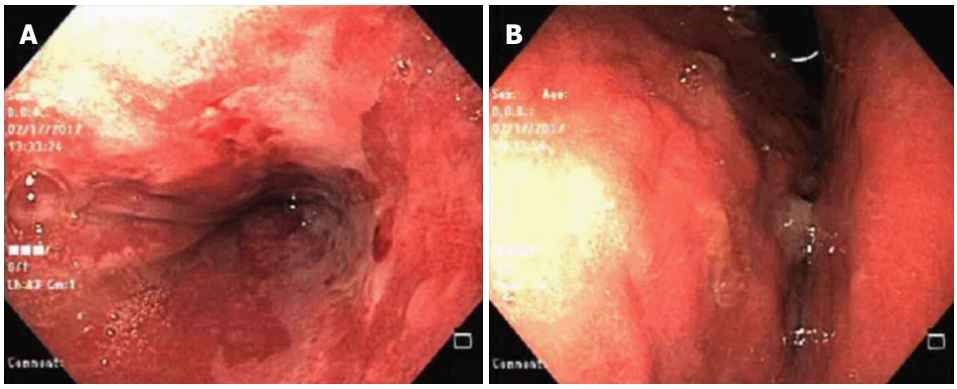

Figure 1 Upper digestive endoscopy showing intense erosive distal esophagitis and extrinsic compression of the stomach. A: Intense erosive distal esophagitis; B: Extrinsic compression of the stomach.

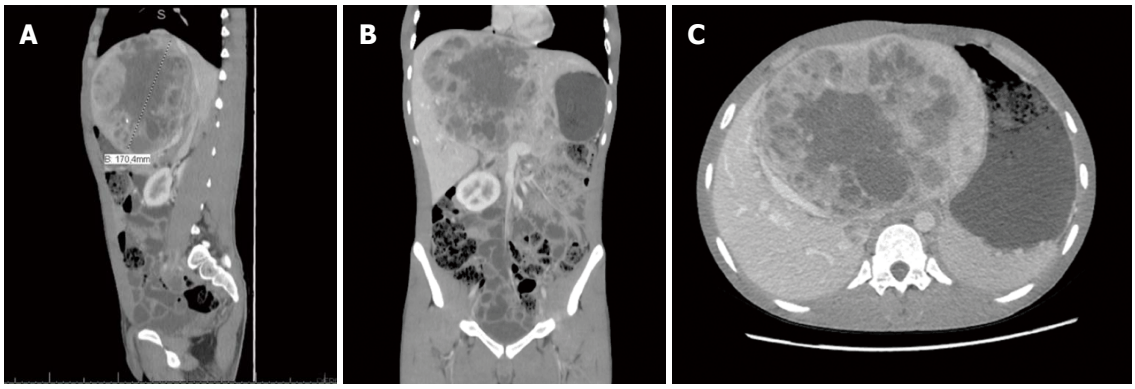

Figure 2 The computed tomography of hepatic lesion. A and B: Computed tomography showing a voluminous heterogeneous hepatic lesion, with a neoplasic aspect and dimensions 19.5 cm × 12.5 cm × 17.0 cm; C: Compression of the right hepatic vein.

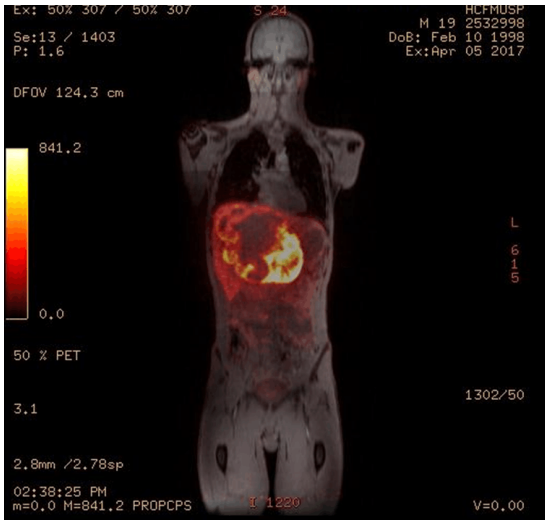

Figure 3 Positron emission tomography associated with magnetic resonance with somatostatin analogue confirmed that the liver was the primary site of the tumor.

value of 6709.0 pg/mL.

Considering that the liver is a common location for metastasis, it was important to define whether or not the tumor was primary. To do this, positron emission tomography associated with magnetic resonance was carried out - PET-MR - with somatostatin analogue (Figure 3). The examination was carried out following the administration of 2.7 mCi of Galium-68-DOTA-Octreotate (68Ga-DOTATATE) intravenously. The examination showed a relatively well-defined expansive lesion on the liver, with lobulated contours, situated in the left lobe. Heterogeneity in T2 and an anomalously high concentration of radiopharmacus was noted. It was heterogeneously distributed, with central and peripheral areas with hypoconcentration of radiotracer (which can correspond to necrosis/liquefaction), measuring around 18.1 cm × 11.3 cm at the largest axes. Contact and constriction of the inferior vena cava, which was permeable, were sustained. There were no signs of invasion of the head or body of the pancreas.

From these examinations it was possible to confirm the diagnosis of primary hepatic gastrinoma and to recommend surgical resection. Prior to the surgical procedure, there were a number of intracavity adherences. Hepatic trisegmentation (Ⅱ, Ⅲ, Ⅳ, Ⅴ, Ⅷ) was carried out (Figure 4), identifying a large volume mass, mainly occupying the left lobe of the liver. Segments Ⅴ and Ⅷ were most affected, with atrophy in segment Ⅱ, as well as compensatory hypertrophy in segments Ⅵ and Ⅶ. The remaining liver maintained approximately 80% of the volume of a functional liver. The

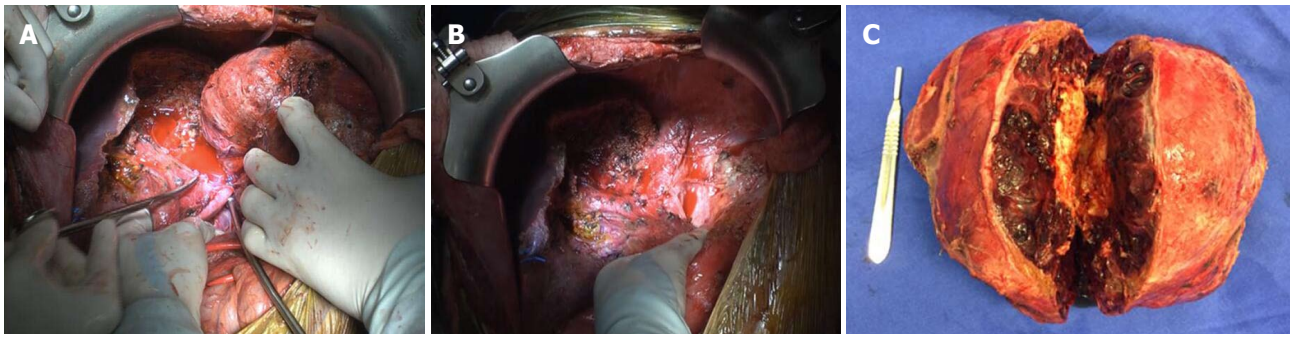

Figure 4 Intraoperative appearances. A: After hepatic posterior sector mass separation; B: Aspect of the remaining liver after surgical removal; C: Surgical piece.

patient was readmitted in the early postoperative phase with abdominal pains and leaking of bile through the drain due to a biliary fistula. The patient was submitted to a surgical procedure with a good outcome. Six months after the resection, the patient was well, had no relapses, and had a gastrin level of 40.5 pg/mL. At 1 year after the surgery, exam showed a gastrin level of 89.2 pg/mL. The normal range of gastrin is 13 to 115 pg/mL.

DISCUSSION

Zollinger-Ellison syndrome (ZES) is predominantly sporadic, although in 20%-30% of cases its origin is type 1 multiple endocrine neoplasia (MEN1). MEN 1 is a genetic disease that occurs in the gene MEN1, leading to the formation of various neoplasias; pancreatic endocrine tumors, pituitary adenomas, and hyperplasia of the parathyroid are the most prevalent. Despite the evidence of ulcers in this patient at the age of nine, ZES should be considered, principally because of the patient's history and hypergastrinemia. There was no report of the occurrence of MEN1 together with primary hepatic gastrinoma, including in our case.

Gastropancreatic neuroendocrine neoplasias, which can cause ZES, are classified according to their prognosis. Well-defined neuroendocrine tumors have a clinical course that is much less aggressive and can be subclassified as G1 (Ki-67 < 3) and G2 (Ki-67 of 3%-20%), according to the Ki-67 index that assesses the level of differentiation of cells in tissue. Poorly defined tumors are in category G3, presenting Ki-67 > 20%. Histopathological analysis of the patient's tumor revealed a Ki-67 of 5% (G2)[8]. The most common gastrinomas, duodenal and pancreatic, present a mean diameter of 1 and 3 cm, respectively[9]. The tumor in this case had dimensions of 19.5 cm × 12.5 cm × 17.0 cm.

Primary ectopic gastrinomas are neuroendocrine tumors located outside of the triangle of gastrinoma and are responsible for less than 10% of cases. One specific site in which they occur is the liver, with only 26 cases described in the literature in a review from 2012[10]. In recent years, there were a few more cases described, but the total number is still very low. According to a recent study[11], in ZES patients, primary gastrinomas of the liver were the second most common extrapancreatic, extraintestinal site for a primary gastrinoma and may metastasize to regional lymph nodes. Compared with typical ZES patients, the epidemiology of primary hepatic gastrinoma is a little different. The patients are generally affected much younger, with a predominance of males and no association with MEN 1, as it was in our case[12].

This rare hepatic gastrinoma is slow growing, with around 65% of cases malignant and 30%-40% of those cases being metastatic at initial presentation. This relatively high rate of metastases arises from the difficulty of diagnosis, especially in palliative care and hepatic metastases[13]. Despite this, our patient did not present metastases in radiological examinations.

A clinical presentation of ZES similar to gastroesophageal reflux can lead to treatment with proton-pump inhibitors. This medication masks the symptoms and alters gastrin levels, prolonging the time necessary to identify the real cause of the disease. A study[14] showed that prior to widespread use of proton-pump inhibitors (before 1985), the rate of metastases was only 19%. Ten years later, this number had risen to 55%. Prior to the advent of proton-pump inhibitors, partial and full gastrectomies were common to reduce the incidence of ulcers. In the report of this case, the fact that the patient had previously undergone antrectomy with vagotomy meant that his symptoms were less apparent than they would otherwise have been.

A second factor that can complicate diagnosis is related to the morphofunctional characteristics of the liver. It is known that the liver is a common site of metastasis for a variety of tumors, and classifying the neoplasia as primarily hepatic requires some examinations to confirm the absence of other primary sites. One of the examinations currently used is PEC/CT with 68 gallium-dotatate. This radiopharmacum is associated with computerized tomography and is a somatostatin analogue, which allows the identification of tumors[15]. The sensitivity of the examination was 93% (95%CI: ± 2%) and the specificity was 91% (95%CI: 82%-97%), as identified in a meta-analysis[9]. Once the neuroendocrine tumors present somatostatin receptors, it is possible to confirm whether the hepatic

gastrinoma is primary or the result of metastasis[16,17]. In this case, the existence of a single focus in the liver combined with a high level of gastrin highly suggested it as a primary site. Is important to emphasize the importance of the follow up of gastrin levels to ensure that there is not a small small duodenal primary gastrinoma that could not have been detected in the PET/CT and surgery[11].

Despite few reported cases, surgical resection should be the treatment of choice and, furthermore, the only chance of a cure. The rate of success in these cases is 86%, and 60% present early postoperative eugastrinemia, 40% after five years. In the event that surgery is not possible, such as in cases of diffuse metastasis or comorbidities, it is possible to follow conventional treatment: Ablation by radiofrequency, chemotherapy (doxorubicin, streptozocin, 5-fluorouracil), interferon, and transplantation[13]. A study with 160 patients with gastrinoma showed that 15-year disease-related survival was 98% for operated and 74% for unoperated (P = 0.0002)[18].

In conclusion, primary hepatic gastrinomas are extremely rare tumors that cause Zollinger-Ellison syndrome. Due to their clinical presentation and the liver being a significant site of metastases, diagnosis of the disease is time consuming and difficult. Following diagnosis, the treatment of choice is surgery, and, in cases where there are no metastases, the prognosis is good. Therefore, it is important to diagnose properly the primary gastrinoma.

ARTICLE HIGHLIGHTS

Case characteristics

Primary hepatic gastrinoma in a 19 years old hypergastremic patient who underwent hepatic trisegmentectomy to remove the tumor.

Clinical diagnosis

Primary hepatic gastrinoma.

Differential diagnosis

Gastrointestinal tumors, MEN 1.

Laboratory diagnosis

Gastrin levels were 6709.0 pg/mL before surgery. After the procedure it was 40.5 pg/mL.

Imaging diagnosis

Computed tomography (CT) showed a voluminous heterogeneous hepatic lesion, with a neoplasic aspect and dimensions 19.5 cm × 12.5 cm × 17.0 cm.

Pathological diagnosis

Histopathological analysis of the patient's tumor revealed a Ki-67 of 5% (G2).

Treatment

Hepatic trisegmentectomy.

Term explanation

Gastrinoma: A gastrinoma is a tumor that secretes an excess of gastrin, leading to ulceration in the duodenum, stomach, and the small intestine.

Experiences and lessons

Primary hepatic gastrinoma is a very difficult to diagnose. Meticulous examination is necessary for appropriate diagnosis and treatment.

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CASE REPORT

Hepatectomy for gallbladder-cancer with unclassified anomaly of right-sided ligamentum teres: A case report and review of the literature

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Author contributions: Goto T, Terajima H and Uchida Y managed the patient and performed the operation; Goto T collected the clinical data, designed this report and wrote the paper; Terajima H and Yamamoto T helped draft and revise the manuscript.

Informed consent statement: The patient provided informed written consent for publication of this case report.

Conflict-of-interest statement: The authors declare that no conflicts of interest are associated with this manuscript.

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Abstract

Right-sided ligamentum teres (RSLT) is a congenital anomaly in which the right umbilical ligament becomes dominant and anomalous ramifications of the hepatic vessels and biliary system are present. A male patient in his 70s was diagnosed with advanced gallbladder cancer directly infiltrating the right hepatic duct (RHD), together with RSLT. Preoperative three-dimensional simulation of the liver based on multiple detector computed tomography images after cholangiography revealed ramifications of all segmental portal veins from the portal trunk and discordance of the arterial and biliary branching patterns of segment 8. Fusion analysis of the biliary architecture and segmental volumetry showed that the RHD drained segments 1r, 5, 6, and 7. We successfully performed a modified right-sided hepatectomy sparing segment 8 (*i.e.*, resection of the RHD drainage territory), with negative surgical margins. This report is the first to describe major hepatectomy for advanced gallbladder cancer with RSLT.

Key words: Right-sided ligamentum teres; Hepatectomy; Gallbladder cancer; Preoperative liver simulation; Anomaly of the portal vein

Core tip: Right-sided ligamentum teres (RSLT) is a congenital anomaly that involves anomalous ramifications of the

hepatic vessels and biliary system. A male in his 70s with RSLT was diagnosed with advanced gallbladder cancer directly infiltrating the right hepatic duct (RHD). Based on preoperative liver simulation, we successfully performed a modified right-sided hepatectomy sparing segment 8, which corresponded to resection of the RHD drainage territory (segments 1r, S5, S6, and S7), with negative surgical margins. This report is the first to describe major hepatectomy for advanced gallbladder cancer with RSLT.

Goto T, Terajima H, Yamamoto T, Uchida Y. Hepatectomy for gallbladder-cancer with unclassified anomaly of right-sided ligamentum teres: A case report and review of the literature. *World J Hepatol* 2018; 10(7): 523-529 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i7/523.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i7.523

INTRODUCTION

Right-sided ligamentum teres (RSLT) is a rare congenital anomaly in which the umbilical ligament on the left side atrophies and the right ligament becomes dominant at the 7-mm embryo stage[1]. The incidence of RSLT has been reported to be 0.2%-1.2% in adults[1,2]. RSLT is associated with anomalous ramifications in the arterial, portal vein, and biliary systems; therefore, precise evaluation of the branching patterns of the vascular and biliary architecture is required to avoid injuring the hepatic vasculature of the remnant liver[3-5]. Recent papers have demonstrated the significance of three-dimensional (3D) liver simulation before hepatectomy in RSLT patients to determine surgical strategies, such as the hepatectomy procedure and the cutting plane[6-8]. For gallbladder cancer, macroscopically complete surgical resection with negative microscopic margins remains the only potentially curative treatment, and major hepatectomy, such as extended right hepatectomy, is usually required for locally advanced gallbladder cancer[9]. This case report is the first to describe major hepatectomy for advanced gallbladder cancer concomitant with RSLT.

CASE REPORT

A male patient in his 70s was incidentally diagnosed with a gallbladder tumor by computed tomography (CT) during a preoperative examination of an inguinal hernia. He had no appreciable disease and had a good exercise tolerance. Laboratory tests showed normal levels of carcinoembryonic antigen and CA19-9. Hyperbilirubinemia was not observed. The indocyanine green (ICG) retention rate at 15 min was 3.6%; the normal range is less than 10%[10]. The patient's Child-Turcotte-Pugh score was 5 points, Grade A[11]. An abdominal contrast-enhanced CT scan showed an atrophied gallbladder with tumor-like localized wall thickness enhanced by contrast medium, strongly suggesting the possibility of gallbladder cancer. The gallbladder was attached to the left side of the liver and complicated variations of the intrahepatic vascular architecture were detected, suggesting RSLT (Figure 1A-E). Endoscopic retrograde gallbladder drainage (ERGBD) was performed for cytological examination of bile in the gallbladder and adenocarcinoma was detected two consecutive times (Figure 1F). To prevent obstructive jaundice and to evaluate horizontal extension of the tumor along the bile duct, ERGBD was switched to endoscopic nasobiliary drainage (ENBD) one week later. Two ENBD tubes were placed both in the right hepatic duct (RHD) and the left hepatic duct (LHD). ENBD tube cholangiography demonstrated a severe stenotic change of the RHD, and the confluence of the LHD and the common bile duct was intact (Figure 1G-I). No metastases of lymph nodes and distant organs were detected by CT scanning. Based on these findings, the patient was diagnosed with advanced gallbladder cancer with direct infiltration of the RHD rather than hilar cholangiocarcinoma. The gallbladder carcinoma was classified as cStage ⅢA (T3; right bile duct, N0, M0) according to the Union for International Cancer Control system[12]. Considering the patient's eligibility for radical surgery, we finally determined that right-sided major hepatectomy with resection of the RHD drainage territory was required.

Preoperative simulation was performed to plan the hepatectomy using multiple detector CT (MDCT) with a slice thickness of 1 mm after contrast cholangiography through an ENBD tube. After a quarto-phase (non-contrast, arterial, portal venous, and hepatic venous phases) contrast study was performed, two-dimensional CT images were transferred to a workstation (SYNAPSE VINCENT: FUJIFILM Medical Co., Ltd., Tokyo, Japan), and 3D images were automatically constructed for some areas and manually constructed for other areas. The intrahepatic vasculature was reconstructed at the 4th order division level (Figure 2A) and was verified by 4 liver surgeons independently. We labeled each sub-segment following the Couinaud classification[13] and the Brisbane 2000 terminology of Liver Anatomy and Resections[14] to define the anatomical divisions of the liver (Figure 2B and C). All segmental portal branches were separately ramified from the portal trunk (Figure 2D). No arterial anomalies were detected (Figure 2E). Bile duct analysis demonstrated that the bile duct of segment 8 (B8) was ramified from the LHD, and the RHD drained segments 5, 6, and 7. The bile duct of the right side of segment 1 (B1r) was ramified from the RHD, and the bile duct of the left side (B1l) was ramified from the LHD (Figure 2F).

Based on this simulation, resection of the gallbladder bed and the RHD drainage territory (segments 1r, 5, 6, and 7) was planned (Figure 3). The future liver remnant (FLR) volume was 605 mL. The plasma clearance rate of ICG in the FLR (calculated as the plasma clearance rate of ICG × the proportion of the FLR) was 0.131; 0.05 is the cut-off value for predicting mortality and morbidity[15]. The functional count rate of the FLR according to technetium-99m diethylenetriamine pentaacetic acid galactosyl human serum albumin scintigraphy was 50.2%[16]. The hepatic artery of segment 8 (A8) branched from the

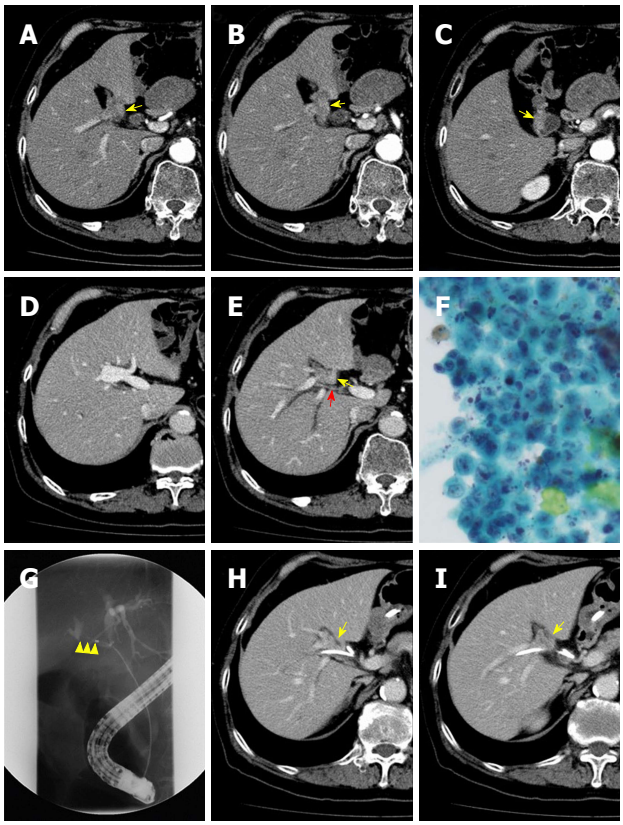

Figure 1 Contrast-enhanced computed tomography, cytological examination of bile, and endoscopic retrograde cholangiography. A, B and C: The highly atrophied gallbladder (yellow arrow) had tumor-like localized wall thickness enhanced by contrast medium; D: The cul-de-sac of the right-sided umbilical portion is shown; E: The gallbladder (yellow arrow) is located on the left-sided liver bed. Direct infiltration of the tumor into the right hepatic artery (red arrow) is not evident; F: Cytological examination of bile obtained from endoscopic retrograde gallbladder drainage demonstrated atypical cells with a high nucleo-cytoplasmic ratio, strongly suggesting adenocarcinoma; G: Endoscopic retrograde cholangiography shows severe stenosis of the right hepatic duct (yellow arrowhead); H and I: The gallbladder tumor (yellow arrow) directly spreads to the nearby right hepatic duct where an endoscopic nasobiliary tube was placed.

right hepatic artery, while B8 branched from the LHD.

During laparotomy, the gallbladder was located on the left side of the RSLT (Figure 4). After mobilization of the right lobe, lymphadenectomy of the hepatoduodenal ligament was performed. The common bile duct and LHD were divided, resulting in pathologically negative surgical margins, followed by parenchymal dissection of the gallbladder bed. All portal and arterial branches of segments 5 to 8 were extrahepatically separated and taped. Each pedicle was temporarily clamped, and the intrahepatic blood flow was simultaneously monitored via ultrasonography. After dividing all inflow vessels of segments 5 to 7, the demarcation line was detected as preoperatively simulated (Figure 5). The liver parenchyma was transected using a Cavitron ultrasonic surgical aspirator and saline-coupled bipolar electrocautery[17], with an intermittent Pringle maneuver. After dividing the right hepatic vein, the specimen was excised (Figure 6). Hepaticojejunostomy to the LHD and jejunojejunostomy were conducted, and the operation was successfully completed as planned (Figure 7). The surgical time was 682 min, and the estimated blood loss was 430 g.

Pathological examinations demonstrated well-differentiated tubular adenocarcinoma of the gallbladder with direct invasion of the liver parenchyma and the RHD (Figure 8). The tumor cell invaded to the RHD wall, but not into the lumen. Surgical margins were pathologically negative, and the tumor was classified as pStage Ⅲ A (T3; liver bed and right bile duct, N0, M0). Postoperative contrast-enhanced CT showed no ischemic regions in the remnant liver, including the spared segment 8. The patient was discharged 8 d after surgery without any complications more severe than a Grade Ⅱ Clavien-Dindo classification[18]. Five months after surgery, solitary liver metastasis in segment 2, far from the surgical stump, was detected during adjuvant chemotherapy with gemcitabine. The liver metastasis disappeared on imaging analysis after 2 cycles of chemotherapy with gemcitabine plus cisplatin (GC). The patient underwent ileocecal resection for primary ascending colon cancer 7 months after surgery; thereafter, an additional 13 cycles of GC therapy were continued. Multiple mediastinal lymph node metastases were identified 18 mo after surgery, and second-line chemotherapy with S-1 was introduced. The patient has currently survived 26 months after hepatectomy.

DISCUSSION

This report is the first to describe major hepatectomy for curative resection of advanced gallbladder cancer with RSLT. Preoperative simulation enabled visualization of the complicated anatomical findings of the intrahepatic vascular and biliary architecture and provided valuable assistance in performing resection of the RHD drainage territory without injuring the hepatic vasculature of the remnant liver.

The anomalies associated with RSLT may lead to several surgical problems during hepatectomy. In the most common cases (*e.g.*, an independent ramification of the right lateral portal pedicle), a surgeon's misunderstanding and ligation of the left portal trunk result in a lack of portal flow in two-thirds of the whole liver during left hepatectomy[3]. Therefore, awareness of the type of anomaly is important before planning hepatectomy for patients with RSLT[1,3,4]. In 1997, Nagai *et al*[1] reported two types of portal vein classifications of RSLT, and later, Shindoh *et al*[3] and Nishitai *et al*[4] demonstrated the classification of arterial, portal, and biliary ramifications. Portal ramification patterns were classified into three types: Independent right lateral, bifurcation, and trifurcation. Our case may be defined as an unclassified type because four sectional portal branches did not exist, and all subsegmental branches were directly ramified from the portal trunk. In the bifurcation type, P4 runs from the right paramedian branch to the left paramedian area beyond the middle hepatic vein (MHV). However, our case showed that P4 was ramified from the main portal trunk and did not run beyond the MHV. Therefore,

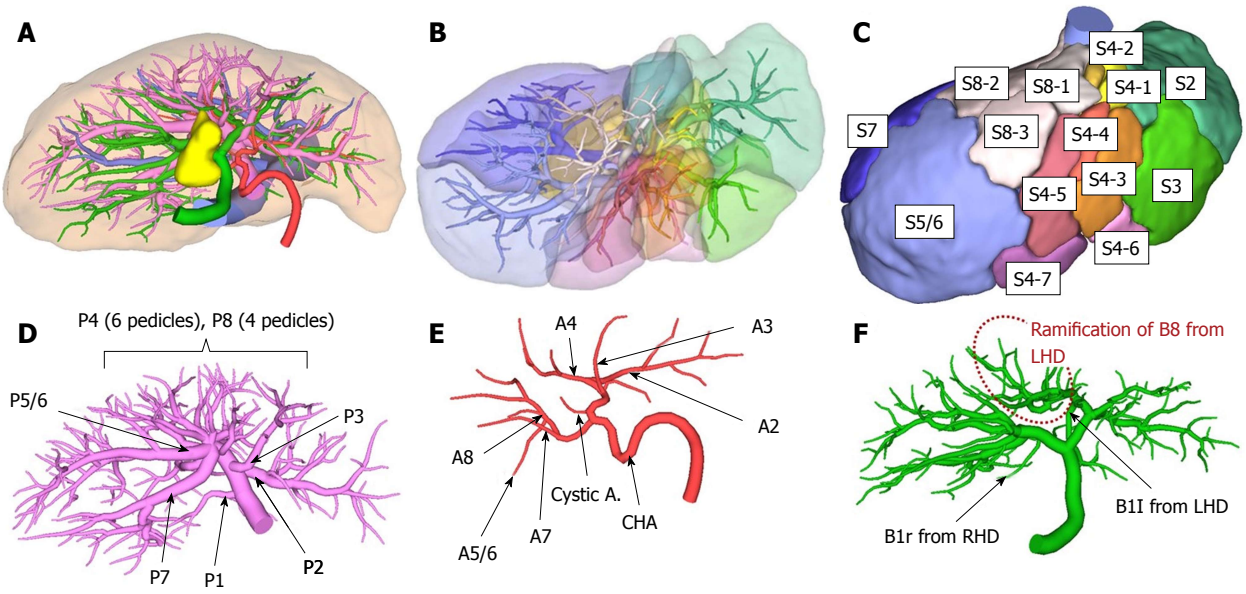

Figure 2 Preoperative simulation. A: An "all-in-one" simulation image. The intrahepatic vasculature was reconstructed at the 4th order division level (red: hepatic artery, pink: portal vein, green: bile duct, yellow: Gallbladder, blue: hepatic vein); B and C: Simulated segmentation based on the portal venous flow. Couinaud's definition was referred to for the naming of each segment; D: All segmental portal branches were ramified from the portal trunk. Interestingly, a common trunk of P5 and P6 was present; E: Hepatic arterial ramification without anomalous anatomy; F: The bile duct of segment 8 (B8) was ramified from the left hepatic duct, not from the right hepatic duct. RHD: Right hepatic duct; LHD: Left hepatic duct.

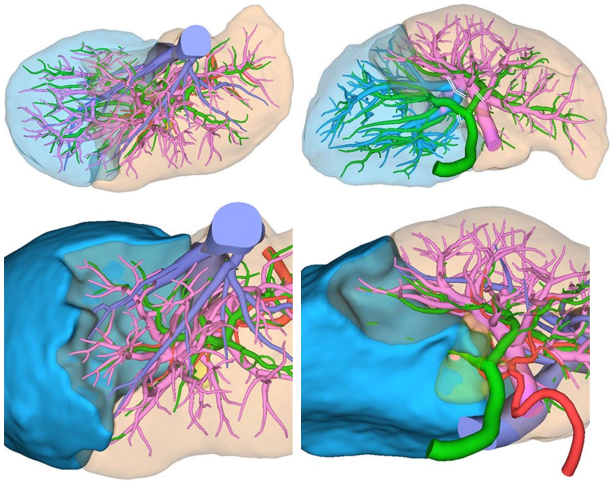

Figure 3 Simulation of modified right-sided hepatectomy. The resection area corresponded to the drainage territory of the right hepatic duct (segments 1r, 5, 6, and 7, blue area).

performing a conventional anatomical hepatectomy was difficult, and each subsegmental portal vein branch from the portal trunk had to be identified and divided. Hepatic arterial ramification patterns are classified into 3 types: Independent ramification of the left hepatic artery, common trunk formation between the left hepatic artery and the ventral branch of the right paramedian artery, and replaced left hepatic artery from the left gastric artery. Our case is classified as the first type. Intrahepatic bile duct confluence patterns are classified into 4 types: Symmetrical, independent right lateral, total left, and total right. Our case corresponds to the independent right lateral type, in which the LHD drains the right paramedian section.

No specific relationship has been reported among the patterns of the biliary, portal, and arterial ramifications in RSLT livers[4]. Embryologically, the portal ramifications induce restructuring of the ductal plate that surrounds them in the future intrahepatic ducts, which results in a periportal position of the intrahepatic bile ducts[19]. In principle, the formation of hepatic arteries follows the portal venous tributaries, and dissociations between the portal venous system and bile duct system have been reported[20]. Therefore, an uncorrelated pattern of portal vein and bile tree ramifications is possible. In our case, however, the arterial ramification pattern differed from the biliary confluence pattern; A8 was ramified from the right hepatic artery, and B8 entered into the LHD. This report is the first to describe an artery and bile duct that were not parallel. If the vascular and biliary anatomies of the expected remnant liver in major hepatectomy with hilar lymphadenectomy for hepatobiliary malignancies are extremely complicated, as in this case, hepatic resection with the Glissonian approach[6] is difficult to perform. Step-by-step extrahepatic division of all vasculature through hilar dissection based on precise preoperative simulation is the safest and most secure surgical strategy, as described in this paper.

Anomalies of the intrahepatic portal vein are not rare[2,21]. However, in our case, all subsegmental portal veins were ramified from the portal trunk extrahepatically. Therefore, we could intentionally encircle all portal branches in accordance with the detailed preoperative simulation. Although conventional right hepatectomy was also planned as an alternative, the infiltration of the tumor to the right hepatic artery was not evident from

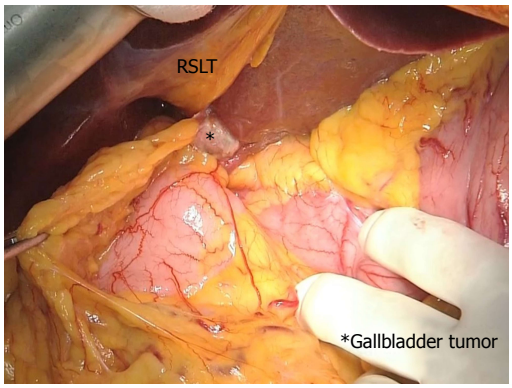

Figure 4 Hepatic hilar view during the operation. The gallbladder was located at the left side of the right-sided ligamentum teres (RSLT).

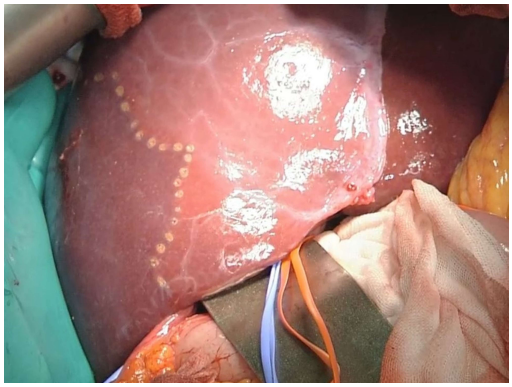

Figure 5 Demarcation line after dividing all arterial and portal branches of segments 1r, 5, 6, and 7.

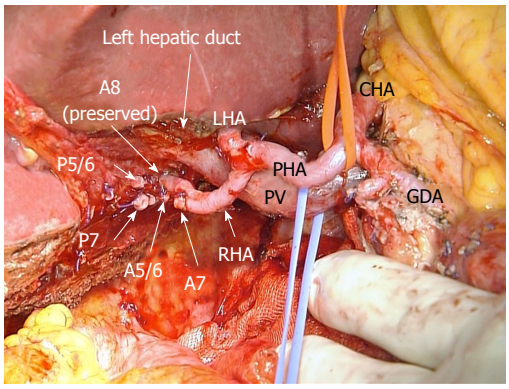

Figure 6 Final view of the hepatic hilum after hepatectomy. The portal and hepatic arterial branches of segment 8 were preserved. A common trunk of P5 and P6 was detected during the operation, which was confirmed by ischemic changes in S5 and S6 after clamping the target branch.

the intraoperative findings; therefore, modified right-sided hepatectomy was possible, as simulated.

Liver simulation, particularly 3D modeling and virtual planning, offers a new approach to liver resection, as reported by several groups[22-24]. Liver simulation has led to significantly shorter surgical times, and the predicted liver volume to be resected appears to correlate well with the actual resected liver weight[23]. Preoperative

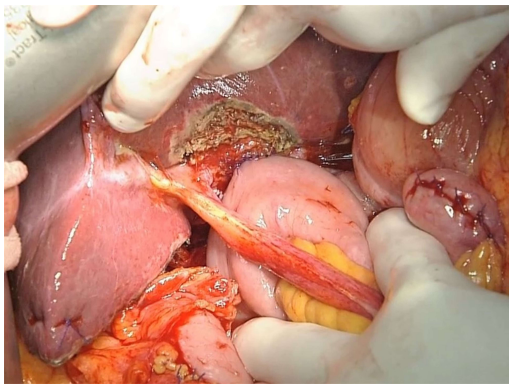

Figure 7 Final view after hepaticojejunostomy. The ligamentum teres originated from the main portal trunk and ran between S4-6 and S4-7.

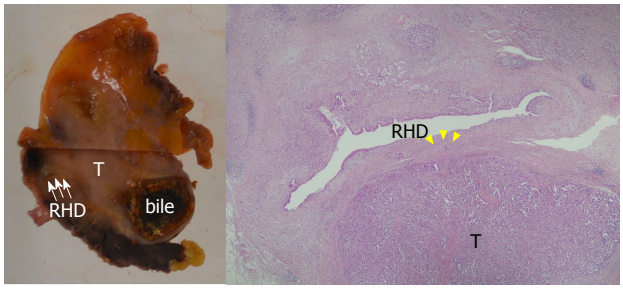

Figure 8 Pathological examination. Pathological examination shows well-differentiated tubular adenocarcinoma of the gallbladder (T) with direct invasion of the liver parenchyma and the right hepatic duct (RHD: white arrow). The tumor cell invaded to the RHD wall, but not into the lumen (yellow arrowhead) (hematoxylin eosin saffron, original magnification × 20).

simulation has become a common practice in Japan because simulation software has been developed and has become commercially available, and the cost of preoperative liver simulation has been covered by national health care insurance since 2012[25]. The fusion image of the portal vein and bile duct is useful for hepatectomy with RSLT because both the symmetrical type and the total left type of bile duct confluence patterns are similar to the ramification pattern of the normal liver, and cholangiography alone may provide misleading information for the operation[4]. The fusion image from one examination modality is referred to as an "all-in-one" (bile ducts, arteries, and portal veins) 3D image, in which MDCT is performed after cholangiography using carbon dioxide or iodine[24]. We strongly recommend structuring "all-in-one" 3D simulation images when planning hepatectomy with RSLT to evaluate the horizontal and perpendicular spreading of a tumor and its influence on the surrounding intrahepatic vasculature.

A systematic review of preoperative liver simulation and navigation has demonstrated the characteristics of the ideal simulation and navigation tool[26]. Simulation can accurately predict the liver volume to be resected, even by complicated anatomical resection procedures, and permits stereotactic measurement of the length of the surgical margin, which could not be estimated previously[27]. In this case, the most useful contribution

of liver simulation was the combination of volumetry with the 3D structure of the intrahepatic vasculature. Using volumetry, we could not only calculate the cubic content but also visualize the parenchymal shape that each subsegmental portal branch dominated. By comparing each subsegmental area with the 3D structure of the right-sided biliary trees, we could exactly determine the hepatic territory to be resected and divide the portal pedicles in the proper sequence, as simulated. Simulation also enabled us to perform a parenchyma-preserving hepatectomy, which may contribute to safe and curative resection in selected cases with liver neoplasm in the right anterior resection region[28]. Takamoto *et al*[29] demonstrated accurate, completed drawings and surgical strategies for malignant liver tumors to execute anatomic segmentectomy and subsegmentectomy using 3D simulation. We also successfully performed parenchyma-preserving anatomical resection at the 3rd order division level for oncologically curative resection based on a precise preoperative analysis. The current 3D simulation system cannot be used as a real-time navigation tool during surgery[29], and the development of new technology is ongoing.

In conclusion, 3D simulation based on precise intrahepatic vascular and biliary analysis enabled accurate and oncologically curative hepatic resection, even in a patient with rare anatomical anomalies.

ARTICLE HIGHLIGHTS

Case characteristics

A healthy and asymptomatic male in his 70s was incidentally diagnosed with a gallbladder tumor by preoperative computed tomography (CT) scanning for an inguinal hernia.

Clinical diagnosis

The patient, who had right-sided ligamentum teres (RSLT) with abnormal vascular anomaly, was diagnosed with advanced gallbladder cancer invading the right hepatic duct (RHD, cStage Ⅲ A, T3; right bile duct, N0, M0).

Differential diagnosis

Based on 2 important findings, including a solid tumor-like appearance of the highly atrophied gallbladder by CT scanning and cytological evidence of adenocarcinoma from bile obtained by endoscopic retrograde gallbladder drainage rather than endoscopic nasobiliary drainage, advanced gallbladder cancer with infiltration of the RHD was more convincing than hilar cholangiocarcinoma as a preoperative diagnosis.

Laboratory diagnosis

Laboratory tests revealed that tumor markers and liver function were within the normal ranges.

Imaging diagnosis

Preoperative three-dimensional liver simulation based on multiple detector computed tomography scanning combined with endoscopic nasobiliary tube cholangiography revealed that all segmental portal veins were independently ramified from the portal trunk and that the RHD drained the right-sided liver except for segment 8 (segments 1r, 5, 6, and 7).

Pathological diagnosis

Pathological examination showed well-differentiated tubular adenocarcinoma of the gallbladder invading the hepatic parenchyma and the RHD.

Treatment

The patient was treated with extended hepatectomy of the RHD drainage territory (segments 1r, 5, 6, and 7) without injuring the hepatic vasculature of the remnant liver based on preoperative liver simulation.

Related reports

Shindoh *et al* demonstrated anomalous vascular architecture with right-sided ligamentum teres (RSLT) and Ome *et al* reported major hepatectomy for RSLT patients. Shindoh J, Akahane M, Satou S, Aoki T, Beck Y, Hasegawa K, Sugawara Y, Ohtomo K, Kokudo N. Vascular architecture in anomalous right-sided ligamentum teres: Three-dimensional analyses in 35 patients. *HPB* (Oxford) 2012; **14**: 32-41 [PMID: 22151449 DOI: 10.1111/j.1477-2574.2011.00398.x]. Ome Y, Kawamoto K, Park TB, Ito T. Major hepatectomy using the glissonean approach in cases of right umbilical portion. *World J Hepatol* 2016; **8**: 1535-1540 [PMID: 28008345 DOI: 10.4254/wjh.v8.i34.1535].

Term explanation

RSLT is a congenital anomaly in which the umbilical ligament on the left side atrophies, and it is associated with anomalous ramifications in the artery, portal vein, and biliary systems.

Experiences and lessons

Preoperative 3D liver simulation based on precise intrahepatic vascular and biliary analysis enabled accurate and oncologically curative hepatic resection, even in a patient with rare anatomical anomalies.

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REVIEW

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REVIEW

Current status of imaging in nonalcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common diffuse liver disease, with a worldwide prevalence of 20% to 46%. NAFLD can be subdivided into simple steatosis and nonalcoholic steatohepatitis. Most cases of simple steatosis are non-progressive, whereas nonalcoholic steatohepatitis may result in chronic liver injury and progressive fibrosis in a significant minority. Effective risk stratification and management of NAFLD requires evaluation of hepatic parenchymal fat, fibrosis, and inflammation. Liver biopsy remains the current gold standard; however, non-invasive imaging methods are rapidly evolving and may replace biopsy in some circumstances. These methods include well-established techniques, such as conventional ultrasonography, computed tomography, and magnetic resonance imaging and newer imaging technologies, such as ultrasound elastography, quantitative ultrasound techniques, magnetic resonance elastography, and magnetic resonance-based fat quantitation techniques. The aim of this article is to review the current status of imaging methods for NAFLD risk stratification and management, including their diagnostic accuracy, limitations, and practical applicability.

Key words: Simple steatosis; Non-alcoholic fatty liver disease; Ultrasonography; Computed tomography; Nonalcoholic steatohepatitis; Elastography; Magnetic resonance

Core tip: Patients with non-alcoholic fatty liver disease (NAFLD) are at risk of steatohepatitis and progressive

liver fibrosis culminating in cirrhosis, typically over a period of decades. Early diagnosis and risk stratification are essential for effective management. Current imaging methods such as ultrasound, computed tomography, and magnetic resonance elastography have demonstrated their values to serve as noninvasive imaging biomarkers to evaluate NAFLD progression, but they are still relatively limited in the detection of inflammation, which is more important than steatosis in terms of its high risk for fibrosis, cirrhosis, and hepatocellular carcinoma.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is increasingly becoming a disease of clinical importance, largely due to increasing prevalence, better understanding of pathophysiology, and rapid development of therapeutics[1]. In this article, we review imaging methods for NAFLD screening, diagnosis and risk stratification, with a focus on diagnostic accuracy, limitations, and practical applicability.

EPIDEMIOLOGY AND CLINICAL FEATURES OF NAFLD

NAFLD is a chronic liver disease defined as the pathological presence of hepatic steatosis (> 5% of the cross-sectional area of the liver occupied by fat vacuoles) in the absence of any secondary cause for hepatic fat accumulation, such as alcohol use, steatogenic medication, and hereditary disorders[1]. It has an estimated worldwide prevalence ranging from 20% to 46%, varying with study population and diagnostic criteria used[2]. In the United States, NAFLD is estimated to affect approximately 30% (100 million) of the population[3,4]. The prevalence is even higher amongst obese (70%) and diabetic (90%) individuals[5]. NAFLD-related liver impairment is expected to be the dominant cause of end stage liver disease (ESLD) requiring transplantation in the United States by 2020[4].

NAFLD comprises a spectrum of disease that can be simplified into two categories: (1) Simple Steatosis (SS), 70%-75% of cases, defined by excess liver fat without inflammation or cellular injury; and (2) nonalcoholic steatohepatitis (NASH), 25%-30% of cases, defined by the presence of excess liver fat with inflammation and cellular injury[1,2]. It is important to appreciate that SS and NASH are not entirely distinct, with many patients falling along a spectrum of fatty accumulation, inflammation, and hepatocyte injury. Nonetheless, this simplification facilitates prognostication and assessment of clinical significance. In most cases, SS is non-progressive, and does not result in liver fibrosis or progressive liver disease. However, recent longitudinal paired biopsy studies have shown that some patients with SS can progress to develop inflammation and fibrosis[6], and up to 20%–30% can progress to NASH[7]. Patients with NASH have a 20%–50% risk of developing progressive inflammation or liver fibrosis[8,9] and have a 2%–20% 5-year cumulative incidence of hepatocellular carcinoma[10]. In addition, NAFLD is an independent cardiovascular disease risk factor with a 70% overall mortality increase, driven by a about 300% increase in cardiovascular disease mortality[11].

PATHOPHYSIOLOGY OF NAFLD

The underlying pathophysiology of NAFLD is the accumulation of hepatic free fatty acids and triglycerides[12]. The pathogenesis of progression from simple fatty liver to NASH is not fully understood. The widely popular "two-hit hypothesis," suggests a "first hit" involves lipid accumulation in the hepatocytes, which increases the risk for a "second hit," comprising several factors, which result in hepatic injury, inflammation, and fibrosis[13]. The "multiple hit" hypothesis considers multiple insults, such as insulin resistance, hormones secreted from the adipose tissue, gut microbiota, genetic, epigenetic factors and nutritional factors, many of them acting together in genetically predisposed subjects to induce NAFLD[14,15].

Irrespective of the underlying pathophysiological process, adequate characterization of the NAFLD spectrum for prognostication necessitates the evaluation of three disease components. (1) Hepatic steatosis: The diagnosis and quantification of hepatic fat is the threshold criterion for the diagnosis of NAFLD. Steatosis has also been recognized as a risk factor for diabetes and other cardiovascular risk factors[14] (Figure 1); (2) Inflammation and cellular injury: steatohepatitis is distinguished from steatosis by the presence of inflammation in conjunction with a particular form of hepatocyte injury termed hepatocyte ballooning, which distinguishes the progressive form of NAFLD from the non-progressive forms[16]. Other histologic features of NASH are variably present and include necrosis, glycogen nuclei, Mallory bodies and fibrosis[17]; and (3) Fibrosis: Clinical outcomes have been shown to be correlated with fibrosis, and hence identification and quantification of fibrosis is crucial for effective risk stratification and disease management[18].

ROLE OF LIVER BIOPSY

Adequate NAFLD risk stratification and management requires assessment and preferably quantification of hepatic parenchymal fat, inflammation, and fibrosis. The current gold standard remains liver biopsy. Liver biopsy specimen evaluation in NAFLD patients can presently be accomplished using standardized pathologic staging systems, including the Kleiner modification of the Brunt scoring system or the NASH-CRN (Nonalcoholic Steatohepatitis Clinical Research Network) scoring system[19]. For example, the Brunt criteria include evaluation

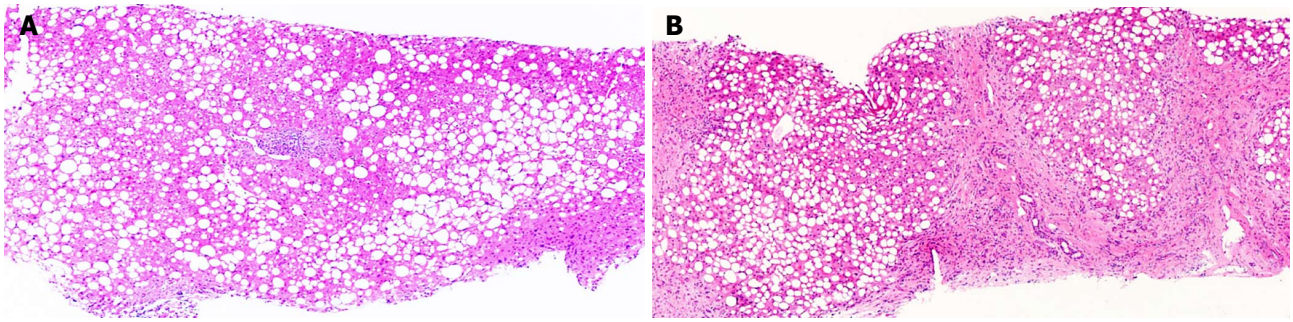

Figure 1 Pathological changes of liver simple steatosis and cirrhosis. A: 45-year-old man with simple steatosis. The liver biopsy shows marked macrovesicular steatosis without inflammation or fibrosis (H and E x4); B: 48-year-old man with cirrhosis due to non-alcoholic fatty liver disease. In addition to marked macrovesicular steatosis, there is loss of normal hepatic architecture and replacement by regenerative nodules surrounded by bands of fibrous tissue, a characteristic feature of cirrhosis (H and E x4).

of fat, fibrosis, and necroinflammation, and according to this system, NASH is diagnosed by the presence of fibrosis (grade 1 or more) or necroinflammation (grade 2 or more)[20].

Patients with a higher likelihood of NASH based on clinical criteria will usually be referred for liver biopsy. Limitations of liver biopsy include sampling error, inter-observer variability, patient anxiety, and procedure-related morbidity and mortality[21]. Ultimately, the high prevalence of NAFLD implies that liver biopsy is not a viable tool for widespread NAFLD risk stratification.

NON-INVASIVE METHODS FOR NAFLD EVALUATION

The limitations of liver biopsy have driven a search for non-invasive NAFLD screening and risk stratification methods. Since advanced fibrosis has been proven to be prognostic of poor outcomes in NAFLD, multiple surrogate fibrosis markers have been studied, including clinical predictors, serum biomarkers, and imaging methods[22]. One of these methods, the NAFLD fibrosis score, is used to assess advanced fibrosis risk. In this method, clinical parameters such as age, body mass index, albumin, AST/ALT ratio, *etc*., are used to calculate a score. A score of > 0.676 has an 82% positive predictive value in diagnosing advanced liver fibrosis (stage $\geqslant$ 3 in a 5-stage fibrosis scoring system) in patients with histology-proven NAFLD[19]. Serum biomarkers such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) have been shown to be elevated in patients with NAFLD/NASH, although normal aminotransferase levels do not exclude the diagnosis of SS or NASH[23]; patients with advanced NAFLD have been reported to have normal ALT levels[3].

A variety of imaging modalities are increasingly used for NAFLD evaluation and include conventional imaging techniques as well as newer technologies. Conventional imaging techniques consist of B-mode ultrasonography (US), computed tomography (CT), and magnetic resonance (MR) imaging. Findings in NAFLD patients with these techniques are based on lipid accumulation. However, evaluation of inflammation and degrees of fibrosis less than cirrhosis are not possible with conventional imaging techniques. Newer imaging technologies are being increasingly used in combination with conventional technologies and include ultrasound elastography (USE), quantitative ultrasound-based techniques, magnetic resonance elastography (MRE), and magnetic resonance-based fat quantitation techniques[24].

IMAGING ASSESSMENT OF NAFLD

Imaging evaluation of the liver has several advantages over liver biopsy and serum biomarkers in the evaluation of NAFLD, including: (1) non-invasiveness; (2) evaluation a greater volume of liver parenchyma than biopsy, which reduces sampling error in heterogeneously distributed diffuse liver disease processes; and (3) less variability and more quantitative than histopathologic liver biopsy specimen evaluation with some techniques[25].

Ultrasonography

Conventional US is often the first imaging modality used to evaluate fatty liver clinically[26], especially for screening of suspected NAFLD, due to its lack of invasiveness, wide availability, and relatively low cost[27]. NAFLD sonographic features include increased echogenicity, hepatomegaly, and intra-hepatic vascular blurring[28]. Fatty liver has higher echogenicity than renal cortex and splenic parenchyma owing to intracellular fat vacuole accumulation[29], Figure 2 with increased acoustic wave reflection. Steatosis is reported to be detectable by US when more than 20% of hepatocytes contain histologically visible fat droplets, with a reported sensitivity of 79.7% and specificity of 86.2%[30].

There are several limitations of conventional US for NAFLD evaluation: (1) It is qualitative and therefore subjective. The value of conventional US to evaluate NAFLD is limited by the subjective nature of the criteria used to differentiate fatty from normal liver and a lack of sonographic criteria for different degrees of steatosis; (2) Sensitivity is limited when there are few steatotic hepatocytes[30]; (3) The sensitivity and specificity of B mode sonography decreases as BMI (body mass index)

Table 1 Summary table for the value of conventional and elastographic imaging modalities in non-alcoholic fatty liver disease stratification

Modality	Steatosis assessment	Fibrosis assessment	SS / NASH differentiation
Conventional imaging			
US	Not quantitative[1][30]: Sensitivity 79.7%, Specificity 86.2%	No for fibrosis, but can detect cirrhosis with high sensitivity	No
CT	Quantitative[2][38]: Sensitivity 82%, Specificity 100%	Semi-quantitative for fibrosis, but can detect cirrhosis with high sensitivity[39]	No
MRI	Quantitative[3]: Sensitivity 76.7%-90.0%, Specificity 87.1%-91%[40,41]	No for fibrosis, but can detect cirrhosis with high sensitivity	No
Elastographic imaging			
TE / CAP	Sensitivity 82%, Specificity 91%[4][42]	Advanced Fibrosis[43]: Sensitivity 91%, Specificity 75%	No
USE	-	Advanced Fibrosis[44]: Sensitivity 100%, Specificity 91%	No
MRE	Sensitivity 90%, Specificity 93.3%[5][42]	Advanced Fibrosis[45]: Sensitivity100%, Specificity 92%	Yes[5][46]: Sensitivity 94%, Specificity 73%

[1]Gray-scale US detecting steatosis (more than 20% of hepatocytes involved in fat infiltration); [2]Non-contrast CT detecting steatosis; [3]MRI detecting liver histological steatosis; [4]Evaluated by CAP based on TE; [5]MRE discriminating steatosis from NASH using a threshold of 2.74 kPa. SS: Simple steatosis; NASH: Non-alcoholic steatohepatitis; US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging; TE: Transient elastography; CAP: Controlled attenuation parameter; USE: Ultrasound elastography; MRE: Magnetic resonance elastography.

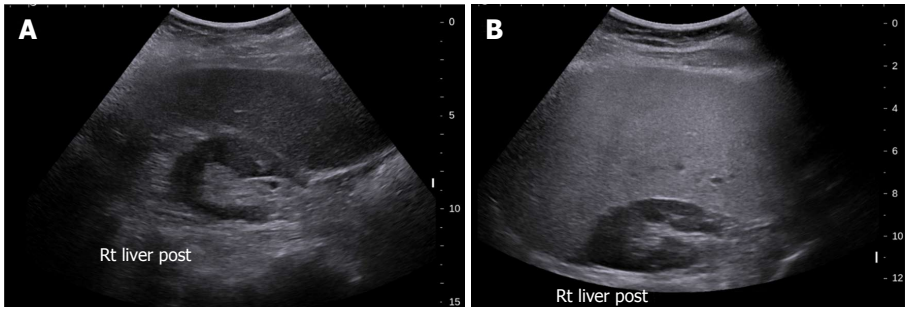

Figure 2 Grey-scale ultrasound in non-alcoholic fatty liver disease. A: 47-year-old female with increased echogenicity of the liver relative to the right kidney, a classic sonographic finding of hepatic steatosis. The patient had elevated serum liver enzymes and underwent a liver biopsy for NASH evaluation; B: 51-year-old female who underwent liver biopsy as part of clinical follow-up. On evaluation of liver pathology, there was no steatosis. Ultrasound image shows normal echogenicity of the liver parenchyma, which is only slightly hyperechoic relative to the renal parenchyma.

increases, varying between 49%-100% and 75%-95%[31]; and (4) Conventional sonography cannot differentiate steatosis and steatohepatitis or stage fibrosis[32,33].

Quantitative ultrasound (QUS) parameters, including attenuation coefficient and backscatter coefficient (BSC), have been developed for liver fat quantification. Attenuation coefficient measures ultrasound energy loss in tissue and provides a quantitative parameter analogous to the qualitative loss of view of deeper structures observed in severe fatty liver[34]. BSC measures the returned ultrasound energy from tissue and provides a quantitative parameter analogous to echogenicity[35]. One study compared the diagnostic performances of attenuation coefficient, BSC, and MRI proton density fat fraction (MRI-PDFF) to differentiate three histological steatosis grades (grade 1, 2, and 3), and revealed AUROCs (area under receiver operating characteristic) ranges of 0.779–0.804, 0.811–0.860, and 0.929–0.962, respectively[36]. In another study, BSC showed excellent diagnostic performance for quantification of hepatic steatosis compared to MRI-PDFF, with AUROCs ranging from 0.90-0.97 at various degrees of hepatic steatosis (MRI-PDFF $\geqslant$ 4%, 5%, 6%, and 8%)[35]. Although QUS parameters have shown potential for accurate hepatic steatosis quantitation, assessment of variation across different scanner manufacturers and operators is warranted to further investigate accuracy, reproducibility, and repeatability[36].

Ultrasound elastography (USE) quantitatively evaluates liver stiffness to make noninvasive evaluation of liver fibrosis and NASH clinically possible. The rationale is that fibrosis acts as a parenchymal scaffold that imparts rigidity. Estimated tissue stiffness therefore provides information on the presence and degree of fibrosis. Current state-of-the-art quantitative elastographic methods do not measure stiffness directly; rather, they assess the propagation of shear waves through tissue from which that tissue's stiffness can be inferred.

USE can be broadly categorized into two methods; (1) Transient elastography (a non-imaging ultrasound-based technique); and (2) imaging-based elastography techniques. Two broad categories of imaging-based sonoelastography are currently in clinical use: (1) strain elastography (SE), which is dependent on operator or physiologic forces to produce tissue deformation; and (2) SWE (shear wave elastography), which is dependent on acoustic radiation force induced tissue displacement[37-43] (Table 1).

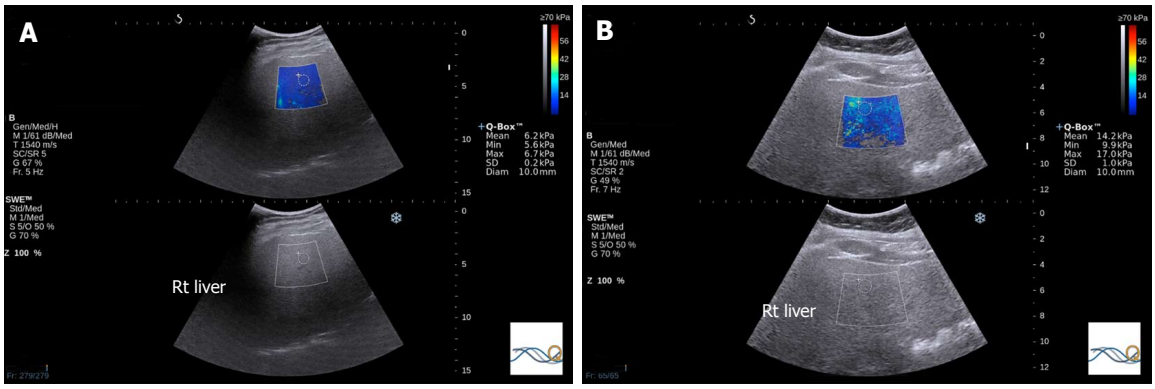

Figure 3 Share wave elastography in non-alcoholic fatty liver disease. A: 54-year old female who underwent liver biopsy for NASH evaluation. The biopsy demonstrated steatosis only with no inflammation or fibrosis. SWE median value of 7.05 kPa; B: SWE in a 52-year old female that underwent liver biopsy for the evaluation of NASH. A median SWE value of 11.5 kPa was significantly higher than normal liver with steatosis only. On biopsy, the subject had fibrosis stage 0 according to the METAVIR system and fibrosis stage 1a as per the NAS CRN criteria. The non-alcoholic fatty liver disease activity score in this patient was 6, consistent with NASH. NASH: Non-alcoholic steatohepatitis; SWE: Share wave elastography.

Fibroscan employs ultrasound transient elastography (TE) to measure hepatic elasticity by quantifying the shear wave speed with pulse-echo ultrasound from low frequency vibrations that are transmitted into the liver[44-46]. It is able to detect liver cirrhosis with high accuracy, and liver stiffness measurements correlate with liver fibrosis stages[47]. In a NAFLD study with 246 subjects, the AUROCs for the detection of F $\geqslant$ 2 and F $\geqslant$ 3 were 0.84 and 0.93, respectively, and the sensitivity and specificity for F $\geqslant$ 3 were 91% and 75% at a cutoff value of 7.9 kPa[48]. A lower TE value appears to reliably exclude advanced fibrosis[49]. Because transient elastography requires transmission of a mechanical wave that originates at the skin, obesity is a significant cause of technical failure and unreliable measurements. To address this problem, the Fibroscan XL probe was developed for obese patients. Controlled attenuation parameter (CAP) is another technique implemented on the Fibroscan device. The reduction in ultrasound amplitude can be estimated as the sound wave traverses liver tissues using the same radiofrequency[50]. In a study of 183 patients, CAP showed good capability in discriminating NASH from simple steatosis, with an AUROC of 0.812 (95%CI: 0.724-0.880)[51].

Two-dimensional SWE (2D-SWE) is an ultrasound-based technique that provides visualization of viscoelastic properties of soft tissue in real time[52]. These techniques employ acoustic radiation force impulses to induce tissue movements at a microscopic level, which in turn produces tissue shear waves. Shear wave speed is algebraically related to tissue stiffness under simple assumptions, expressed as Young's modulus[53]. Unfortunately, vendors of ultrasound elastography equipment use technical terminology inconsistently, which can cause confusion. Researchers have attempted to use this technique for evaluating liver fat content, however, a recent study showed that the SWE value was essentially independent of hepatic liver fat content and was not comparable with the subjective evaluation of liver echogenicity in terms of hepatic steatosis evaluation[54].

Acoustic radiation force impulse (ARFI) imaging is a technique of point shear wave elastography, which refers to the use of acoustic energy to create shear waves in tissue and quantitatively measures the shear wave velocity as a marker of elasticity. It has been shown that ARFI-induced liver shear wave velocity increases with increasing hepatic fibrosis. A study of 54 patients with NAFLD showed that, at a cutoff of shear wave speed (SWS) of 1.77 m/s, AUROC, sensitivity, and specificity for the diagnosis of stage $\geqslant$ 3 fibrosis have been reported to be as high as 0.973, 100%, and 91%, respectively[44]. The relationship between shear wave speed and steatosis is unclear. In one study, SWS was reduced in NAFLD patients, and SWS was lower in patients with simple steatosis than in healthy volunteers. If true, this would make it difficult to distinguish between simple steatosis and NASH with mild fibrosis[49]. However, in other studies, steatosis had no effect on SWS elasticity estimates[48].

Inflammation is known to increase shear wave velocity, confounding fibrosis staging. Nonetheless, the information obtained may permit diagnosis of NASH[55] (Figure 3).

A large body habitus with BMI > 28 kg/m^2 also predisposes to failure of shear wave measurements in NAFLD. In one study, successful SWE measurements were captured in only 75% of obese patients (BMI $\geqslant$ 28 kg/m^2) using a standard probe (78). To improve diagnostic performance, combined ultrasound elastographic methods have been used for liver fibrosis assessment. For example, shear wave elastography and FibroS can were both used in patients with chronic hepatitis C, resulting in high performance in fibrosis staging when used in combination[56].

In summary, among the different ultrasound techniques for differentiating NASH and simple steatosis, TE is studied much more extensively than SWE, but both

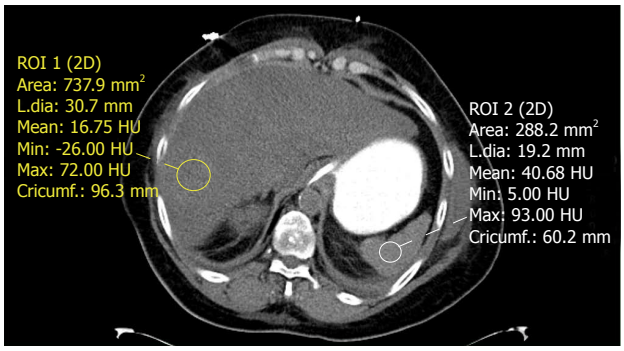

Figure 4 Unenhanced computed tomography of the abdomen in a patient with fatty liver disease. Regions of interest placed within the liver and spleen demonstrate a hepatic attenuation of 16.75 Hounsfield units (less than 40) and a splenic attenuation of 40.68 Hounsfield units. This meets the definition of fatty liver on CT by absolute value, liver/spleen attenuation difference, and liver/spleen attenuation ratio criteria.

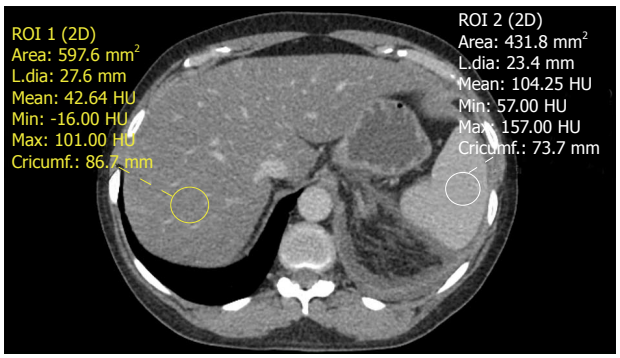

Figure 5 Contrast-enhanced computed tomography of the abdomen demonstrating a fatty liver. The liver has an attenuation value of 42.64 Hounsfield units while the spleen has an attenuation value of 104.25 Hounsfield units. An attenuation difference of 62 HU is highly suggestive of fatty liver disease.

appear to have similar accuracy[57].

CT

Unenhanced CT is more specific than US for NAFLD detection[58]. The mechanism of unenhanced CT to assess NAFLD utilizes attenuation values to evaluate the liver triglyceride content. Hepatic steatosis manifests as reduced attenuation in the liver parenchyma[29], which correlates with the degree of intrahepatic fat accumulation[59]. CT parameters to evaluate fatty liver include (1) the absolute attenuation value (HU, Hounsfield units); (2) the attenuation value difference between the liver and spleen; and (3) ratio of the liver to the splenic attenuation values.

The normal attenuation value of the unenhanced liver parenchyma is 50-65 HU, which is typically 8-10 HU higher than the spleen[26]. The sensitivity and specificity of unenhanced CT to assess ⩾ 30% macrovesicular steatosis has been reported to be 100% (95%CI: 70-100) and 95% (95%CI: 90-98), respectively, at a cutoff of 58 HU and 73% (95%CI: 43-91) and 100% (95%CI: 97-100), respectively, at a cutoff of 42 HU[38]. A recent longitudinal study following NAFLD patients using CT showed no progression of moderate-to-severe hepatic steatosis to symptomatic fatty liver disease over a 5-10-year time period[60]. This may be ascribed to the variations of liver fat content changes over time. BMI changes were identified as an influencing factor, which was negatively correlated with changes in attenuation[61].

Using these normal values on unenhanced CT, various combinations of liver parenchyma versus spleen parenchyma attenuation values have been studied to evaluate fat accumulation in the liver[38]. The normal difference of hepatic and splenic attenuation on unenhanced CT (CT_{L-S}) ranges from 1-18 HU. An unenhanced CT_{L-S} of less than 1 HU can diagnose ⩾ 30% hepatic steatosis, with a sensitivity of 67%[62]. The liver/spleen attenuation ratio is another helpful parameter, which has an acceptable diagnostic performance for steatosis detection[63] (Figure 4). Unenhanced CT assessment of hepatic steatosis can be confounded by deposition of materials other than fat, including iron, copper, glycogen, and amiodarone[64].

CT has limited diagnostic performance for quantitative assessment of mild steatosis; it is sensitive for detecting moderate to advanced steatosis. CT can evaluate for pre-cirrhotic liver fibrosis, with parameters such as the caudate-to-right-lobe ratio and decreased diameter of the hepatic veins. The combination of both of these parameters showed good diagnostic performance for pre-cirrhotic liver fibrosis (sensitivity 83%, specificity 76%) and liver cirrhosis (sensitivity 88%, specificity 82%)[39].

Voxel-based CT attenuation values are subject to be influenced by the contents in the dedicated voxel, so CT values may be influenced by other materials in the liver, such as iron or glycogen, even having the same fat content[65]. Other sources of variations in attenuation values include CT scan settings (voltage, tube current, pitch, *etc*.) and patient parameters (BMI, iodinated contrast agents, *etc*.), all of which limit the reliability of unenhanced CT for quantitative measurement[54,66,67].

Contrast-enhanced CT: Both hepatic and splenic attenuation can be influenced by altered perfusion, acquisition timing, contrast type, dosage, and injection rate. On contrast-enhanced CT, a difference of liver/spleen attenuation of at least 20 HU between 80-100 s after intravenous contrast injection or at least 18.5 HU between 100-120 s after contrast administration (Figure 5) have sensitivity ranging from 54% to 93% and specificity ranging from 87%-93% for hepatic steatosis[68]. These criteria are confounded by variability in contrast-enhanced CT protocols and timing differences related to peripheral injection site variation[69], consequently reliability of the technique is limited. In general, the diagnosis of hepatic steatosis should be rendered only with caution or if the findings are unequivocal on contrast-enhanced CT.

Dual-energy CT: Recent advances in dual-energy CT (DECT) have introduced the ability to perform material decomposition, which has been shown to more accurately quantify hepatic steatosis and potentially permit fibrosis staging[70]. In DECT, the liver is imaged at two

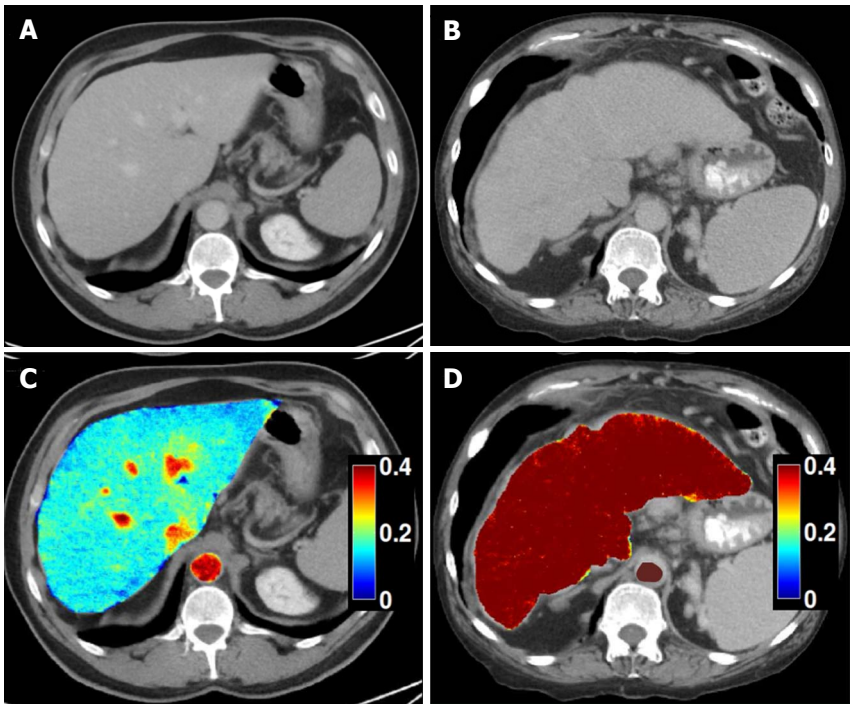

Figure 6 Dual-energy CT images for the assessment of liver fibrosis. A: Delayed phase axial CT images from a patient with mild fibrosis; B: Severe fibrosis; C-D: DECT color overlay contrast agent maps. Iodine concentration within the liver parenchyma in reference to that in the aorta [NIC (normalized iodine concentration) in mg/mL = I Liver / I Aorta] on 5 min delayed acquisitions can be seen on the images. Since contrast media is retained within fibrotic tissues, the NIC on delayed-phase images increases with the severity of liver fibrosis; D: Patients with severe cirrhosis have higher parenchymal contrast media retention on delayed images in relationship to the aorta, as compared to the mild retention in patients with lesser grades of liver fibrosis (C).

different energy levels (typically 80 kVp and 140 kVp). Attenuation differences of tissue at different energies levels are a function of tissue composition, allowing for post-processing of images into "material decomposition images." Material separation enables the generation of iodine, water, and fat-weighted images. DECT has the potential to quantitate liver fat content independent of ROI (region of interest) placement[71] (Figure 6). In a recent study of 50 patients, DECT measurement of fat content showed good correlation with magnetic resonance spectroscopy (MRS), indicating its potential to quantify steatosis[54].

Single-energy CT: Unenhanced single-energy CT (SECT) attenuation values has shown good correlation with quantitative MRI in animal NAFLD models[72]. In a human study, SECT attenuation values acquired at 120 kVp had good correlation with MRS (r^2 = 0.855) in overall patients, but the correlation was significantly lower with MRS in patients with less than 5.56% fat at MRS (r^2 = 0.07), which may be ascribed to the fact that minimal fat in the liver is not CT-detectable[54]. This study also showed that unenhanced SECT (120 kVp) had high performacne for fat quantification compared with DECT (80 and 140 kVp). This was likely bacause DECT was presumably used with contrast enhancement, which accentuated the differences between iodine and the liver but not between fat and water.

MRI

MRI is regarded as the most definitive imaging tool to qualitatively and quantitatively evaluate hepatic steatosis. Both fat and water contribute to the signal observed in the liver on MRI. Differences in the precession of protons in fat and water allow for detection of fat through various MR techniques (Table 1)[36,73]. The sensitivity and specificity of MRI for detecting histologically confirmed steatosis (⩾ 5%) are 76.7%-90.0% and 87.1%-91%[40,41]. Frequency-selective MRI, chemical-shift-encoded MRI, and MR spectroscopy are three techniques that exploit fat-water precession differences to assess fatty liver disease[74].

Frequency-selective imaging applies a saturation radiofrequency pulse to the fat or water frequency range to selectively suppress (or excite) fat or water signals. Fat saturation is a common option for many clinical imaging sequences, including most spin-echo and gradient-echo based sequences at 1.5 T and higher. With fat saturation, the images coincide with the water signal alone; without fat saturation, they represent the sum of fat and water signals. Therefore, hepatic fat may be assessed by comparing these two sets of images. In hepatic steatosis, the fat-saturated images show relative signal loss compared to unsaturated images. In normal liver, fat saturation has no effect and the two sets of images have similar signal intensities. This approach is reasonable for qualitative assessment of liver fat, but does not lend itself to accurate or reliable fat quantification because it is

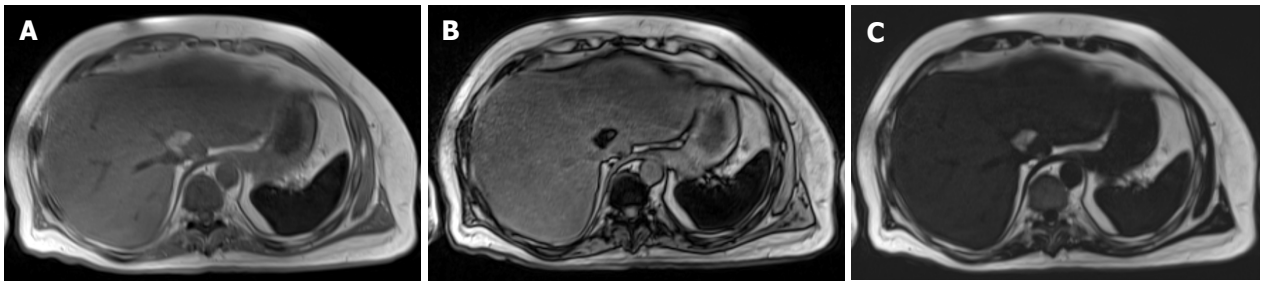

Figure 7 Hepatic steatosis on magnetic resonance imaging. A, B: In phase (A) and out of phase (B) gradient echo T1-weighted images of the abdomen demonstrate signal dropout on the out of phase image due to the presence of microscopic (intracellular) fat deposition in the liver; C: A fat only image via the Dixon method demonstrates diffuse fat accumulation as evidenced by increased T1 signal within the liver.

not possible to achieve perfect fat suppression (or water excitation), and there is usually unsuppressed fat signal.

Dixon methods

Chemical-shift-encoded imaging methods, also termed Dixon methods, use the echo time-dependent phase-interference effect between fat and water gradient-echo signals to accurately detect and quantify fat content[63]. Because fat and water molecules process at different frequencies, they undergo phase interference at predictable intervals. Fat and water signals cancel at out-of-phase (OP) and add at in-phase (IP) echo times. Due to this phenomenon, hepatic fat may be assessed by comparing sequential OP and IP images. In fatty liver disease, the OP images show relative signal loss due to signal cancellation (Figure 7). In normal liver, the OP and IP images have similar intensities[75]. The OP pulse sequence should be obtained prior to the IP sequence to ensure that perceived signal loss is due to fat deposition and not T2* decay, which may occur in the setting of hepatic parenchymal iron deposition. Fat-only and water-only images can be computed using advanced chemical-shift-encoded imaging data. These techniques allow for reliable and reproducible detection of liver fat[76] but are unsuitable for fat quantification because of the presence of confounding factors.

Dixon pulse sequences are offered as a standard fat-suppression technique by nearly every major vendor. Currently, GE (Milwaukee, WI) offers a three-point Dixon technique called IDEAL (Iterative Decomposition of water and fat with Echo Asymmetry and Least-squares estimation), which is a single acquisition technique that results in inherent registration between in-phase and out-of-phase sequences, allowing for reduced scan times and increased reproducibility[77]. The robust fat suppression of IDEAL also allows for imaging of challenging body parts, which is why it is widely utilized in musculoskeletal radiology. Another GE product, named Flex, is a two-point Dixon technique that, while providing less robust fat suppression than its IDEAL counterpart, offers faster acquisition for use in dynamic contrast-enhanced imaging and patients prone to motion artifact. Siemens (Erlangen, Germany) has two and three-point techniques under the generic name DIXON, which utilize multi-echo fat and water separation for detection of small concentrations of fat with increased contrast resolution. Philips (Best, the Netherlands) offers multi-point Dixon (mDixon) in a single breath hold to decrease acquisition time and maximize in-plane and through-plane resolution while offering flexibility in echo time settings. Hitachi (Tokyo, Japan) offers a two-point Dixon sequence called FatSep. Advantages of this technique include uniform fat suppression over a large field-of-view, particularly in the presence of a metal implant, as well as customizable levels of fat suppression (light, medium, or heavy). Toshiba (Tochigi, Japan) also has a 2-point Dixon technique called WFOP (Water-Fat Opposed Phase). Regardless of the implementation details, all of the current Dixon-type sequences produce four sets of images, including water only, fat only, in-phase, and out-of-phase[78] (Figure 8).

Magnetic resonance spectroscopy (MRS) evaluates proton signals as a function of their resonant frequency, which shows multiple peaks at different locations within a specified volume of the liver[71]. The MR spectrum describes the intensity of MR signal as a function of precession frequency, with fat and water producing the most visible peaks. However, water occurs as a single peak and fat shows as multiple peaks due to its multiple chemical components[29]. Thus, in fatty liver disease, both water and fat spectral peaks are present. In normal (non-fatty) liver, only the water peak is seen. To be performed correctly, confounding factors need to be addressed including T1 bias (addressed by use of long repetition time $\geqslant$ 3000 ms), T2 relaxation (addressed by collecting spectra at multiple echo times and correcting for T2 decay), and J-coupling (minimized by using appropriate spectroscopic sequences and using the shortest possible mixing time).

Current state-of-the-art MR techniques for quantifying hepatic steatosis include confounder-corrected chemical-shift-encoded MRI, which can estimate the PDFF. Unlike US and CT, which use surrogate measures of fat in the form of altered echogenicity and attenuation, PDFF measures the fraction of MRI-visible protons bound to fat divided by all MRI-visible protons in the liver (fat and water)[79]. Using this technique, the liver signal on MRI is divided into water and fat signal components by acquiring gradient echoes at appropriately spaced echo times, so as to quantify the percentage of liver fat[80]. Images are acquired with a low flip angle to minimize T1 bias and at multiple echo times to measure and

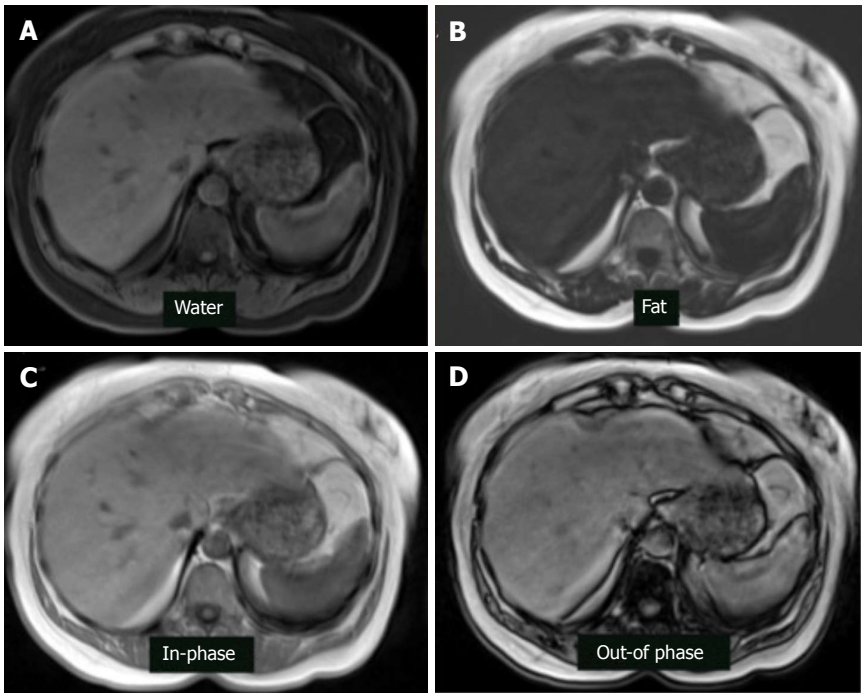

Figure 8 Magnetic resonance imaging dixon technique in the patient with hepatic steatosis. A: Water only sequence; B: Fat only sequence; C: In-phase sequence; D: Out-of-phase sequence.

correct for T2* decay. The signal model mathematically incorporates a multi-peak fat spectrum to address the multi-frequency interference effects of protons in fat[81]. In a study of 50 patients, it was found that complex three-echo chemical-shift-encoded MRI is equivalent to MRS for quantifying liver fat, but only after correction for T2* decay and T1 recovery and spectral fat modeling[82]. MRI-PDFF has demonstrated a high diagnostic accuracy (AUROC = 0.989, 95%CI: 0.968-1.000) for detecting histological steatosis grade 1 or higher, and can be measured reproducibly across field strengths and scanner manufacturers[83].

Magnetic resonance elastography (MRE) uses a modified phase-contrast MRI sequence and an external mechanical actuator to induce and non-invasively visualize propagating tissue shear waves[84]. It estimates the degree of fibrosis throughout the liver by analyzing the resulting wavefield using a so-called inversion algorithm from which the magnitude of the complex shear modulus (often described as the "shear stiffness" for simplicity) is computed[31]. Similar to ultrasound elastography, MRE-estimated stiffness increases along with fibrosis stage. MRE can differentiate fibrosis between F $\geqslant$ 2 and F1 (sensitivity 89.7%, specificity 87.1%) at a stiffness cutoff value of 3.05 kPa, and it can also discriminate severe fibrosis (F3) from liver cirrhosis (sensitivity 100%; specificity 92.2%) with a cutoff value of 5.32 kPa[45]. In addition to staging advanced fibrosis, MRE has also shown high accuracy for discriminating SS from NASH in a retrospective study in a cohort of patients with a relatively high frequency of advanced disease (cutoff value 2.74 kPa, AUROC 0.93, sensitivity of 94%, and specificity 73%). The performance of MRE to diagnose NASH in the absence of significant fibrosis is unknown[46] but is thought to be limited. These studies indicate that MRE-measured hepatic stiffness has the potential to identify NASH before fibrosis onset. Finally, MRE shows superior diagnostic performance than TE for liver fibrosis evaluation, but low availability and high cost limit its widespread clinical adoption[29,42].

IMAGING ASSESSMENT OF NAFLD IN PEDIATRIC PATIENTS

The estimated prevalence of NAFLD in overall children is 10%, but increases to 40%-70% in the obese pediatric population[85]. The value of MRE and SWE in the pediatric population has been compared with that in adult patients. MRE has shown comparable sensitivity and specificity to detect any stage of fibrosis ($\geqslant$ F1) from no fibrosis, and its optimal threshold to differentiate $\geqslant$ F3 fibrosis is reported 3.03-3.33 kPa, which were lower than in adults with NAFLD (3.45-4.8 kPa) (Figure 9)[37,86,87]. SWE is also an accurate technique to detect significant liver fibrosis ($\geqslant$ F2) in pediatric patients with NAFLD (AUC = 0.97, 95%CI: 0.95-0.99) and for mild liver fibrosis ($\geqslant$ F1, AUC = 0.92, 95%CI: 0.86-0.98)[88]. Given the variated thresholds in MRE and SWE, further investigation is warranted to validate and standardize optimal cutoffs for fibrosis staging. Additionally, current imaging techniques still have limitations to accurately discriminate pediatric early stage fibrosis, which is important to be monitored for early treatments.

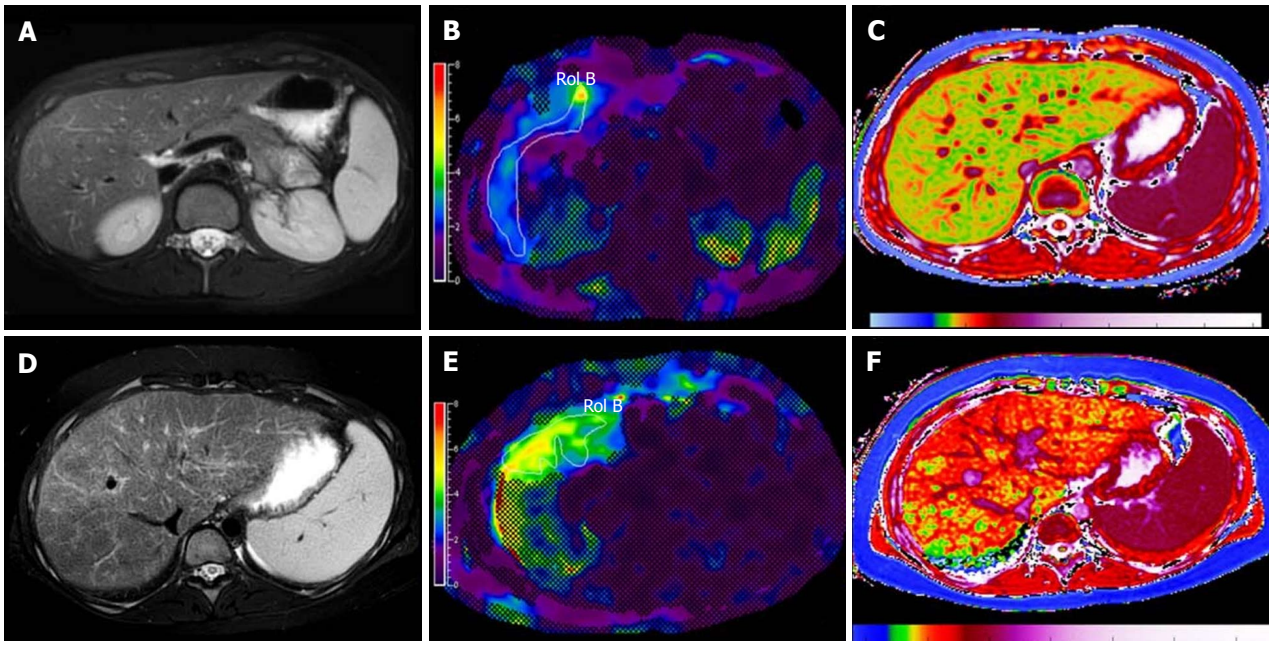

Figure 9 Examples of multiparametric magnetic resonance imaging measurements in pediatric patients[87]. A-C: MRI images in a healthy child without evidence of liver disease. A: Conventional T2-weighted fast spin echo (A); B: MRE with normal hepatic shear stiffness of 2.22 kPa (B), and corrected T1 time of 879 ms (C); D, F: Corresponding images in a child with biopsy-confirmed primary sclerosing cholangitis. D: The T2-weighted fast spin echo image shows heterogeneously increased liver signal due to fibrosis that can be quantified using texture mapping; E: MRE image shows an increased hepatic shear stiffness of 3.96 kPa; F: The corrected T1 time was increased to 1048 ms, likely due to a combination of fibrosis and inflammation.

CONCLUSION

Patients with NAFLD are at risk of steatohepatitis and progressive liver fibrosis culminating in cirrhosis, typically over a period of decades. Early diagnosis and risk stratification are essential for effective management. Current imaging methods such as ultrasound, CT, and MRI have demonstrated their values to serve as noninvasive imaging biomarkers to evaluate NAFLD progression, but they are still relatively limited in the detection of inflammation (NASH), which is more important than steatosis in terms of its high risk for fibrosis, cirrhosis, and HCC. Detection of NASH by imaging remains the future direction in NAFLD.

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EDITORIAL

Hepatitis C resistance to NS5A inhibitors: Is it going to be a problem?

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Abstract

Treatment of hepatitis C virus (HCV) infection has evolved greatly through the recent decade. The availability of direct-acting antiviral agents (DAAs) targeting the functional proteins of HCV has resulted in the introduction of DAA-based combination therapies, providing an optimal rate of treatment success. Among the DAAs, NS5A inhibitors are used in most of the introduced and approved HCV antiviral regimens. Resistance-associated substitutions (RASs) are amino acid substitutions in HCV protein sequences that result in decreased antiviral efficacy of the HCV DAAs. Among the HCV RASs, the NS5A RASs were found to effectively modify and decrease treatment response to NS5A inhibitor-containing regimens. As a baseline predictor of treatment response, NS5A RAS draws attention for pretreatment testing in targeted patient groups. Given NS5A RASs are either naturally-occurring or DAA-selected, the application of NS5A RAS testing can be considered in two settings of NS5A inhibitor-naïve patients and NS5A inhibitor-experienced patients. Less than 5% of NS5A inhibitor-naïve patients harbor naturally-occurring NS5A RAS with high resistance level (> 100X resistance fold-change). In NS5A inhibitor-naïve patients, NS5A RAS testing accompanied by treatment optimization cannot increase treatment response more than 2%-3%, while in NS5A inhibitor-experienced patients, > 75% are found to have NS5A RASs > 100X and NS5A RAS testing in this group of patients seems to be reasonable. This editorial will address the debate on the application of NS5A RAS testing and will discuss if the NS5A RAS testing has any role in clinical management of hepatitis C.

Key words: Direct-acting antiviral agent; Hepatitis C; NS5A; Resistance; Treatment

Core tip: Hepatitis C virus resistance to NS5A inhibitors

is one of the main problems of treatment with NS5A inhibitors. While the treatment of NS5A inhibitor-naïve patients is feasible and efficient, retreatment of NS5A inhibitor-experienced patients is challenging. In the context of failure following treatment with NS5A inhibitor-containing regimens, NS5A resistance-associated substitution testing can help in clinical decision-making, while the usefulness of baseline NS5A resistance-associated substitution testing in NS5A inhibitor-naïve patients is in question.

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INTRODUCTION

Antiviral therapy of hepatitis C virus (HCV) infection has been the mainstay of hepatitis C management since application of conventional interferon (IFN) was introduced as the first HCV antiviral agent in 1990s[1,2]. HCV antiviral therapy has transformed greatly over time, from use of immunomodulatory agents, such as IFN, to use of direct-acting antiviral agents (DAAs), such as NS3 protease inhibitors, NS5A inhibitors and NS5B polymerase inhibitors[1]. While the IFN-based regimens result in suboptimal (30%-70%) virologic response, the DAA-based all-oral regimens have efficacy of > 95% in most patient groups[3-5]. Moreover, the treatment response to IFN-based regimens was modified by a large number of different host and virus parameters, such as age, sex, host genetics, liver fibrosis, HCV ribonucleic acid (RNA) level, HCV genotype, variations in HCV genes, *etc*[3,6,7].

On the other hand, with an increase of response rate to the DAA-based regimens, the modifiers of treatment response have been limited to few parameters, including cirrhosis, history of previous treatment (especially failure with DAA-based regimens), and finally naturally-occurring and DAA-selected resistance-associated substitutions (RASs)[8,9]. Currently, in most of the approved regimens for treatment of HCV infection, NS5A inhibitors are one of the components of the combination therapy with DAAs. The currently available and approved NS5A inhibitors are ledipasvir (LDV), daclatasvir (DCV), ombitasvir, elbasvir, velpatasvir (VEL) and pibrentasvir (PIB). In the context of treatment with NS5A inhibitor-containing regimens, the NS5A RAS is one of the baseline predictors of treatment response and, also, the major finding after treatment failure with these regimens[8,10].

NS5A RASs

Among the nonstructural proteins of HCV, the roles of NS5A protein are not fully known yet; however, it has been found that NS5A binds the RNA-dependent RNA polymerase NS5B and modulates RNA-dependent RNA polymerase activity[11]. The inhibitory activity of NS5A inhibitors on replication of HCV shows the pivotal role of NS5A protein in replication of HCV. The substitutions in the amino acid sequence of NS5A protein causing decreased binding of NS5A inhibitor molecules to NS5A protein can be selected under pressure of the treatment with NS5A inhibitors and subsequently decrease the inhibitory activity of NS5A inhibitors[12].

Generally, the NS5A amino acid substitutions causing > 2.5 resistance fold-change are considered as NS5A RASs. A subset of these NS5A RASs cause > 100 resistance fold-change, and are considered NS5A RASs > 100X[13]. It is worth noting that the resistance fold-change should be defined with consideration to the HCV genotype and subtype (*i.e.,* 1a, 1b, 3a, *etc.*), NS5A inhibitor type (*i.e.,* LDV, DCV, VEL, *etc.*) and also the specific amino acid substitution (*i.e.,* Y93H, Y93F, Y93L, *etc.*). The NS5A RASs are observed as naturally-occurring substitutions in a proportion (< 10% to > 50%) of NS5A inhibitor-naïve patients[13-16]. The rate of detection of NS5A RASs is determined mainly by HCV genotypes and subtypes, and by the method for detection of NS5A RASs (*i.e.*, deep sequencing *vs* Sanger sequencing)[17,18]. Among patients with NS5A RASs, only a small number (< 5% of NS5A inhibitor-naïve patients) of patients harbor isolates with naturally-occurring NS5A RASs > 100X.

The main finding regarding the prevalence of RASs in NS5A inhibitor-naïve patients with HCV genotype-1 (HCV-1) is the higher prevalence of NS5A RASs in patients with HCV-1b than in those with HCV-1a[19,20]. In the study by Dietz *et al*[19], the prevalence of NS5A RASs was 7.1% in patients with HCV-1a infection and 17.6% in those with HCV-1b infection. These NS5A RASs could be found at different levels of a mixed population of the mutated and wild-type viruses[21,22]. In NS5A inhibitor-experienced patients, especially those who have undergone a complete course of 12-wk or > 12-wk treatment, DAA-selected NS5A RASs are observed in > 75% of patients following treatment failure[10,23-25]. Most of these patients with failure to NS5A inhibitor-containing regimens harbor isolates with NS5A RASs > 100X[24,26]. Unfortunately, these DAA-selected NS5A RASs are persistent and they are observed even in the follow-up, 96 wk after treatment failure[27].

The impact of naturally-occurring baseline NS5A RASs has been assessed in many studies. These studies showed the impact of NS5A RASs on treatment response to NS5A inhibitor-containing regimens such as LDV/sofosbuvir[23,28], grazoprevir/elbasvir[29-31], ombitasvir/paritaprevir/ritonavir[21] and DCV/asunaprevir[22]; however, treatment with regimens containing the second-generation NS5A inhibitors with medium resistance barrier, including sofosbuvir/VEL[26,32] and glecaprevir/PIB[33], was either not impacted or only minimally modified by the NS5A RASs. In terms of HCV genotypes, it seems that the treatment of patients with HCV-3 infection

using first-generation NS5A inhibitors is more influenced by NS5A RASs than that of patients with HCV-1 infection using same NS5A inhibitor[13,26]. This finding can be a result of different resistance fold-change of NS5A RASs by HCV genotypes with higher resistance fold-change for NS5A RASs in HCV-3 isolates than that for NS5A RASs in HCV-1 isolates[34]. Finally, it can be concluded that the higher response rate observed with the optimized treatment using a second-generation NS5A inhibitor with higher resistance barrier (*i.e.*, VEL, PIB, *etc.*) effectively inhibiting NS5A protein of all HCV genotypes (pan-genotypic regimen) results in a lower chance for observation of treatment response modification by NS5A RASs.

With the knowledge that most of the patients with failure following treatment with NS5A inhibitor-containing regimens harbor NS5A RASs, especially those which confer a high level of resistance, the impact of these DAA-selected NS5A RASs on the efficacy of retreatment were evaluated in a small number of studies; the results showed that NS5A RASs are prominent treatment response predictors, especially when the regimen of the retreatment was not intensified or optimized for eradication of the virus[35,36].

TESTING RESISTANCE TO NS5A INHIBITORS: WHEN AND HOW?

There is a great debate on the application and usefulness of NS5A RAS testing prior to treatment. Generally, NS5A RAS testing can be considered under two different conditions: (1) prior to treatment with NS5A inhibitor-containing regimens in NS5A inhibitor-naïve patients; and (2) prior to retreatment with NS5A inhibitor-containing regimens in patients with a history of treatment failure using NS5A inhibitor-containing regimens. In Figure 1, the strategies with and without pretreatment NS5A RAS testing for treatment of HCV infection using NS5A inhibitor-containing regimens in NS5A inhibitor-naïve patients are presented. Since the overall response rate to the currently available and standard NS5A inhibitor-containing regimens is high (> 95%) and also the prevalence of NS5A RASs conferring high resistance level is low (< 5%), the strategy of pretreatment NS5A RAS testing and optimization of treatment in terms of treatment prolongation, adding ribavirin (RBV) and targeting all three HCV protease, NS5A and polymerase proteins can result in 2%-3% increase of the overall treatment response rate. Considering the costly ($100-$500) and time-consuming (7-30 d) process of NS5A RASs testing, we do not recommend NS5A RAS testing in NS5A inhibitor-naïve patients prior to treatment with NS5A inhibitor-containing regimens.

In patients with previous history of treatment with NS5A inhibitor-containing regimens, the scenario is much different with the fact that > 75% of them harbor NS5A RASs. As shown in Figure 2, the NS5A RAS testing can be implemented in the management of patients with previous history of treatment with NS5A inhibitor-containing regimens. If the patient harbors the NS5A RAS, they should be treated with the most intensified and optimized available treatment regimen (adding RBV, prolongation of treatment to 24 wk, and targeting all three protease, NS5A and polymerase proteins). However, the small portion of patients with previous history of treatment with NS5A inhibitor-containing regimens and without detectable NS5A RAS can be treated by treatment prolongation and/or adding RBV without targeting additional HCV protein.

Currently, there are services available for NS5A RAS testing in diagnostic laboratory centers. These services are based on Sanger direct population sequencing or deep sequencing using next-generation sequencing platforms. The main difference between these two available technologies is the limit of detection of the RAS (mutated nucleotide) among the population of the virus without the RAS (wild-type nucleotide). Sanger sequencing can detect the NS5A RASs comprising 20%-30% of the whole population of the virus, while deep sequencing can even detect NS5A RASs comprising < 1% of the whole HCV population. It figures that deep sequencing is much more advanced for detection of NS5A RASs than Sanger sequencing; however, it was found that a small (< 10%-20% of the whole genetic pool of virus) population of HCV with RAS in a patient has no or minimal impact on treatment success[21,22,37]. With this finding, it seems that in the clinical management of hepatitis C, the NS5A RAS testing using Sanger sequencing is reasonable, and the reports of NS5A RAS testing using deep sequencing methods should be accompanied by the percentage of the NS5A RAS in the whole virus population.

CONCLUSION

DAA-based treatment of HCV is one of the greatest recent achievements in the field of hepatology and liver diseases, making hepatitis C a curable disease; however, the main problem with most of the targeted therapies is resistance. With a combination of NS5A inhibitor with an NS5B polymerase inhibitor and/or an NS3 protease inhibitor, the problem of HCV resistance has been mostly resolved in DAA-naïve patients and only a small (< 5%) population of treated patients relapsing after treatment termination. While pretreatment NS5A RAS testing cannot help in management of DAA-naïve patients, it can help in treatment decision-making for patients with failure to NS5A inhibitor-containing regimens. Currently, with the introduction of second-generation NS5A inhibitors, VEL and PIB, and the upcoming NS5A inhibitors in the pipeline with a high barrier to resistance, the problem of resistance to NS5A inhibitors is going to fade. However, unsolved concerns remain, such as transmission of NS5A RASs in populations with high-

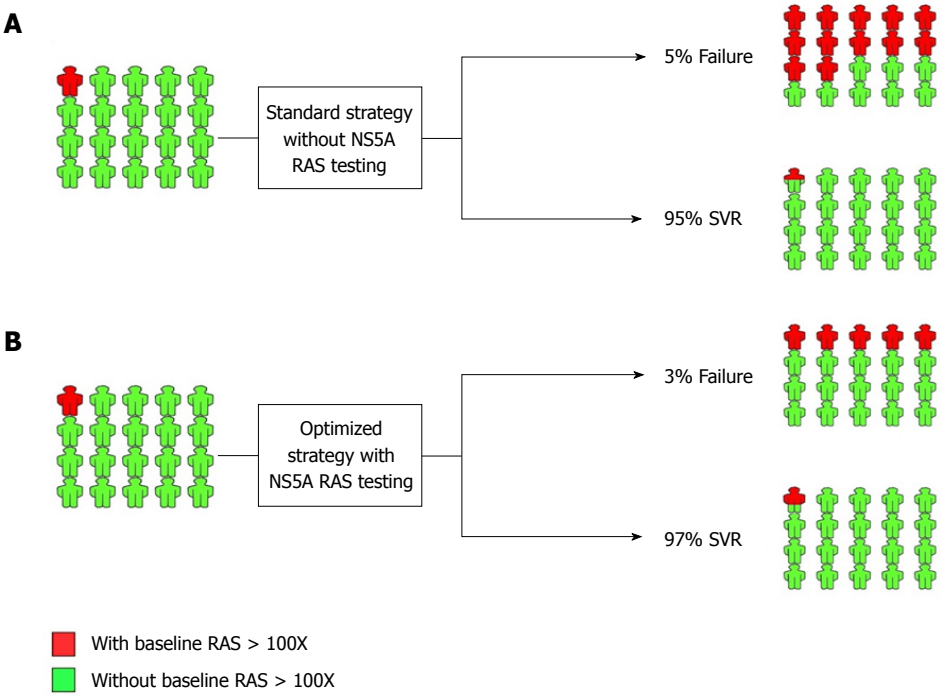

Figure 1 NS5A resistance-associated substitution testing in NS5A inhibitor-naïve patients prior to treatment with NS5A inhibitor-containing regimen. A: Standard treatment strategy and outcome without NS5A resistance-associated substitution (RAS) testing; B: Optimized treatment strategy and outcome with NS5A RAS testing. The implementation of NS5A RAS testing is developed by decision-making based on the presence of RAS > 100X. SVR: Sustained virologic response.

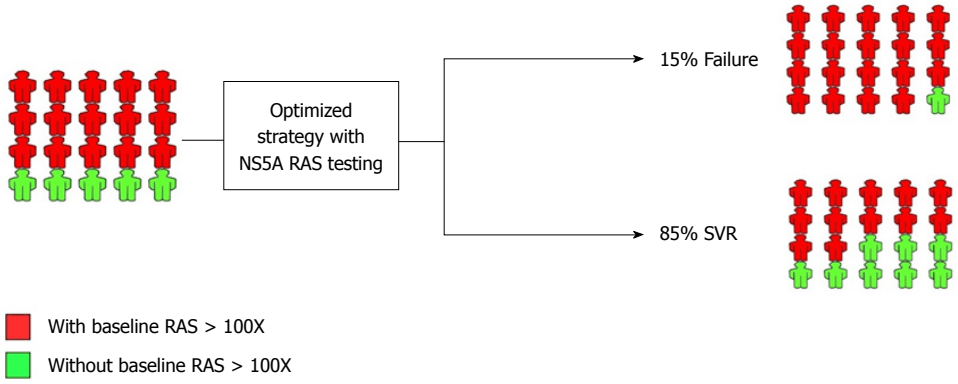

Figure 2 NS5A resistance-associated substitution testing in NS5A inhibitor-experienced patients prior to retreatment with NS5A inhibitor-containing regimen. Optimized treatment strategy with NS5A resistance-associated substitutions (RAS) testing and the outcome is shown. The implementation of NS5A RAS testing is developed by decision-making based on the presence of RAS > 100X. SVR: Sustained virologic response.

risk behaviors, including people who inject drugs, which should be considered by clinicians and researchers carefully.

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REVIEW

Challenge of hepatitis C in Egypt and hepatitis B in Mauritania

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Abstract

Egypt has one of the highest prevalence rates of hepatitis C virus (HCV) in the world, mostly with genotype 4 that is highly associated with severe fibrosis. As a consequence, hepatocellular carcinoma has become the leading cause of cancer in this country. Mauritania is a highly endemic area for hepatitis B virus (HBV). HBV and HCV could both be iatrogenically transmitted through infected blood products, infected needles, and medical equipment improperly sterilized. Adequate and efficient healthcare and public health measures with good surveillance programs, access for screening, prevention strategies, and successful treatment are needed to halt the spread of these diseases. Herein, we have reviewed the epidemiology, modes of transmission, predisposing factors, and novel treatment modalities of these viruses. We have proposed practices and interventions to decrease the risk of transmission of HCV and HBV in the affected countries, including strict adherence to standard precautions in the healthcare setting, rigorous education and training of patients and healthcare providers, universal screening of blood donors, use of safety-engineered devices, proper sterilization of medical equipment, hepatitis B vaccination, as well as effective direct-acting antiviral agents for the treatment of HCV.

Key words: Hepatitis B virus; Hepatocellular carcinoma; Hospital acquired infection; World Health Organization; Hepatitis delta virus; Hepatitis C virus

Core tip: Hepatitis C virus (HCV) and hepatitis B virus (HBV) are major public health concerns in Egypt and Mauritania. HCV and HBV can both be transmitted through medical and surgical procedures (healthcare-associated transmission) among others. Screening, prevention, and treatment strategies should be emphasized in Egypt and Mauritania to prevent the spread of these diseases. Direct-acting antivirals for the treatment of HCV are highly effective and well tolerated and should be made accessible and affordable to patients.

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HEPATITIS C VIRUS IN EGYPT

Over the last several years, Egypt continues to have one of the largest epidemics of hepatitis C virus (HCV), with an estimated prevalence of 10%. A prevalence above 4% is considered high by World Health Organization (WHO) standards[1]. A study published in the Proceeding of the National Academy of Science (PNAS) reports more than 500000 new HCV cases yearly[2]. However, others recently estimated a lower number of new cases every year, but still it remains high compared to other areas in the world[3]. Hence, viral hepatitis caused by HCV genotype 4 continues to represent the most serious public health threat currently facing Egypt[1,2].

To estimate the prevalence of HCV in Egypt, a national survey was conducted in 2015 known as the Egyptian Health Issues Survey (EHIS). This survey was a cross-sectional household survey of 16003 who were identified between the ages of 15 and 59 and had blood testing for HCV[1]. The results of this survey were compared to another cross-sectional national survey conducted in 2008 known as the Egyptian Demographic Health Survey (EDHS) in which 12008 Egyptian were interviewed[4]. The 2015 survey (EHIS) reported a prevalence of 10% for HCV antibody and 7% for HCV RNA. This reflected an estimated 29% reduction in HCV RNA prevalence compared to the 2008 national survey (EDHS). This reduction could be related to the disappearance of the highly infected group that was treated with reused syringes during the shistosomiasis treatment campaign in the 1960s and 1970s[1]. Hence, by 2015, this highly infected older age group disappeared due to differential age related migration and mortality (particularly since the survey excluded individuals that are 60 years or older). Furthermore, when a shift adjustment was made (by 7 years), the age specific prevalence of HCV RNA positivity was matched and was comparable in the 2008 surveillance (EDHS) and the 2015 surveillance (EHIS). Hence, these surveys represent an underestimate of the true prevalence of the HCV infections in Egypt, particularly since the rate of infection is among elderly patients above 60 years who acquired the infections in the 1960s and 1970s whereas these surveys were limited only to individuals with an age range of 15-59 years [1].

Based on the above and given the population of Egypt, which exceeds 95 million, we estimate that more than 6.5 million Egyptians are infected with the HCV virus, and most of those are caused by a genotype 4. Several genotypic studies have shown that genotype 4 of HCV accounts for 93.1% of HCV infections, and most (80.6%) of the Egyptians infected with HCV4 belong to subtype 4A[5-7]. It is likely that the subtype 4A was the main strain associated with antischistosomal therapy epidemic that occurred in the 1960s and 1970s and which was associated with the reuse of needles[8,9]. In fact HCV4 is the predominant HCV genotype in the Middle East and North Africa, which accounts for 59% of HCV infections in Syria, 53% in Iraq, 54% in Kuwait, and 64% in Palestine. It is also reported to be a dominant genotype in Qatar, Saudi Arabia, and Libya[5,6,10,11] and is common in other parts of the Middle East such as Lebanon, Oman, and UAE. This may possibly be related to the migration of Egyptians, who represent a major workforce in some of these Middle Eastern countries, to these areas[5,11].

Furthermore, there is evidence that HCV4 is a highly pathogenic virus. Wali *et al*[12] demonstrated that HCV4 was significantly more associated with fibrosis progression, severe fibrosis development, and confluent necrosis than other non-genotype 4 HCV infected patients. In addition, hepatocellular carcinoma (HCC) in Egypt is a leading cause of cancer and cancer mortality among men, whereby more than 84% of Egyptian patients with HCC are positive with HCV4[13,14]. Chronic infection that could lead to severe fibrosis and cirrhosis can occur in 50%-85% of HCV patients[15,16]. Given the decline in the economic and healthcare conditions, nationwide efforts to control the spread of HCV infection have been disrupted for several years.

The iatrogenic spread of HCV genotype 4 in Egypt due to the improper dental instruments sterilization has been a major concern[17]. Furthermore, between 46%-100% of hemodialysis patients and 11%-82% of patients who received multi-transfusions were found to be seropositive for HCV[18].

Despite all the effort done by the Ministry of Health and Population (MOHP) in Egypt to control infection, healthcare-associated infection (HAI) remains one of the most common cause of HCV infection in Egypt,

Table 1 Risk factors of the transmission of hepatitis C in Egypt through the healthcare system and proposed interventions

Risk factors	Proposed interventions
Needle stick injuries or other injuries	Institute infection control and occupational health programs in all healthcare facilities to reduce occupational exposure, protect against needle stick, and other healthcare related injuries Adequate education and training of healthcare providers Use of safety-engineered devices, such as needleless intravenous medication systems, blunted suture needles Use of needle disposal containers
Surgical or invasive interventions, dental procedures	Appropriate sterilization of surgical and dental instruments Good aseptic techniques practiced during invasive procedures Provide personal protective equipment, such as gloves, gowns, face/eye shields, to be used during procedures with anticipated blood exposure
Exposure to medical equipment, hemodialysis machines and procedures	Strict infection control and prevention policies Universal precautions should be used when caring for all patients
Injection and Ⅳ insertion	Use of self-sheathing needles, needleless connectors, needleless intravenous medication system, and needle disposal containers
Blood transfusion from poorly screened individuals (false negative anti-HCV)	Universal screening of all donors
Organ donation	Universal screening of all donors

HCV: Hepatitis C virus.

related to the overuse of contaminated needles and syringes[2]. In addition, healthcare workers in Egypt and many Middle Eastern countries have the highest rates of needle stick injuries worldwide[19,20]. Hence, unsafe medical and dental practices, including reuse of medical devices, inadequate sterilization of surgical and interventional equipment, poor aseptic techniques practiced during invasive procedures, circumcisions or deliveries of neonates by providers, unsafe injections, and limited testing of blood product transfusions for HCV have all contributed to high iatrogenic transmission of HCV in Egypt[21-23]. These factors play an important role in the transmission of HCV in Egypt. Table 1 outlines the risk factors for the transmission of HCV in Egypt through the healthcare system, and the proposed infection prevention interventions that could control this transmission.

To control this self-perpetuating HCV epidemic in Egypt, a health initiative was started in 2017 to screen all governorates of Egypt called "Egypt without virus C, 2020" in cooperation with Tahya Misr Fund[24]. A concerted effort should be made towards universal screening of all patients going through the healthcare system, early detection of HCV, early treatment using direct acting antiviral (DAA) regimen with simultaneous implementation of strict infection control and prevention policies within the healthcare system.

Egypt also developed a strategy for prevention and control of viral hepatitis (2008-2014) in collaboration with WHO, Centers for Disease Control (CDC), and Pasteur[25]. The National Committee for Control of Viral Hepatitis (NCCVH) in affiliation to the MOHP established a large model of care in Egypt since 2006 that aimed at elimination of HCV in Egypt and delivering DAAs for all patients at the expense of the government[25,26]. However, the limited resources, the unmet needs and suboptimal access to care, and the low rates of patients' follow-up hindered the success of the program[27,28].

Most of the individuals living with viral hepatitis caused by HCV are asymptomatic and, hence, remain unaware of their illness for decades, even though liver damage is occurring. Given the high prevalence of HCV in Egypt, universal surveillance through rapid enzyme immunoassay testing of all patients going through the healthcare system would detect a large number of asymptomatic patients. With the advent of DAA and the demonstration of high cure rates (sustained virologic response or SVR) of over 90%, including genotype 4 infected patients. The DAAs are also associated with minimal side effects and relatively short duration of treatment, when compared to interferon based treatments.

Hence, it is possible to virologically cure the majority of these patients, particularly if the patient is treated in the early phase when they are not suffering from decompensated liver disease or cirrhosis[29-32]. Curing these patients would preempt the morbidity and mortality associated with subsequent liver cirrhosis and HCC. SVR after non-interferon DAA regimens is associated with improvement in liver fibrosis and necrosis in up to 73% of patients, with reversal of cirrhosis in 49% of the cases[33]. Emerging data with DAAs show significant improvement of liver stiffness that has been reported in patients with HCV-associated advanced liver disease[34]. A recent study showed that DAA-induced SVR was associated with 71% reduction in HCC[35].

Early treatment of HCV infection, regardless of the infecting genotype, may also reduce the risk of extrahepatic complications, including mixed cryoglobulinemia, porphyria cutanea tarda, diabetes mellitus, cardiovascular disease, renal diseases, and B-cell non-Hodgkin lymphoma[36-38].

Given the availability of generic versions of DAAs

(particularly sofosbuvir at an affordable low cost), early treatment can be widely used and, hence, could effectively contribute to the elimination of HCV viral hepatitis in Egypt[3]. The WHO reported that after generic sofosbuvir became available in Egypt at a cost of $153 for a 12-wk course, approximately half a million patients with chronic HCV were treated with this drug since January 2016[3,39]. Our team conducted a large surveillance study on patients evaluated at Harpur Memorial Hospital in the Delta area of Menouf, Egypt, involving 729 adult patients (18-65 years) who were evaluated for health-related illnesses not associated with hepatitis or liver diseases between January 2012-January 2013. All patients who consented were screened by a rapid enzyme immunoassay (ELISA) to detect the presence of HCV antibodies and determine the prevalence of HCV (HCV antibodies). The reactive ELISA rapid test samples were further confirmed for HCV antibody positivity by the chemiluminescent microplate immunoassay (CIA). Subsequently, CIA HCV antibody positive samples were tested further for HCV RNA by quantitative real time polymerase chain reaction (PCR). We identified 146 patients (20%) who were positive for HCV, which was later confirmed by the ELISA antibody test and HCV-RNA levels (viral load). Interestingly, 119 (82% of the 146 patients) with a positive test had no risk factors for developing HCV, such as a surgical procedure, dental extraction, needle stick injuries, blood transfusions, and other risk factors listed in Table 1 in the prior 5 years. All of the 146 patients with positive HCV in the study were asymptomatic with no symptoms of liver disease. Therefore, this study highlights the importance of universal surveillance in the general population, as suggested by WHO, in countries with high HCV antibody seroprevalence (⩾ 2%), which will lead to the early detection of HCV in asymptomatic patients and ultimately lead to high cure rates after using DAA[29-32,40].

In addition to universal surveillance, early diagnosis, and early treatment of HCV in Egypt, promotion of infection prevention and control procedures in a healthcare setting is of paramount importance in controlling HCV transmission[40]. These infection control policies and practices should prohibit reuse of medical devices or needles, emphasize appropriate sterilization of surgical and dental instruments, promote the use of safe injectables, protect against needle stick and other healthcare related injuries, test all blood donors, and adhere to appropriate healthcare waste management. This involves a large scale training of healthcare professionals in Egypt on infection control practices and includes new safeguard technologies, such as the autodisable syringes (Table 1).

The conventional combination regimen of pegylated-interferon and ribavirin alone has demonstrated low SVR rates against genotype 4 of approximately 30% after 48 wk of treatment with poor tolerance of the regimen[41,42]. However, several DAAs have been approved in the United States and Europe that showed highly promising results; and, hence, they have changed the treatment paradigm for chronic HCV infections in general, including genotype 4 (Table 2)[43,44]. The DAA regimen should be individualized, mostly on the basis of the patient's prior antiviral therapy, presence of cirrhosis, availability, cost, and drug-drug interactions with concomitant medications[43]. In most of these regimens, the treatment duration extended between 12-24 wk[29-32,43-45].

One of the first interferon-free regimens used in treatment of genotype 4 HCV, which consisted of sofosbuvir plus ribavirin, showed that 12 wk of treatment was associated with overall lower SVR rates compared to 24 wk, and, hence, this regimen was approved for 24 wk[29].

In particular, several fixed dose combination regimens, such as ledipasvir-sofosbuvir[30,46,47], sofosbuvir-velpatasvir[31,48], and glecaprevir/pibrentasvir[49,50], have demonstrated efficacy with SVR that exceeds 90% after 8-12 wk of treatment of HCV 4. They are unique in that they achieve successful outcome with once daily dosing and without the need to administer ribavirin in the treatment of genotype 4 HCV. Only glecaprevir/pibrentasvir can be used for 8 wk in the subset of treatment-naive or peginterferon/ribavirin-experienced genotype 4 patients without cirrhosis[43,49,50].

Ledipasvir-sofosbuvir is available and widely used in the treatment of genotype 4 HCV patients in Egypt. In an open label phase 2 trial, Kohli *et al*[30] showed that ledipasvir-sofosbuvir treatment of patients with HCV genotype 4 for 12 wk resulted in 100% SVR 12 and was well tolerated with minimal mild adverse events. Crespo *et al*[47] reported on the effectiveness and safety of this once daily oral combination in the treatment of hepatitis C genotype 4 infections, showing an overall 95.4% SVR 12 response with a respective 100% SVR 12 response in patients without cirrhosis. In patients with cirrhosis, 12-wk treatment with ledipasvir-sofosbuvir without ribavirin had an equivalent successful outcome to this once daily combination with ribavirin for 12 wk or 24 wk[47]. However, many of the genotype 4 HCV patients in Egypt are elderly patients with some renal insufficiency or reflux disorder requiring proton pump inhibitors (PPIs), which might limit the activity and the use of the drug in this population[51,52].

Sofosbuvir-velpatasvir has also demonstrated high efficacy in the treatment of patients with genotype 4 HCV infections irrespective of treatment history (treatment naïve or experienced) or the presence of cirrhosis[31,48]. In a study by Feld *et al*[31], 100% of the 116 patients with genotype 4 infection achieved SVR 12 following a 12-wk treatment of sofosbuvir-velpatasvir. Similarly, this regimen was highly effective in genotype 4 HCV infections and was well tolerated, with serious adverse events occurring in only 2% of those who received this regimen[48].

The fixed dose combination of glecaprevir-pibrentasvir given once daily in three pills was uniquely effective in the treatment of HCV genotype 4 infected

Table 2 Direct-acting antiviral regimens available to treat hepatitis C virus genotype 4

[1]Combination regimen[43,44]	Duration (wk)
Sofosbuvir-ledipasvir	12
Sofosbuvir-velpatasvir	12
Glecaprevir-pibrentasvir	8 (without cirrhosis) 12 (with cirrhosis)
Sofosbuvir-velpatasvir-voxilaprevir	12
Ombitasvir-paritaprevir-ritonavir ± ribavirin	12
Elbasvir-grazoprevir ± ribavirin	12-16
Elbasvir-grazoprevir	12 (treatment naïve)
Elbasvir-grazoprevir + ribavirin	16 (treatment experienced)
Sofosbuvir + ribavirin	24
Sofosbuvir + daclatasvir ± ribavirin	12
Sofosbuvir + simeprivir ± ribavirin	12-24

[1]The information from the table is from the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) hepatitis C virus guidelines.

patients when given for only 8 wk in patients without cirrhosis, resulting in an SVR 12 response rate of 93% in this patient population[49,50]. Furthermore, in 16 patients with cirrhosis who received 12 wk of this fixed combination, 100% SVR 12 was achieved. It is important to note that the HCV genotype 4 patients without cirrhosis who did not achieve SVR 12 with the 8 wk regimen were either patients who had incomplete data or discontinued therapy prematurely[50]. The advantage of this fixed dose combination regimen of glecaprevir-pibrentasvir is that it can be given in patients in any degree of renal impairment, unlike ledipasvir/sofosbuvir, sofosbuvir/velpatasvir, and sofosbuvir/velpatasvir/voxilaprevir[49,50].

HEPATITIS B IN MAURITANIA

Hepatitis B virus (HBV) is a major cause for liver disease worldwide, particularly in Mauritania[53]. There are approximately two billion people who have been infected with HBV, of which 400 million people are infected chronically and of whom 65 million reside in Africa. HBV infection leads to 0.5-1.2 million deaths annually due to liver cirrhosis and HCC. Sub-Saharan Africa has a high HBV prevalence rate of 16.16%[54,55]. Mauritania remains a hyper-endemic area for HBV. Studies in Mauritania on the epidemiology of HBV[56] have shown a high prevalence of HBsAg positivity in blood donors, mostly between the age of 21-30, despite HBV vaccination of children and newborns since 2000[57]. Moreover, high HBV DNA levels were shown to be associated significantly and independently with the incidence of cirrhosis and HCC.

Co-infection with hepatitis delta virus (HDV)[58] is also endemic in Mauritania[59]. There is a high prevalence of HBV (20%) and HDV (30%). HDV infection tends to occur early, affecting mainly children and young adults, leading to chronic hepatitis. The natural course of chronic HDV is rapid progression to cirrhosis and liver related complications, including HCC[60,61].

The modes of transmission in Mauritania for HBV and HDV are social close contact, sexual contact, sharing needles, and other forms of blood exchange as well as maternal-fetal transmission during delivery (Table 3). Interfamilial transmission is common and may be facilitated by poor hygiene. Thus, socially and economically disadvantaged populations, as found in Mauritania, are more affected. Children infected perinatally with HBV are asymptomatic, and 25% die in adulthood from cirrhosis complications or HCC[62].

HCC remains a major cause of mortality due to limited therapeutic options. The aim should focus on prevention. Effective vaccine for HBV is available and was recommended by WHO to be included in the national immunization program. However, not all countries have adopted and implemented this recommendation effectively, including Mauritania, which ranks among the countries with the highest mortality of HBV associated HCC[62].

Hepatitis B vaccination is the best strategy to prevent this infection and decrease its incidence in the young population[63]. Vaccination is highly recommended in high prevalence areas like Mauritania, and the vaccines should be given to all infants at the time of birth, children, and adolescents as well as adults with risk factors that are included in Table 3. In addition, booster doses of hepatitis B vaccine are recommended in hemodialysis and in the immunocompromised person. The high cost of the vaccine and the lack of the infrastructure to deliver the vaccines do impact the implementation of a universal vaccination program in Mauritania. Prevalence rates of hepatitis B and children vaccination rates in various African region have been published[64]. However, given the limited data available from the unaccounted home births, the reported vaccination rates could be inflated and the prevalence of the disease understated. Therefore, public health action is urgently needed. A multifaceted approach to improve the socioeconomic conditions, increase the awareness of the risk of transmission, aggressive vaccination campaigns, and public health intervention are strongly needed to prevent viral transmission. Also, a regional

Table 3 Risk factors of transmission of hepatitis B in Mauritania and proposed interventions

Risk factors	Proposed interventions
Direct contact with infected blood and or handling blood or body fluids (job exposure) Sharing needles or other equipment (such as cotton, spoons, and water) to inject drugs Hemodialysis	Rigorous adherence to standard precautions in healthcare settings Completely avoid sharing needles or re-using disposable devices Education of healthcare providers and patients Hepatitis B vaccinations and assessment of response to vaccine (hepatitis B surface antibody) Use of safety-engineered devices and needless infusion systems Use of sharp object disposal containers Strict infection control measures upon cleaning and reusing medical equipment Appropriate screening of blood donors Post-exposure prophylaxis Antiviral therapy
Intimate contact with a person with HBV	Hepatitis B vaccination Avoid sharing toothbrushes, razors, *etc.*
Multiple sex partners or having unprotected sex with someone who is infected with the virus	Hepatitis B vaccination Protected sexual intercourse
Mother-to-Child transmission	Screening pregnant women Antiviral therapy to pregnant women with high DNA levels Passive-active immunization of newborns of mothers with HBV Universal vaccination of newborns
Body piercings, tattoos or acupuncture	Avoid body piercing and tattoos Strict infection control and prevention policies
Ⅳ drug users	Avoid sharing syringes and needles

HBV: Hepatitis B virus.

specific clinical guideline for screening, targeting infection control measures, and using ultra-sonographic tools in the highest risk setting, such as healthcare, dentistry, and personal grooming service centers, is needed. In addition, enhanced access to health care services and providing public funds for treating HBV and HDV may help to optimize management of infected patients.

Patients with acute hepatitis B do not require antiviral medication, as most patients will recover spontaneously. Supportive care with hydration and regular follow up with their physician is recommended. However, patients with chronic hepatitis B are more likely to require antiviral medications. Its purpose is to stop any further damage to the liver by slowing the multiplication of the virus. Therapy should be given to the following patients: patients with chronic hepatitis B with HBV DNA > 20000 IU/mL and with liver alanine aminotransferase > 2 × upper limit of normal, patients with evidence of fibrosis or moderate to severe hepatitis on liver biopsy irrespective of hepatitis B DNA level, and patients with cirrhosis associated with chronic HBV irrespective of hepatitis B DNA level.

Nucleoside analogues are the preferred agents. They are very effective at viral suppression and have a high barrier to drug resistance. The current first line therapy are entecavir 0.5 mg/d × 48 wk orally, tenofovir 300 mg/d × 48 wk orally[65], and pegylated interferon in noncirrhotic HbeAg-positive patients is also an option[66]. However, most patients require lifelong therapy because relapse is common after discontinuation of therapy[67,68]. Unfortunately, most of Mauritanians with chronic HBV infection who are eligible for this therapy cannot afford it. Less than 1% of individuals eligible for antiviral therapy receive HBV treatment. In mothers with a high viral load, the antiviral treatment rate to reduce mother-to-child transmission is even lower[69]. In summary, HCV and HBV are major causes of morbidity and mortality worldwide. Both could be iatrogenically transmitted through infected blood products, infected needles and medical equipment. Egypt has one of the highest prevalence rates of hepatitis C in the world and North Africa which could be controlled by rigorous infection control measures with education of healthcare providers and patients as well as universal screening of blood donors. In addition providing the current efficacious antiviral therapy (DAA) for the Egyptians infected with HCV could also help eliminate this infection. On the other hand, Mauritania is a highly endemic area for hepatitis B and ranks among the North African countries with the highest mortality of HBV and associated HCC. Despite this impressive burden, hepatitis B in Mauritania is now considered a preventable disease with the available vaccine and the proposed implementation of several infection prevention measures. Universal surveillance programs, active infection prevention strategies particularly within the healthcare system and early detection with early treatment and precaution could be the common strategy that will halt the spread of both HCV and HBV diseases in these two countries.

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REVIEW

Micro-RNAs in hepatitis B virus-related chronic liver diseases and hepatocellular carcinoma

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that modulate gene expression at the post-transcriptional level by affecting both the stability and translation of complementary mRNAs. Several studies have shown that miRNAs are important regulators in the conflicting efforts between the virus (to manipulate the host for its successful propagation) and the host (to inhibit the virus), culminating in either the elimination of the virus or its persistence. An increasing number of studies report a role of miRNAs in hepatitis B virus (HBV) replication and pathogenesis. In fact, HBV is able to modulate different host miRNAs, particularly through the transcriptional transactivator HBx protein and, conversely, different cellular miRNAs can regulate HBV gene expression and replication by a direct binding to HBV transcripts or indirectly targeting host factors. The present review will discuss the role of miRNAs in the pathogenesis of HBV-related diseases and their role as a biomarker in the management of patients with HBV-related disease and as therapeutic targets.

Key words: Hepatitis B virus infection; MicroRNAs; Hepatitis B virus pathogenesis; Molecular mechanisms

Core tip: This review article will focus on the emerging puzzle of hepatitis B virus (HBV)-hepatocyte interaction *via* miRNAs, indirectly or directly modulating HBV

replication and pathogenesis, and thus on the role of microRNAs in the natural history of HBV infection. We evaluated the literature on their possible future role as a biomarker in the management of patients with HBV-related disease and as therapeutic targets.

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INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs that modulate gene expression at the post-transcriptional level by affecting both the stability and translation of complementary mRNAs[1]. MiRNAs play crucial roles in a variety of physiological processes, such as cell development and differentiation[2,3]. miRNA mutations, dysregulation of their expression or dysfunction of miRNA biogenesis lead to an interference with biological pathways involved in the development and evolution of human diseases, including cancer, cardiovascular diseases and infectious diseases[4-8].

About the host-virus interplay, various studies have shown that miRNAs are important regulators in the conflicting efforts between the virus (to manipulate the host for its successful propagation) and the host (to inhibit the virus), culminating in either the elimination of the virus or its persistence. In fact, different viruses encode miRNAs that modulate not only the viral mRNA expression to regulate its own lifecycle, but also the host mRNA expression to establish a cellular environment resulting favorable to their replication; on the other hand, host cells encode miRNAs counteracting viral replication[8-10]. An increasing number of studies report a role of miRNAs in hepatitis B virus (HBV) replication and pathogenesis. HBV is a non-cytopathic virus belonging to the Hepadnaviridae family. It has a 3.2 kb partially double-stranded DNA, showing 4 known open reading frames (ORFs): The S region, which contains three in-frame initiator codons, codes for the small, medium and large surface antigen (HBsAg) proteins; the C region, with two initiator codons for the core and "e" antigen (HBcAg, HBeAg); the P frame, coding for an RNA-dependent DNA polymerase; and the X region, coding for a protein regulating the transcription of both viral and cellular genes[11].

The present review will discuss the emerging puzzle of HBV-hepatocyte interaction *via* miRNAs, indirectly or directly modulating HBV replication and pathogenesis, and thus will focus on the role of microRNAs in the natural history of HBV infection and on their possible future role as biomarkers in the management of patients with an HBV-related disease and as therapeutic targets.

HBV INFECTION

Despite the universal vaccination campaigns against HBV infection undertaken in many countries over the last two decades, this infection remains a global health problem. In 2015, the WHO estimated that nearly 3.5% of the world population live with chronic HBV infection[12], with about 800000 deaths per year (460000 for complications of liver cirrhosis and 340000 for hepatocellular carcinoma-HCC)[13]. For non-immune/non-infected subjects any parenteral or mucosal exposure to blood, blood products or blood-contaminated material should be considered a risk for acquiring HBV infection[14]. In addition, being present in semen and cervical secretions at infectious concentrations, HBV is also transmitted by sexual and vertical routes[15]. The age at the time of infection strongly modulates the progression to chronicity, which occurs in around 90% of subjects infected at birth, a rate progressively decreasing with the increase in age at infection, up to 2%-5% in the adult population[16].

The geographical distribution of HBV chronic carriers is highly variable, ranging from 0.7%-1% in developed western countries to 8% or more in some countries in sub Saharan Africa and South-East Asia, depending on environmental factors, earning potential, educational levels and lifestyles. In countries with an intermediate-high level of endemicity, HBV infection is most frequently acquired at birth from an HBeAg-positive mother or through horizontal transmission in early childhood by household contacts (most frequently between siblings); in these cases the rate of progression to chronicity is high, which keeps the prevalence of infection in these geographical areas intermediate or high. Intravenous drug addiction is the major risk factors for acquiring HBV infection in countries with a low HBV endemicity like Western Europe[15] and North America[17], whereas promiscuous unprotected sexual activity is a main risk factor worldwide.

The clinical presentation of chronic HBV infection is variable, ranging from asymptomatic carriage of the virus to liver cirrhosis with or without HCC[18]. Patients with chronic hepatitis develop liver cirrhosis with an incidence of 1-5 per 100 persons/year, with a 5-year cumulative probability of progression ranging from 8% to 20%, depending on the degree of disease activity, HBeAg/anti-HBe status, the HBV load and co-morbidities[19]. The incidence of HCC in patients with HBV-related liver cirrhosis is estimated around 3.7 persons/year[12]. HCC may develop, but with a lower frequency also in patients with chronic HBV infection without cirrhosis, depending on demographic (male sex, older age), viral (higher levels of HBV replication; co-

Table 1 miRNAs involved in hepatitis B virus infection

miRNA	Validated target	Effect on HBV	Ref.
miRNA targeting HBV transcripts			
miR-15a/miR-16-1	HBx	↓	[28,33]
miR-20a/miR-92a-1	Polymerase/HBx	↓	[29]
miR-122	Polymerase/HBc	↓	[27]
miR-125a	HBsAg	↓	[23-26,32]
miR-199a-3p	HBsAg	↓	[22]
miR-205	HBx	↓	[31]
miR-210	HBsAg pre-S1 region	↓	[22]
miR-1231	HBc	↓	[30]
miRNAs targeting HBV regulators			
miR-1	HDAC4	↑	[51-53]
miR-15b	HNF1	↑	[50]
miR-34a	CCL22	↓	[60]
miR-122	Cyclin G1	↑	[43-45]
	HO-1		[45,46]
miR-130a	PGC1 PPAR	↓	[41]
miR-141	PPAR	↓	[24,40]
miR-146a	STAT1	↑	[58]
miR-152	DNMT-1	↑	[54-57]
miR-155	C/EBP	↓	[35-38]
	SOCS1	↓	[39]
miR-370	NFIA	↑	[49]
miR-372/373	NFIB	↑	[48]
miR-501	HBXIP	↑	[42]
miR-548a	IFNλ-1	↑	[59]

MiRNAs are listed according to their increasing number name. ↓: Suppress HBV infection; ↑: Promote HBV infection; HBV: Hepatitis B virus.

infections) and environmental (alcohol abuse) factors[20].

MIRNAS ASSOCIATED WITH HBV INFECTION

So far, there is no experimental evidence confirming the synthesis of miRNAs by HBV, though a computational analysis suggested one HBV pre-miRNA candidate[21]; however, HBV is able to modulate different host miRNAs, particularly through the transcriptional transactivator HBx protein. Conversely, different cellular miRNAs can regulate HBV gene expression and replication by a direct binding to HBV transcripts or indirectly targeting host factors, which in turn modulate viral replication.

Cellular miRNAs directly targeting HBV transcripts

The first miRNAs found to bind viral transcripts and repress HBV gene expression and replication were miR-210, miR-199-3p and miR-125a-5p. They were identified by two different experimental approaches (Table 1). In one procedure, Zhang *et al*[22] systematically screened for cellular miRNAs affecting HBV replication by a loss-of-function approach: Antagomirs targeting 328 miRNAs were transfected into a HepG2.2.15 cell model supporting full HBV replication, and then HBV surface antigen (HBsAg) expression was measured. Among the six miRNAs whose antagomirs caused an increase in HBsAg expression, miR-199a-3p and miR-210 were predicted to bind the HBsAg coding region and the HBV pre-S1 region, respectively; the direct effect of miRNAs on viral transcripts were further validated by GFP reporter assay[22]. In a different approach, Potenza *et al*[23] first predicted the potential targets of human hepatic miRNAs in different HBV sequence subtypes. The most promising targets were then subjected to a validation test based on cultured hepatic cells and luciferase reporter genes, demonstrating that miR-125a-5p was able to bind viral sequences. In particular, miR-125a was shown to be able to interfere with the HBsAg expression, since the transfection of miR-125a mimic or inhibitor into PLC/PRF/5 cell line that secretes HBsAg induced a marked decrease or enhancement in the amount of secreted HBsAg, respectively[23]. Two independent studies then confirmed the ability of miR-125a to inhibit HBsAg translation: in a screening of HBV replication-related miRNAs, a pri-miR-125a expression vector could repress HBsAg synthesis in HepG2 cells[24]; again, miR-125a mimic and inhibitor transfection in HepG2.2.15 cells resulted in an increase or decrease in HBV replication, respectively. The expression analysis of a panel of 814 miRNAs revealed that iron or TGF-α treatments, which increased or decreased HBV replication, respectively, had opposite effects on the expression of miR-125a, supporting its ability to interfere with HBV replication[25,26].

Later, another study reported an inhibitory effect of a cellular miRNA on HBV replication: Chen *et al*[27] focused on the liver-specific microRNA, miR-122, first demonstrating its inhibitory effect on HBV gene expression and replication in cultured cells and then

validating its target sequence located at the coding region of the mRNA for the viral polymerase and the 3' untranslated region of the mRNA for the core protein[27]. In a similar approach, two other studies found that miR-15a and miR-16-1 (differing by only one base outside the seed region) target HBx transcript and miR-20a/miR-92a-1 (belonging to miR-17-92 polycistron) and may inhibit HBV replication by targeting the viral transcripts[28,29].

More recently, miR-1231 has also been shown to suppress HBV replication by targeting the HBV core (HBc) protein. In HBV-transfected HepG2 cells, overexpression of hsa-miR-1231 resulted in the suppression of HBV replication by targeting the HBV core protein[30]. Also miR-205 was found to target a viral transcript, in particular HBx mRNA[31]. miR-125a, miR-205 and miR-15/miR-16-1 expression were found to be modulated by HBx protein, resulting in an upregulation for miR-125a and downregulation for the others[31-33]. These regulatory feedback loops may have an impact on the development of liver disease progressing to HCC, given the crucial role of HBx in hepatocarcinogenesis[34].

Cellular miRNAs targeting regulators of HBV infection

HBV transcripts are under the control of four promoters and two enhancers (enhancer Ⅰ and Ⅱ), interacting with cellular factors that regulate HBV gene expression. Thus, miRNA regulation of these factors results in the modulation of HBV transcription and replication. Some miRNAs suppress HBV replication by targeting positive regulators of HBV (Table 1). One example is miR-155, which suppresses HBV transcription and replication, given its ability to target CAAT enhancer-binding protein (C/EBP), which is a positive regulator of HBV transcription through its binding to HBV enhancer Ⅱ, core promoter and S promoter[35-38]. miR-155 suppresses HBV infection also by modulating the host immune system (see below)[39]. Also miR-141 is able to suppress HBV replication, since it represses at both the transcriptional and translational levels the peroxisome proliferator-activated receptor alpha (PPARα), a transactivator of HBV promoters, with a critical role in HBV replication[24,40]. Similarly, miR-130a reduced HBV replication by targeting two major metabolic regulators PGC1α and PPARγ, both of which can potently stimulate HBV replication[41].

Other miRNAs promote HBV replication by targeting negative regulators of HBV activity or by enhancing a positive regulator. miR-501 promotes HBV replication by targeting HBXIP, a negative modulator of HBV replication, because of its binding to the transactivation domain of HBx protein[42]. A positive effect on HBV replication has also been described for miR-122, in contrast with that reported above. In particular, miR-122 can promote HBV replication in two ways: It prevents cyclin G1 from interacting with p53, which has a suppressive effect on HBV replication, and it targets heme oxygenase-1 (HO-1), an anti-HBV enzyme[43,44]; miR-122 targets heme oxygenase-1 (HO-1), whose anti-HBV activity has been shown in HBV-transfected hepatoma cells and in persistently HBV replicating transgenic mice. HO-1 acts by decreasing stability of HBV core protein, thus blocking refill of nuclear HBV covalently closed circular (ccc)DNA[45,46]. According to these mechanisms, the liver-rich miR-122 may have a similar role in stimulating the replication and gene expression of the two hepatotropic viruses, HCV and HBV[47]. microRNA-372/373 promote the expression of HBV by targeting the nuclear factor I/B (NFIB), a transcription factor able to reduce viral HBsAg and HBeAg protein levels and viral core-associated DNA levels, due to its binding to enhancer Ⅰ and core promoter of HBV[48]. A similar mechanism has been described for miR-370, which suppresses HBV transcription and replication by targeting nuclear factor IA (NFIA)[49]. Also miR-15b is able to target a negative regulator of HBV Enhancer Ⅰ, *i.e.*, hepatocyte nuclear factor 1α (HNF1α) mRNA, thus resulting in the transactivation of HBV Enhancer Ⅰ, in turn causing the enhancement of HBV transcription and replication[50].

Another two miRNAs have a role in HBV transcription and replication by modulating epigenetic modifications such as histone modification and methylation. In particular, miR-1, by targeting histone deacetylase 4 (HDAC4) changes the expression of different genes, including an upregulation of farnesoid X receptor a, which enhances HBV transcription and replication by binding to the HBV core promoter[51-53]. miR-152 has been shown to target DNA methyltransferase (DNMT-1), eventually resulting in a reduced methylation of covalently closed circular DNA (cccDNA), with an impact on the replicative activity of HBV[54,55]. Consistently, miR-152 expression was also shown to be downregulated in the livers of HBx transgenic mice and inversely correlated with DNMT1 expression in HBV-related HCC patients[56,57].

Some miRNAs may also have an indirect effect on HBV infection because of their role in the modulation of the host immune system. From the point of view of HBV, they represent a strategy to suppress antiviral immune responses, thus facilitating viral replication. This is the case of miR-146a and miR-548a, which promote HBV infection by suppressing T cell function through targeting Stat1 and by binding the 3'UTR of IFN-λ1, respectively[58,59]. Conversely, miR-34a and miR-155 suppress HBV infection by inhibiting Treg cell recruitment *via* chemokine CCL22 and augmenting the IFN signaling pathway, respectively[39,60].

Overall, it is clear that the host-virus interaction in HBV infection is mediated not only by immune responses but also by miRNAs; during the co-evolution and adaptation between HBV and humans, complex miRNA-based networks have been established; this complexity may partly explain the opposing effects on HBV transcription and replication reported for some miRNAs (*e.g.*, miR-122).

MICRORNA DYSREGULATION IN HBV-RELATED HEPATOCELLULAR CARCINOMA

An aberrant expression of miRNAs has a causative effect on several pathological conditions, including cancer[5]. In this field, several studies indicate that miRNAs can act as either tumor suppressors by downregulating the expression of oncogenes, or tumor promoters (oncomirs) by limiting the expression of oncosuppressor proteins[61-63]. Cancer cell downregulation of Dicer, an enzyme playing a critical role in the biosynthesis of miRNAs, or mutations in its structure suppress miRNA biogenesis, leading to increased tumor progression[64-67]. This implies that the oncosuppressive effect of miRNAs generally overcomes their oncogenic potential. It is also known that cell differentiation is often accompanied by increased Dicer expression[68-70].

In 2011, Hou *et al*[71] performed an extensive study of the miRNomes of healthy human liver and HCC. In this paragraph, their work will be discussed in some detail since it provides a good example of the experimental procedures currently employed to study the miRNAs with oncogenic or oncosuppressive roles. The authors used a next-generation sequencing (NGS) technique to analyze human liver miRNome and found that 9 miRNAs accounted for about 90% of the liver miRNA content, with miR-122 being the most represented (52%). Other highly expressed miRNAs included miR-192 (16.9%), miR-199a/b-3p (4.9%), miR-101 (3.7%), let-7a (3.3%), miR-99a (2.2%), let-7c (2.1%), let-7b (1.7%), and let-7f (1.5%). MicroRNAs -199a-3p and -199b-3p have an identical nucleotide sequence but are transcribed from three genes, a-1, a-2, and b, with a2 being the most expressed in the liver. The authors then analyzed liver biopsies from patients with hepatocellular carcinoma by comparing tumor samples with adjacent non-cancer tissues. In HBV-related HCCs, a remarkable decrease in 199a-3p was observed. This result was then validated by qRT-PCR in a cohort of 40 HBV-related HCCs. This analysis showed that the miRNA was downregulated in 100% of the patients, with a mean decrease of 8.3-fold. Other miRNAs markedly downregulated were miR-99a and miR-125b, both decreased by about 7-fold in the majority of the patients. MicroRNA-122 and miR-125a were also downregulated (Table 2). On the other hand, miR-21 was upregulated by 4.8-fold in half of the tumor samples. In the same study, genomic analyses of DNA samples from liver biopsies indicated that miR-199a-3p downregulation was not due to gene deletion or promoter DNA methylation but to histone modification and subsequent repression of transcription. Biological assays were then performed showing that transfection of a synthetic miR-199a-3p mimic in cultured HCC cell lines repressed cell proliferation and induced apoptosis, thus suggesting a tumor suppressive role *in vitro*. Experiments *in vivo* were then performed by monitoring human HCC cell growth in nude mice. Intra-tumoral injection of cholesterol-conjugated miR-199a-3p or its over-expression with a recombinant adeno-associated virus system markedly decreased tumor growth, further supporting its tumor suppressive role. The mechanism of action of miR-199a-3p was then studied. A computational analysis with TargetScan was used to identify the human genes whose mRNA sequence may allow binding of miR-199a-3p, possibly leading to gene silencing. Most of them were associated with the mitogen-activated protein kinase (MAPK) pathway, thus providing a possible explanation for the anti-proliferative activity of the miRNA. Among them, PAK4 was downregulated by miR-199a-3p transfection in HCC cells, and this effect was found to be due to a direct interaction with the gene transcript using a luciferase-based reporter assay. Finally, measurement of the miR-199a-3p content in the liver biopsies from two other cohorts of 142 and 152 patients showed that a markedly decreased HCC level of the miRNA correlated with poor survival. It should be noted that a downregulation of miR-199a-3p in HCC had already been observed in 2006 by Murakami *et al*[72] employing much less powerful miRNA detection techniques based on microarrays and Northern blotting analyses. The same study had also shown a tumor downregulation of miR-125a. Since then, other studies have confirmed the tumor-suppressive role of miR-199a-3p in HCC and identified CD44, CD51, c-MET, mTOR, YAP1, and ZHX1 as other direct miRNA targets contributing to its anti-proliferative and pro-apoptotic effects[73-78] (Table 2). The ability of miR-199a-3p to downregulate several target proteins should not surprise given the pleiotropic effects of microRNAs that are able to interact with several mRNAs provided with the same or similar binding sites located in their 3'-UTR. Therefore, a single microRNA often silences several genes with related functions, thus showing a marked effect on the cell physiology.

Other studies conducted with similar experimental approaches, profiling microRNAs by qPCR-arrays on multi-well plates, have confirmed the HCC downregulation of miR-99a, -122, -125a, -125b, and the upregulation of miR-21, also identifying their molecular targets (Table 2). Other miRNAs consistently downregulated in HCC included the let-7 family of microRNAs and miR-29; other examples of upregulated miRNAs were miR-155 and -221 (Table 2). These data have raised substantial interest in microRNAs in the field of molecular and cellular oncology, leading to the publication of several interesting reviews[79-84].

As regards the reasons for deregulated miRNA expression in HCC, it should be noted that the viral HBx protein plays a primary role in HBV cancerogenesis[85]. It is a transcriptional trans-activator lacking a DNA-binding domain but able to interact with several transcription factors[86,87]. Therefore, it is not surprising that HBx can modify the hepatic miRNA expression[88]. It is noteworthy that HBx downregulates the expression of

Table 2 MicroRNAs playing oncogenic or oncosuppressive roles in hepatocellular carcinoma

MicroRNA	Expression in HCC	Cellular effects	Direct targets in HCC	Ref.
let-7 family	Downregulated	Proliferation (-) migration (-) apoptosis (+)	Bcl-xL, c-Myc, collagen type 1α2, RAS, STAT3	[89,132-136]
miR-21	Upregulated	Proliferation (+) migration (+) apoptosis (-)	IL-12, HBP1, PDCD4, PTEN, RECK, TIMP-3	[72,137-141]
miR-29	Downregulated	Proliferation (-) apoptosis (+)	Bcl-2, lncRNA MEG3, Mcl-1, SIRT1	[80,142,143]
miR-99a	Downregulated	Proliferation (-)	AGO2, IGF1R, mTOR	[144-147]
miR-122	Downregulated	Proliferation (-) migration (-)	ADAM17, c-Myc, CUTL1, CCNG1, WNT1	[145,148-153]
miR-125a	Downregulated	Proliferation (-) migration (-) angiogenesis (-)	c-RAF, LIN28B, MMP11, SIRT7, VEGFA, Zbtb7a	[72,123,145,154-159]
miR-125b	Downregulated	Proliferation (-) apoptosis (+)	Bcl-2, LIN28B, SIRT7	[72,145,157,160-162]
miR-155	Upregulated	Proliferation (+) migration (+)	ARID2, C/EBPbeta, PTEN, SOCS1, SOX6	[38,163-165]
miR-199a-3p	Downregulated	Proliferation (-) apoptosis (+)	CD44, CD51, c-MET, mTOR, PAK4, YAP1, ZHX1	[71-78,154]
miR-221	Upregulated	Proliferation (+) apoptosis (-)	BMF, Caspase-3, CDKN1B, CDKN1C, DDIT4	[166-169]

ADAM17: Disintegrin and metalloprotease 17; AGO2: Argonaute-2 protein; Bcl-2: B-cell lymphoma 2 protein; ARID2: AT-rich interactive domain 2; BMF: Bcl-2-modifying factor; C/EBPbeta: CCAT/enhancer binding protein beta; CCNG1: Cyclin-G1; CDKN: Cyclin-dependent kinase inhibitor; DDIT4: DNA-damage inducible transcript 4; HBP1: HMG-box transcription factor 1; IGF1R: Insulin-like growth factor 1 receptor; IL-12: Interleukin-12; Mcl-1: Induced myeloid leukemia cell differentiation protein 1; PAK4: Serine/threonine-protein kinase 4; PDCD4: Programmed cell death protein 4; PTEN: Phosphatase and tensin homolog; RECK: Reversion-inducing-cysteine-rich protein with kazal motifs; SIRT: Sirtuin; SOCS1: Suppressor of cytokine signaling 1; STAT3: Signal transducer and activator of transcription 3; TIMP3: Metalloproteinase inhibitor 3; VEGFA: Vascular endothelial growth factor A; YAP1: Yes-associated protein 1; Zbtb7a: Zinc finger and BTB domain-containing protein 7A; ZHX1: Zinc-fingers and homeoboxes-1.

oncosuppressive miRNAs let-7 and miR-122, whereas it upregulates oncomirs -21 and -221. MicroRNA-125a is induced by HBx[32,89] but is downregulated by its carboxyl-terminal truncated variant that is frequently found in HBV-related HCC[90]. These data indicate that the tumorigenic effect of HBx is partially mediated by microRNAs. Besides HBx, dysregulation of microRNAs in cancer cells may be determined by other genetic or epigenetic factors[91]. Chromosomal abnormalities, deletions, and mutations can downregulate cellular miRNA expression[92,93], and several miRNA genes associated with CpG islands are also transcriptionally repressed by promoter DNA methylation[94].

MICRORNAS AS BIOMARKERS OF HBV-RELATED LIVER DISEASES

Biomarkers of liver damage and treatment response in chronic hepatitis B

As mentioned above, chronic HBV infection is associated with a wide spectrum of clinical manifestations: An inactive carrier state, chronic hepatitis of different grade of activity and liver cirrhosis in different stages of compensation, with or without HCC. The mechanisms of virus/host interactions leading to different outcomes have been only partially clarified and a substantial contribution to their knowledge is expected from the studies on the expression profile of microRNAs and their role in liver fibrogenesis. To this regard, one of the most interesting microRNAs is miR-122, which accounts for 50%-70% of all miRNAs expressed in the human liver. Several investigations[90-97] have shown a higher miR-122 serum concentration in patients with chronic HBV infection than in normal subjects and a correlation between its serum levels and HBV load, HBsAg titers and liver biochemistry. However, the interpretation of these data as a consequence of an upregulation of microRNA requires the exclusion of the possibility that they could be a consequence of an increased release of miR-122 in the blood due to concomitant hepatic cytolysis. To this regard, Wang *et al*[44] showed that the miR-122 expression in the liver was significantly downregulated in 41 Chinese patients with HBV infection compared with 10 healthy controls, and that the miR-122 levels negatively correlated with the intrahepatic viral load and with the degree of necroinflammation, confirming *in vivo* the inhibitory activity of miR-122 on HBV replication.

Interesting information on the correlation between microRNAs and HBV-related liver damage comes from some investigations on miR-29. A study[98] performed on serum samples of 91 HBV-infected patients and 12 healthy controls demonstrated a downregulation of this miRNA in patients with more advanced liver fibrosis. In addition, the serum levels of three microRNAs (miR-29a, miR-143, miR-223) predicted the progression of liver fibrosis better than APRI or FIB-4 tests in 123 Chinese patients with chronic HBV infection[99].

In 2013, we identified the miR-125a-5p as an independent predictor of more severe liver lesions (necroinflammation and fibrosis) in a cohort of 27 treat-

ment-naïve patients with HBeAg-negative chronic hepatitis B[100], findings confirmed by the data of a study by Zheng *et al*[101] on 91 HBV-infected patients. More recently, a Chinese study performed on 211 patients with chronic hepatitis B demonstrated that serum concentrations of miR-125b, a microRNA classified in the same family, correlate with the histological activity and HBV load[102].

The clinical use of microRNAs in chronic HBV infection may go beyond the assessment of liver damage. For example, Brunetto *et al*[103] reported that the use of a serum six miRNAs signature (MiR-B-Index) correctly discriminated 61 HBV subjects in a naturally inactive stage and 84 in a stage of treatment-induced immune-control. More recently, pre-treatment serum levels of two microRNAs predicted the off-treatment biochemical and virological response after a 48-wk combination therapy with Peg-IFN and adefovir, the miR-301a-3p in 41 HBeAg-positive patients and the miR-145-5p in 45 HBeAg-negative patients[104].

Biomarkers and therapeutic targets in HBV-related HCC

HCC is the fifth most common cancer in men and the ninth in women, with respectively 554000 and 228000 new cases per year worldwide[105]. In addition, HCC is the second most common cause of death for cancer worldwide, responsible for nearly 750000 deaths per year, half of which in HBV-infected patients. Despite the great efforts of the scientific communities and Healthcare Authorities, the mortality rate of HCC has not significantly decreased in the last decade, mainly because the diagnosis is very late in most cases. In fact, the level of serum alpha-fetoprotein (α-FP) lacks sensitivity and is no longer indicated for screening[106,107] and the imaging diagnostic techniques require quality of equipment and considerable experience by the radiologists. In addition, the use of sorafenib, the only treatment shown to improve the overall survival of patients in the advanced stages[108] is limited by the high rates of adverse reactions and treatment failures[109].

In this context, many microRNAs have been found dysregulated in serum and liver of HCC patients and therefore considered as possible diagnostic biomarkers and therapeutic targets[84,110-113]. In 2011, Zhou *et al*[111] screened for 723 microRNAs the serum samples of 934 Chinese patients with HBV-related chronic hepatitis, cirrhosis or HCC and identified and validated a panel of 7 miRNAs providing a high diagnostic accuracy for HCC, regardless of cancer stage. Subsequently, the serum level of miR-21 was proposed as a novel biomarker of HCC[112]. The diagnostic accuracy of miR-21 serum level has been further investigated in several subsequent studies[113-115] and in a meta-analysis[116] including 677 patients of different etiologies, with 81.2% sensitivity and 84.8% specificity in the diagnosis of HCC.

In 2010, Li *et al*[117] identified a panel of 13 miRNAs differentially present in serum samples of 120 HBV-related HCC, 135 HBV-infected patients and 210 healthy controls. Using a panel with miR-25, miR-375, and let-7f, they obtained a 97.9% sensitivity and 99.1% specificity in HCC prediction. Furthermore, the miR-375 proved to be of high diagnostic accuracy in two prospective Chinese cohorts[118].

The tissue expression of several miRNAs in HCC tissue has been investigated to identify therapeutic targets. Gao *et al*[119] analyzed the expression profile of 7 miRNAs in 24 dysplastic nodules, 29 HCC tissues and 40 non-tumoral liver tissues surrounding HCC from HBV-infected patients and found a downregulation of miR-145 and miR-199b and an upregulation of miR-224. They also demonstrated that the restoration of miR-145 in both HepG2 and Hep3B HCC cells significantly inhibited cell proliferation and reduced cell migration and invasion. As mentioned above, miR-122 is downregulated in liver tissue of patients with chronic hepatitis B, and its concentration is inversely correlated with the degree of liver fibrosis. This downregulation was reported also in 19 HBV-related HCC tissues by Li *et al*[120] in 2013; they also demonstrated that the pituitary tumor-transforming gene 1 (PTTG1) binding factor (PBF), a validated molecular target of miR-122, enhances the proliferation and invasion of HCC cells, while its silencing induced a significant reduction in tumor growth in a murine HCC model. It has also been demonstrated that the deletion of mouse Mir122 resulted in hepatosteatosis, hepatitis, and the development of tumors resembling HCC[121], while its re-expression reduced disease manifestations and tumor incidence[122]. We recently reported a lower expression of miR-125a-5p in HCC tissues compared with non-tumor tissue in 55 patients with hepatocellular cancer of different etiologies[123]. In addition, we found a significant upregulation of three oncogenes in HCC tissue, MMP-11, c-Raf and Sirt-7, already validated as molecular targets of miR-125a-5p, an observation that provides an explanation for the tumor suppressor activity exerted by this microRNA.

FUTURE PERSPECTIVES: RNA-INTERFERENCE IN THE TREATMENT OF CHRONIC HEPATITIS B AND HEPATOCELLULAR CARCINOMA

Some literature data suggest that microRNA-interference might be useful in the treatment of HBV-related chronic hepatitis and HCC. The RNA-interference directed to inhibit HBV replication has been investigated in several animal models and, more recently, in a clinical study[124]. In a phase 2 clinical trial enrolling entecavir- naïve or -exposed HBsAg-positive patients with chronic hepatitis[125], ARC-520, a mixture of small-interfering RNAs (siRNAs) targeting all viral transcripts, induced HBsAg reduction up to 1.5 log10 in HBeAg-positive and up to 0.5 log10 in HBeAg-negative subjects with a single intravenous administration of up to 4 mg/kg. The reasons for the limited efficacy in HBeAg-

negative patients have been more recently investigated in a preclinical study on chimpanzees[126]. The authors demonstrated a lack of target sites for the siRNAs in the HBV DNA integrated in the host genome, which represents the dominant source of viral transcripts in HBeAg-negative patients. These findings highlight a novel issue that should be addressed by future research on HBV treatment. Other two RNAi-based therapies (TKM-HBV and ALN-HBV) are currently investigated in chimpanzees and mice with promising preliminary results[127].

There is some evidence of the efficacy of miRNAs mimics or inhibitors both in preclinical studies and in a recent phase I clinical trial regarding the treatment of hepatocellular cancer. In an HCC murine model, the systemic administration of miR-26a using an adeno-associated virus resulted in an inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and protection from disease progression[127-129]; a cholesterol-modified isoform of anti-miR-221 can reduce tumor cell proliferation and increase the tumor doubling time and the survival in mice with hepatocellular cancer. An miR-375 mimic delivered in gold nanoparticles has shown therapeutic efficacy without significant toxicity in primary and xenograft tumor mouse models[130]. Finally, MRX34, a liposomal miR-34a mimic, showed anti-tumor activity in a phase I clinical trial enrolling patients with refractory advanced primary liver cancers or other solid neoplasms[131], but this trial has been stopped because of serious adverse events.

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REVIEW

Treatment strategies for advanced hepatocellular carcinoma: Sorafenib *vs* hepatic arterial infusion chemotherapy

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Abstract

Sorafenib is used worldwide as a first-line standard

systemic agent for advanced hepatocellular carcinoma (HCC) on the basis of the results of two large-scale Phase Ⅲ trials. Conversely, hepatic arterial infusion chemotherapy (HAIC) is one of the most recommended treatments in Japan. Although there have been no randomized controlled trials comparing sorafenib with HAIC, several retrospective analyses have shown no significant differences in survival between the two therapies. Outcomes are favorable for HCC patients exhibiting macroscopic vascular invasion when treated with HAIC rather than sorafenib, whereas in HCC patients exhibiting extrahepatic spread or resistance to transcatheter arterial chemoembolization, good outcomes are achieved by treatment with sorafenib rather than HAIC. Additionally, sorafenib is generally used to treat patients with Child-Pugh A, while HAIC is indicated for those with either Child-Pugh A or B. Based on these findings, we reviewed treatment strategies for advanced HCC. We propose that sorafenib might be used as a first-line treatment for advanced HCC patients without macroscopic vascular invasion or Child-Pugh A, while HAIC is recommended for those with macroscopic vascular invasion or Child-Pugh A or B. Additional research is required to determine the best second-line treatment for HAIC non-responders with Child-Pugh B through future clinical trials.

Key words: Treatment strategy; Hepatic arterial infusion chemotherapy; Sorafenib; Hepatocellular carcinoma

Core tip: In Japan, sorafenib and hepatic arterial infusion chemotherapy (HAIC) are described as treatment options for hepatocellular carcinoma (HCC). Although no randomized controlled trials have compared these treatments, retrospective analyses have shown similar survival between them. Sorafenib is generally used for Child-Pugh A, while HAIC is indicated for Child-Pugh A or B. Compared to sorafenib, HAIC shows better responses in cases exhibiting macroscopic vascular invasion. After reviewing treatment strategies for advanced HCC, we recommended sorafenib as first-line treatment for cases without macroscopic vascular invasion or Child-Pugh A, and HAIC for those with macroscopic vascular invasion or Child-Pugh A or B.

Saeki I, Yamasaki T, Maeda M, Hisanaga T, Iwamoto T, Fujisawa K, Matsumoto T, Hidaka I, Marumoto Y, Ishikawa T, Yamamoto N, Suehiro Y, Takami T, Sakaida I. Treatment strategies for advanced hepatocellular carcinoma: Sorafenib *vs* hepatic arterial infusion chemotherapy. *World J Hepatol* 2018; 10(9): 571-584 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i9/571.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i9.571

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second most common cause of cancer related death worldwide[1]. According to the Global Burden of Disease 2015 study of 195 countries, the number of liver cancer cases increased by 75% between 1990 and 2015, and hepatitis B virus (HBV) was responsible for 33% of global liver cancer mortality compared to 30% from alcohol, 21% from hepatitis C virus (HCV), and 15% from other causes[2]. However, the incidence of both HBs antigen-negative and HCV antibody-negative HCC (non-B, non-C HCC) has recently increased in Japan[3,4]. As there are not yet any established surveillance programs for non-B, non-C HCC patients, it is difficult to diagnose such patients at an earlier disease stage. Therefore, the number of advanced HCC patients at the time of diagnosis may be increasing in Japan. Additionally, even if an earlier disease stage of HCC is detected, many patients progress to an advanced stage because of frequent recurrence of the disease. Therefore, it is now more important than ever to develop a treatment for advanced HCC. In this review, we review the treatment strategies for advanced HCC, particularly sorafenib and hepatic arterial infusion chemotherapy (HAIC).

GUIDELINES FOR ADVANCED HCC

The results of the global investigation of therapeutic decisions in HCC and of its treatment with sorafenib (GIDEON) study show differences in the management of HCC, including diagnosis, treatment, and monitoring, among several regions. In consequence, there have been regional differences in patient outcomes[5]. Although several guidelines for the clinical management of HCC have been established worldwide, there are some differences in the treatment algorithms among these guidelines. Table 1 shows the major recent guidelines from Asia, Europe and the United States[6-13]. The Barcelona clinic liver cancer (BCLC) staging system, which stratifies patients by tumor stage and underlying liver disease, is widely accepted in clinical practice[14]. Among the five HCC stages (BCLC 0, A, B, C and D), the advanced BCLC C stage includes symptomatic patients with performance status (PS) 1-2, vascular invasion, extrahepatic spread, or a combination thereof[14]. For patients with BCLC C and good liver function (Child-Pugh A), sorafenib is the preferred first-line treatment according to guidelines from Europe and the United States[11-13]. According to guidelines from Asia[7-9], systemic therapy (molecular-targeted drugs) or transcatheter arterial chemoembolization (TACE) is recommended as standard treatment for such patients. However, HAIC is not generally recommended as a standard of care in the above-mentioned guidelines.

Whereas sorafenib and HAIC are indicated for the patients with minor portal vein invasion (so-called Vp1, 2) or portal invasion at the first portal branch (so-called Vp3) in the Japan Society of Hepatology and Liver Cancer Study Group of Japan (JSH-LCSGJ) Consensus-based Treatment Algorithm for HCC revised in 2014, HAIC, but not sorafenib, is recommended for portal invasion at the main trunk of the portal vein (so-called Vp4)[6]. Fur-

Table 1 Guidelines for the clinical management of hepatocellular carcinoma

	Publishing year	Guidelines	Drafted by	Treatment algorithm for advanced HCC (BCLC C)	Ref.
Asia	2014	JSH-LCSGJ	Japan Society of Hepatology and Liver Cancer Study Group of Japan	HAIC (Vp1-4), Sorafenib (Vp1-3), TACE (Vp1, 2), Resection (Vp1, 2)	[6]
	2014	KLCSG-NCC	Korean Liver Cancer Study Group and National Cancer Center	TACE, Sorafenib	[7]
	2014	HKLC	Hong Kong Liver Cancer	Systemic therapy, Supportive care	[8]
	2017	APASL	Asian-Pacific Association for the Study of the Liver	Systemic therapy (sorafenib and regorafenib), TACE for patients with no extrahepatic metastasis	[9]
	2017	JSH	Japan Society of Hepatology	TACE, Resection, HAIC, Molecular targeted agents	[10]
Europe	2018	EASL	European Association for the Study of the Liver	Sorafenib (sorafenib, lenvatinib, regorafenib, and cabozantinib)	[11]
	2012	ESMO-ESDO	European Society for Medical Oncology and European Society of Digestive Oncology	Sorafenib	[12]
United States	2011	AASLD	American Association for the Study of Liver Disease	Sorafenib	[13]

HAIC: Hepatic arterial infusion chemotherapy; Vp1-4: Portal vein invasion according to JSH-LCSGJ; TACE: Transcatheter arterial chemoembolization; BCLC: Barcelona clinic liver cancer.

thermore, according to the most recent version (2017) of the Clinical Practice Guidelines for HCC proposed by JSH, TACE, resection, HAIC, and molecular-targeted agents are equally recommended for HCC patients with portal invasion. It has also been argued that the treatment should be selected after considering all of the patient's conditions as a whole[10].

Finally, the 2017 version of the National Comprehensive Cancer Network (NCCN) Guidelines supports HAIC for unresectable HCC; however, its use in the context of a clinical trial is preferred[15].

SORAFENIB FOR ADVANCED HCC

Current status of sorafenib

Sorafenib is an oral multi-targeted kinase inhibitor that suppresses tumor growth, and it was the first drug to demonstrate a survival benefit in patients with advanced HCC. In two large-scale Phase Ⅲ trials, although the response rate of sorafenib was only 2%-3.3% according to the Response Evaluation Criteria in Solid Tumors (RECIST), sorafenib treatment significantly improved overall survival (OS) [sorafenib *vs* placebo median survival time (MST): 10.7 mo *vs* 7.9 mo, hazard ratio (HR): 0.69, $P < 0.001$ in the SHARP trial; and MST: 6.5 mo *vs* 4.2 mo, HR: 0.68, $P = 0.014$ in the Asia-Pacific trial] and the time-to-progression (TTP) (sorafenib *vs* placebo median TTP: 5.5 mo *vs* 2.8 mo, HR: 0.58, $P < 0.001$ in the SHARP trial; and TTP: 2.8 mo *vs* 1.4 mo, HR: 0.57, $P = 0.0005$ in the Asia-Pacific trial) in patients with advanced HCC[16,17]. Therefore, sorafenib is utilized as a standard first line agent for the treatment of advanced HCC worldwide[6-13]. Recently, Rimola *et al*[18] reported that 1% of patients treated with sorafenib (12/1119) exhibited complete response (CR), according to RECIST, and the MST for those patients was 85.8 mo.

For several years, antiangiogenic tyrosine-kinase inhibitors other than sorafenib have failed in Phase Ⅲ clinical trials[19,20]. However, recent studies have demonstrated the efficacy of two oral multi-kinase inhibitors, the second-line agent regorafenib, which is used for sorafenib-resistant HCC, and the first-line agent lenvatinib, which has been shown to be non-inferior to sorafenib for OS[21,22].

Regorafenib has been reported as a second-line agent following sorafenib because of improvement in OS (regorafenib *vs* placebo MST: 10.6 mo *vs* 7.8 mo, HR: 0.63, $P < 0.0001$) (RESORCE trial)[21]. According to the results of this study, regorafenib was approved in the United States and Japan in 2017.

Lenvatinib is an oral multi-target inhibitor of vascular endothelial growth factor (VEGF) receptors 1-3, fibroblast growth factor receptors 1-4, platelet-derived growth factor receptor alpha, KIT, and RET[23]. A comparative global Phase Ⅲ trial of lenvatinib in the first-line setting (REFLECT trial) demonstrated non-inferiority to sorafenib in advanced HCC patients (lenvatinib *vs* sorafenib MST: 13.6 mo *vs* 12.3 mo, HR: 0.92)[22]. In addition, the progression-free survival (PFS), TTP, and overall response rate (ORR) were significantly better in patients treated with lenvatinib than in those treated with sorafenib (lenvatinib *vs* sorafenib, median PFS: 7.4 mo *vs* 3.7 mo, HR: 0.66, $P < 0.0001$; median TTP: 8.9 mo *vs* 3.7 mo, HR 0.63, $P < 0.0001$; ORR: 24.1% *vs* 9.2%, $P < 0.0001$). Lenvatinib is approved for unresectable thyroid cancer and has been usable for HCC in Japan prior to it being approved in the rest of the world. However, HCC patients with 50% or higher liver occupation, bile duct invasion, or main portal invasion met the exclusion criteria of the REFLECT trial. Such HCC patients may be candidates for general usage of sorafenib.

Predictive factors for response and survival

Bruix *et al*[24] conducted analyses of two large trials

(827 patients, SHARP and Asia-Pacific trials) and reported prognostic factors. According to this report, vascular invasion, high alpha-fetoprotein (AFP), and high neutrophil-lymphocyte ratio (NLR) were prognostic factors for poorer OS, while lack of extrahepatic spread, HCV, and low NLR were predictive factors for greater sorafenib benefit[24]. Among serum and plasma factors, VEGF[25-27], angiopoietin-2 (Ang-2)[25,26], AFP[25,26,28-31], NLR[32,33], TIE-2 expressing monocytes (TEMs)[34], microRNA[35-37], and circulating tumor cells (CTCs)[38] have been identified as potential biomarkers (Table 2). The expression of phospho-ERK[39-41], phospho-c-Jun[42], and VEGFR-2[41], and amplification of FGF3/FGF4[43], have been identified as possible predictive biomarkers in tissues (Table 3). In studies of imaging biomarkers, it has been reported that decreased blood flow after sorafenib treatment[44] and low pretreatment standardized uptake values of ^{13}F-fluorodeoxyglucose (FDG) in positron emission tomography (PET)[45] are associated with prolonged OS. Although there have been several reports of a correlation between adverse effects (hypertension, skin toxicity, diarrhea, *etc.*) and sorafenib efficacy, it has been difficult to establish conclusions because of difference in the frequencies of these adverse effects among patients of different races. However, Howell *et al*[46] reported that patients with sorafenib-related toxicity such as diarrhea, hypertension, and hand-foot syndrome, had good prognoses in a large, multicenter prospective cohort study. Furthermore, the potential of other biomarkers has been explored[47]. Although several studies have investigated predictive biomarkers for response and survival associated with sorafenib, no such biomarkers have been established.

HAIC FOR ADVANCED HCC

Current status of HAIC

In HAIC, as it is theoretically possible to accumulate local concentrations of anti-cancer drugs in the liver and to reduce their systemic distribution, it is believed to have a stronger antitumor effect and lower incidence of adverse reactions compared with systemic chemotherapy. On the other hand, one disadvantage is the need to master the HAIC procedure, and several adverse effects are associated with HAIC including inflammation of blood vessels, arterial obstructions, peptic ulcers due to drug leakage, and infections or obstructions of reservoir catheters.

According to the 2017 version of the treatment algorithm for HCC produced by JSH[10], HAIC is recommended as a second-line treatment for patients with ⩾ 4 HCCs and an absence of portal invasion, while HAIC is considered a first-line treatment for those with portal invasion.

HAIC has become widely used in Asia, especially Japan, where the main HAIC regimens are low-dose cisplatin (CDDP) combined with 5-fluorouracil (5-FU) (low-dose FP)[48-51], interferon (IFN) in combination with 5-FU (FAIT)[50,52,53], and CDDP alone[51,54-56] (Table 4). In both low-dose FP and FAIT regimens, the key drug is 5-FU. In addition, CDDP or IFN exert their own effects to amplify the effect of 5-FU, and they are therefore considered biochemical modulators of 5-FU. Moreover, one benefit of the CDDP alone regimen is that a catheter is inserted each time, making the troublesome implantation of a reservoir catheter unnecessary. The regimens using low-dose FP or FAIT have response rates of approximately 30%-40%, while the CDDP alone regimen has rates of approximately 20%-30% (Table 4)[48-53,55-57]. Survival is significantly better in patients with radiological response [CR or partial response (PR)] (so-called responders) than in patients with radiological no-response (stable or progressive disease) (so-called non-responders).

The principal reasons for low clinical recognition of HAIC are the small sample size of almost all studies and the lack of large randomized trials. However, effective results have been demonstrated by previous studies. In a report comparing the FAIT regimen of HAIC with historical controls, HAIC was shown to significantly improve survival[53]. A Japanese nationwide survey supported the efficacy of the low-dose FP regimen of HAIC for treating advanced HCC[49]. After adjusting for known risk factors, survival benefits of this therapy were evident (HR: 0.48, 95%CI: 0.41-0.56, $P < 0.0001$). In a propensity score-matched analysis, the MST was longer in patients who received HAIC ($n = 341$, 14.0 mo) than in those who did not receive active treatment ($n = 341$, 5.2 mo) (HR: 0.60, 95%CI: 0.49-0.73, $P < 0.0001$). In cases of Child-Pugh A or B disease with more than three tumors (370 propensity score-matched patients), the MST was longer in patients treated with HAIC (13.9 mo) than in those with no therapy (3.7 mo) ($P < 0.0001$). In cases of Child-Pugh A or B disease with portal vein tumor thrombus (378 propensity score-matched patients), the MST was also longer in patients treated with HAIC (7.9 mo) than in those with no therapy (3.1 mo) ($P < 0.0001$).

Predictive factors for response and survival

As HAIC is selected for advanced HCC patients with poor prognoses, it is important to identify predictive factors for response and survival (Table 5)[48,49,53,58-61].

The predictive factors for poor response to HAIC include the presence of vascular invasion[58], the presence of extrahepatic metastasis[58], NLR ⩾ 2.87[58], a concentration of serum VEGF ⩾ 100 pg/mL[60], a negative HCV antibody test result[61], and a platelet count ⩾ $15 \times 10^4/\mu L$[61], and a negative des-gamma-carboxy prothrombin (DCP) response [defined as a reduction of < 20% or an increase from baseline after a half course of HAIC (2 wk)][48].

Survival benefits for HAIC have been reported in HAIC responders[53,60,61]. However, therapeutic effect is not an effective prognostic predictor. The poor prognostic predictors include not only tumor-associated factors,

Table 2 Serum and plasma biomarkers of sorafenib response and survival

Biomarkers	Ref.	Publishing year	Case number	Predictive factors for response	Predictive factors for survival	Others
VEGF	Llovet *et al*[25]	2012	299	No predictive value	Not prognostic value	
	Miyahara *et al*[26]	2013	120	No predictive value	Not prognostic value	
	Tsuchya *et al*[27]	2014	63	No predictive value	VEGF response (a > 5% decrease during 8 wk of treatment): Better OS	
Ang-2	Llovet *et al*[25]	2012	299	No predictive value	Low Ang-2: Better OS	
	Miyahara *et al*[26]	2013	120	High Ang2: PD	Low Ang-2: Better OS	
Changes of AFP	Personeni *et al*[28]	2012	85	AFP response (a > 20% decrease during 8 wk of treatment): Better ORR, DCR	AFP response: Better OS	
	Yau *et al*[29]	2011	94	AFP response (a > 20% decrease during 6 wk of treatment): Better DCR	AFP response: Better PFS	
	Kuzuya *et al*[30]	2015	47	-	High AFP ratio (a > 1.2 at 2 wk relative to baseline): Poor OS	High poor prognostic score (the absence of disapperance of arterial tumor enhancement on CE-CT, AFP ratio of > 1.2, and two or more increments in CP score after 2 wk of Treatment): Poor OS and DCR
	Nakazawa *et al*[31]	2013	59	AFP increase (more than 20% from baseline during 4 wk of treatment): PD	AFP increase: Better OS and PFS	
AFP	Llovet *et al*[25]	2012	299	-	AFP > 200 ng/mL: Poor OS	
	Miyahara *et al*[26]	2013	120	-	Not prognostic value	
	Kuzuya *et al*[30]	2015	47	-	Not prognostic value	
NLR	Zheng *et al*[32]	2013	65	-	High NLR (> 4): Poor OS and TTP	
	Howell *et al*[33]	2017	175	-	High NLR (> 2.52): Poor OS	
TEMs	Shoji *et al*[34]	2017	25	High ΔTEMs (changes in TEMs before and at 1 mo after therapy): PD	High ΔTEMs (changes in TEMs before and at 1 mo after therapy): Poor OS	
MicroRNA	Stiuso *et al*[35]	2015	39	Upregulation of miR-423-5p after treatment: SD or PR	-	
	Yoon *et al*[36]	2017	24	-	Low miR-10b-3p: Poor OS	
	Nishida *et al*[37]	2017	53	High miR-181a-5p: PR + SD	High miR-181a-5p: Better OS	
CTCs	Li *et al*[38]	2016	59	pERK+/pAkt- CTCs: Better DCR	pERK+/pAkt- CTCs: Better DCR	

Ang-2: Angiopoietin-2; CE-CT: Contrast-enhanced computed tomography; NLR: Neutrophil-to lymphocyte ratio; AFP: Alpha-fetoprotein; CTC: Circulating tumor cells; TEMs: TIE-2-expression monocytes; VEGF: Vascular endothelial growth factor; PD: Progressive disease; OS: Overall survival; DCR: Disease control rate; ORR: Overall response rate; PFS: Progression-free survival; CP: Child-Pugh; pERK: Phosphorylated extracellular signal-regulated kinase; PR: Partial response; SD: Stable disease; TTP: Time to progression.

such as more than three tumors[49], large tumors (> 3 cm)[49], the presence of vascular invasion[49,53], the presence of extrahepatic metastasis[49,58,61] and high AFP levels[49,58,61], but also those associated with the patient, including dysfunction of the liver reserve[48,49,53,58-61], ECOG PS 1-2[58,61], and a positive HBs antigen test result[49]. Additionally, poor prognostic predictors include negative responses of AFP or DCP[48], high levels of inflammation-related markers such as NLR and CRP[58], low transferrin levels (< 190 mg/dL)[59] and high VEGF levels (⩾ 100 pg/mL)[60].

A new assessment score: Assessment for continuous treatment with HAIC

It is important to identify the effective benefit of early HAIC treatment in HCC patients. Therefore, we developed a new therapeutic assessment score to guide decisions regarding HAIC treatment, the Assessment for Continuous Treatment with HAIC (ACTH)[48]. The ACTH score (range, 0-3) is calculated from simple three parameters: Child-Pugh score before HAIC (A = 0, B = 1), AFP response (yes = 0, no = 1), and DCP response (yes = 0, no = 1). The tumor markers' responses are

Table 3 Tissue biomarkers of sorafenib response and survival

Biomarkers	Ref.	Publishing year	Case number	Predictive factors for response	Predictive factors for survival
Expression of p-ERK	Abou-Alfa *et al*[39]	2012	33	-	High pERK: Longer TTP
	Chen *et al*[40]	2013	54	-	High pERK: Longer TTP
	Negri *et al*[41]	2015	77	-	High pERK: Shorter OS and PFS
Expression of p-c-Jun	Hagiwara *et al*[42]	2012	39	High p-c-jun: Poor response	High p-c-jun: Shorter TTP and OS
Expression of VEGFR-2	Negri *et al*[41]	2015	54	-	High VEGFR-2: Shorter OS and PFS
FGF3/FGF4 amplification	Arao *et al*[43]	2013	48	FGF3/FGF4 amplification: Responder	-

ERK: Extracellular signal-regulated kinase; FGF: Fibroblast growth factor; TTP: Time to progression; OS: Overall survival; pERK: Phosphorylated extracellular signal-regulated kinase; PFS: Progressive-free survival; VEGFR: Vascular endothelial growth factor receptor.

Table 4 Regimens of hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma

Ref.	Publishing year	Case number	Vascular invasion (%)	Regimens	Response rate (%)	Median survival time (mo)
Saeki *et al*[48]	2015	90	ND	Low-dose FP, including the combination of LV/IV or IV plus IFN	34.4	10.6
Nouso *et al*[49]	2013	476	44.1	CDDP + 5-FU	40.5	14.0 (341 patients)
Monden *et al*[50]	2012	34	90	IFNα, 5-FU	26.7	8.4
		35	90.3	Low-dose FP/CDDP	25.8	11.8
Yamashita *et al*[52]	2011	57	26.7	IFNα, CDDP, 5-FU	45.6	17.6
		57	50	IFNα, 5-FU	24.6	10.5
Nagano *et al*[57]	2011	102	100	IFNα, 5-FU	39.2	9
Obi *et al*[53]	2006	116	100	IFNα, 5-FU	52	6.9
Ikeda *et al*[54]	2013	25	100	CDDP powder (IA call)	28	7.6
Iwasa *et al*[55]	2011	84	31	CDDP powder (IA call)	3.6	7.1
Kim *et al*[51]	2011	41	83.3	CDDP	12.2	7.5
		97		CDDP, 5-FU	27.8	12
Yoshikawa *et al*[56]	2008	80	27.5	CDDP powder (IA call)	33.8	ND

ND: Not described; Low-dose FP: Low-dose 5-FU plus Cisplatin; LV: Leucovorin; IV: Isovorin; IFN: Interferon; CDDP: Cisplatin.

assessed as the difference between the baseline and 2 wk after HAIC induction (positive response: A reduction of ⩾ 20% from the baseline). ACTH score could stratify patients' survival (score ⩽ 1 *vs* score ⩾ 2, 15.1 mo *vs* 8.7 mo; $P = 0.003$)[48]. A validation study similarity showed that this score is useful for therapeutic assessment[62]. Therefore, the ACTH score makes it possible to provide an early prediction of the prognosis of advanced HCC patients receiving HAIC, and can improve treatment efficiency by switching to other treatments, such as sorafenib or an experimental treatment in a clinical trial, for patients with a score ⩾ 2 (Figure 1).

Modified HAIC and the combination approach

Nagamatsu *et al*[63] developed a modified procedure for administering a low-dose FP regimen: HAIC using 5-FU after lipiodol-transcatheter arterial infusion chemotherapy (Lip-TAI) with CDDP; a multicenter phase Ⅱ study showed that the MST and response rate were 27.0 mo and 75% for advanced HCC patients with portal vein thrombosis, respectively[64]. Although this regimen produced a favorable outcome, it has not become widespread owing to the high level of proficiency needed for the procedure.

A multicenter open-labeled randomized Phase Ⅱ trial was conducted to evaluate the effect of combining the CDDP regimen of HAIC with sorafenib for treating advanced HCC. The results showed that survival was significantly better for patients receiving sorafenib plus HAIC (MST, 10.6 mo) than those receiving sorafenib alone (MST, 8.7 mo) (HR: 0.60, $P = 0.031$)[65]; however, there was not a significant difference in survival between patients receiving sorafenib plus HAIC using low-dose FP and those receiving sorafenib alone[66]. Therefore, further investigation is required.

Radiotherapy (RT) has become recognized as an optional treatment for HCC in the APASL and NCCN guidelines[9,15], but it is not recommended in the AASLD and EASL guidelines[11,13]. For advanced HCC patients with intravascular tumor thrombus, a combination of HAIC with RT is a reasonable approach. Compared to HAIC alone, a beneficial effect of 3-D conformal radiotherapy (3D-CRT) for major portal vein tumor thrombosis combined with HAIC has been demonstrated, although these results came from retrospective cohort studies[67,68].

SORAFENIB *VS* HAIC

Sorafenib is recommended as a first-line treatment worldwide for advanced HCC patients (those with

Table 5 Predictive factors for response and survival of hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma

Ref.	Publishing year	Case number	Regimens	Poor predictive factors for response	Poor predictive factors for survival
Saeki *et al*[48]	2015	90	Low-dose FP with/without LV, IV, or IV plus IFN	DCP reduction or increase of < 20% from baseline to 2 wk after HAIC	Child-Pugh B, AFP reduction or increase of < 20% from baseline to 2 wk after HAIC, DCP reduction or increase < 20% from baseline to 2 wk after HAIC
Terashima *et al*[58]	2015	266	IFNα, 5-FU with/without CDDP	NLR ⩾ 2.87 (cut-off, median value), presence of vascular invasion, presence of extrahepatic metastasis	NLR ⩾ 2.87 (cut-off, median value), ECOG PS 1/2, Child-Pugh score 8-9, presence of extrahepatic metastasis, CRP ⩾ 0.8 mg/dL, AFP ⩾ 235.5 ng/mL
Zaitsu *et al*[59]	2014	44	Low-dose FP with/without IV, or IV plus IFN	ND	Child-Pugh B, serum transferrin < 190 mg/dL
Nouso *et al*[49]	2013	476	CDDP + 5-FU	ND	HBs antigen positive, Child-Pugh B, tumor number > 3, tumor size > 3 cm, presence of extrahepatic metastasis, Vp3/4, AFP > 400 ng/mL
Niizeki *et al*[60]	2012	71	Low-dose FP	VEGF ⩾ 100 pg/mL	Child-Pugh B, VEGF ⩾ 100 pg/mL, therapeutic effect SD + PD
Miyaki *et al*[61]	2012	249	Low-dose FP (106 patients); IFNα, 5-FU (143 patients)	HCV antibody negative, platelet count ⩾ 15 × 104/μL	ECOG PS 1-2, Child-Pugh score 8-9, presence of extrahepatic metastasis, AFP ⩾ 1000 ng/mL, abcence of additional therapy, theraputic effect SD + PD + DO
Obi *et al*[53]	2006	116	IFNα, 5-FU	Not detect	Vp4, Total bilirubin ⩾ 1.0 mg/dL, theraputic effect PR + SD + PD

Low-dose FP: Low-dose 5-FU plus Cisplatin; LV: Leucovorin; IV: Isovorin; IFN: Interferon; CDDP: Cisplatin; DCP: Des-gamma-carboxy prothrombin; NLR: Neutrophil-to-lymphocyte ratio; ND: Not described; VEGF: Vascular endothelial growth factor; PS: Performance status; SD: Stable disease; PD: Progressive disease; DO: Drop-out; CR: Complete response.

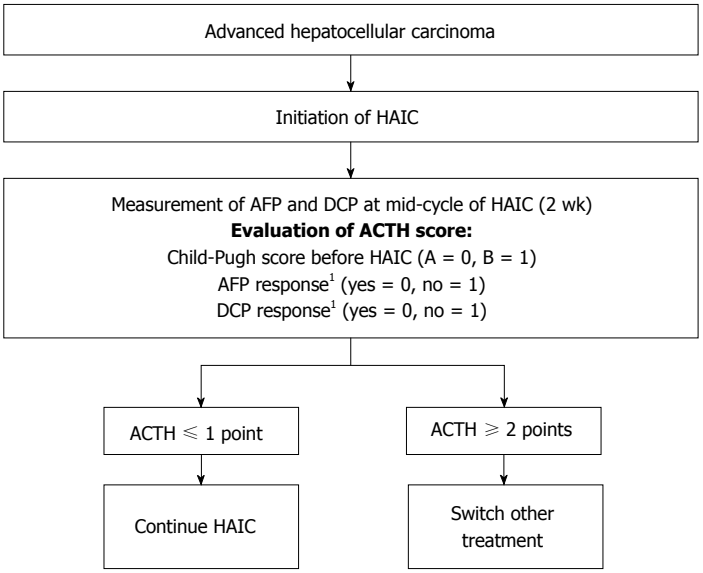

Figure 1 Treatment strategy for advanced hepatocellular carcinoma according to the hepatic arterial infusion chemotherapy score to assess continuous treatment. The score (range, 0-3) was calculated as follows: Child-Pugh score before hepatic arterial infusion chemotherapy (HAIC) (A = 0, B = 1), alpha-fetoprotein (AFP) response (yes = 0, no = 1), and des-gamma-carboxy prothrombin (DCP) response (yes = 0, no = 1). For patients with a score ⩽ 1, HAIC treatment would be continued, while for patients with a score ⩾ 2, a second-line therapy such as sorafenib and/or participation in a new clinical trial would be a better option. [1]The AFP and DCP responses were assessed 2 wk after HAIC induction; a positive response is defined as a reduction of ⩾ 20% from baseline. ACTH: Arterial infusion chemotherapy.

BCLC-C HCC)[11-13]. Because of the low response rate to sorafenib, we suggest that maintaining the stability of HCC by suppressing tumor growth can significantly improve survival. Sorafenib therapy also worsens survival in patients with Child-Pugh B, unlike those with Child-Pugh A[69]. Therefore, advanced HCC patients with Child-Pugh A are candidates for general usage of sorafenib.

Table 6 Clinical characteristics of three advanced hepatocellular carcinoma patients with complete response who have survived over 10 years

Age diagnosed as HCC	Sex	Etiology	Child-Pugh	Tumor stage[1]	Previous treatment	Maximum tumor size (mm)	Vascular invasion[1]	Regimen	Therapeutic effect	AFP (ng/mL)	DCP (mAU/mL)	HCC recurrence	Prognosis	Cause of death
67	Male	HCV	A (5)	IVA	None	110	Vp4, Vv0	Low-dose FP	CR	120700	260	62 mo	151 mo (dead)	Hepatic failure
66	Male	HCV	A (5)	III	None	50	Vp0, Vv0	Low-dose FP + IV	CR	6.4	2970	None	176 mo (dead)	Larynx cancer
44	Male	HBV	B (7)	III	None	150	Vp3, Vv3	Low-dose FP + IV + Peg IFN	CR	7145	233640	None	148 mo (alive[2])	-

[1]According to the Liver Cancer Study Group of Japan; [2]The follow-up period ended on January 31, 2018. HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; DCP: Des-γ-carboxyprothrombin; HCV: Hepatitis C virus; HBV: Hepatitis B virus; CR: Complete remission; Low-dose FP: Low-dose cisplatin combined with 5-FU; IV: Isovorin; Peg IFN: Pegylated interferon.

On the other hand, HAIC is not widely recommended as a standard of care for advanced HCC patients. As HAIC is thought to be one of the most effective treatment options for such patients, HAIC has become widely used in Asia, especially Japan. We propose that HAIC might be used as a treatment for achieving CR or PR. If patients with PR after HAIC receive additional therapies such as surgical resection, local ablation, or radiation, it is possible for those who show a disappearance of viable HCC to have a long survival time[64]. In addition, although liver reserve dysfunction is a poor prognostic factor[48,49,53,58-61], advanced HCC patients with Child-Pugh B are candidates for HAIC[6,10].

Currently, no criteria have been established for selecting advanced HCC patients to receive either sorafenib or HAIC. According to the results of two largescale randomized controlled trials (RCTs), sorafenib indeed improved the survival of patients with macroscopic vascular invasion[16,17]. However, these HCC patients with macroscopic vascular invasion have poorer prognoses than those without such invasion[16,17,70,71]. Moreover, there have been no RCTs comparing sorafenib with HAIC. In a retrospective cohort study, while there was no significant difference in survival between the sorafenib group and the HAIC group, survival was significantly better in the HAIC group than in the sorafenib group among patients with macroscopic vascular invasion (14 mo *vs* 7 mo, $P = 0.005$)[72]. A propensity score matched analysis also showed no significant differences in survival or disease progression between the two groups, while PFS was significantly longer in the HAIC group than in the sorafenib group, particularly for patients with portal vein invasion and/or without extrahepatic spread[73]. On the other hand, survival was favorable in patients with HCC refractory to TACE treated with sorafenib rather than HAIC[74]. Furthermore, it is important to preserve liver function during and after chemotherapy in advanced HCC patients. It has been reported that liver function after therapy was not significantly reduced in patients treated with HAIC compared with those treated with sorafenib[75], and the Child-Pugh score of HAIC responders with deteriorated liver function was significantly improved after HAIC[76]. According to our report[62], most HAIC responders showed no deterioration of liver function. It was interesting to note that the Child-Pugh class of some responders with deteriorated live function improved from B to A after HAIC, but this did not occur in non-responders. Therefore, we conclude that HAIC may be well tolerated by advanced HCC patients with deteriorated liver function.

As of 2017, only 10 years have passed since sorafenib was first shown to be efficacious against advanced HCC. As such, it is impossible to assess survival longer than 10 years. However, we can examine survival rates from shorter-duration studies. As previously mentioned, Rimola *et al*[18] reported a CR rate and MST for CR patients under sorafenib of 1% and 85.8 mo, respectively. Shiba *et al*[77] reported that the CR rate was below 0.6% (18/3047 patients) in a nationwide study from Japan. By contrast, the CR rate for HAIC was 4.0% (19/476 patients) in a nationwide survey in Japan[49]. According to our previous report[78], the CR rate under HAIC using a low-dose FP-based regimen was 5% (6/114 patients), and overall 1-, 3-, 5-, 7-, and 10-year cumulative survival rates were 43.9%, 10.0%, 5.6%, 2.8%, and 2.8%, respectively (MST, 10.2 mo). Three of six CR patients from our study survived over 10 years, though 2 patients have since died and only one is still alive (Table 6 and Figure 2). Further investigations are

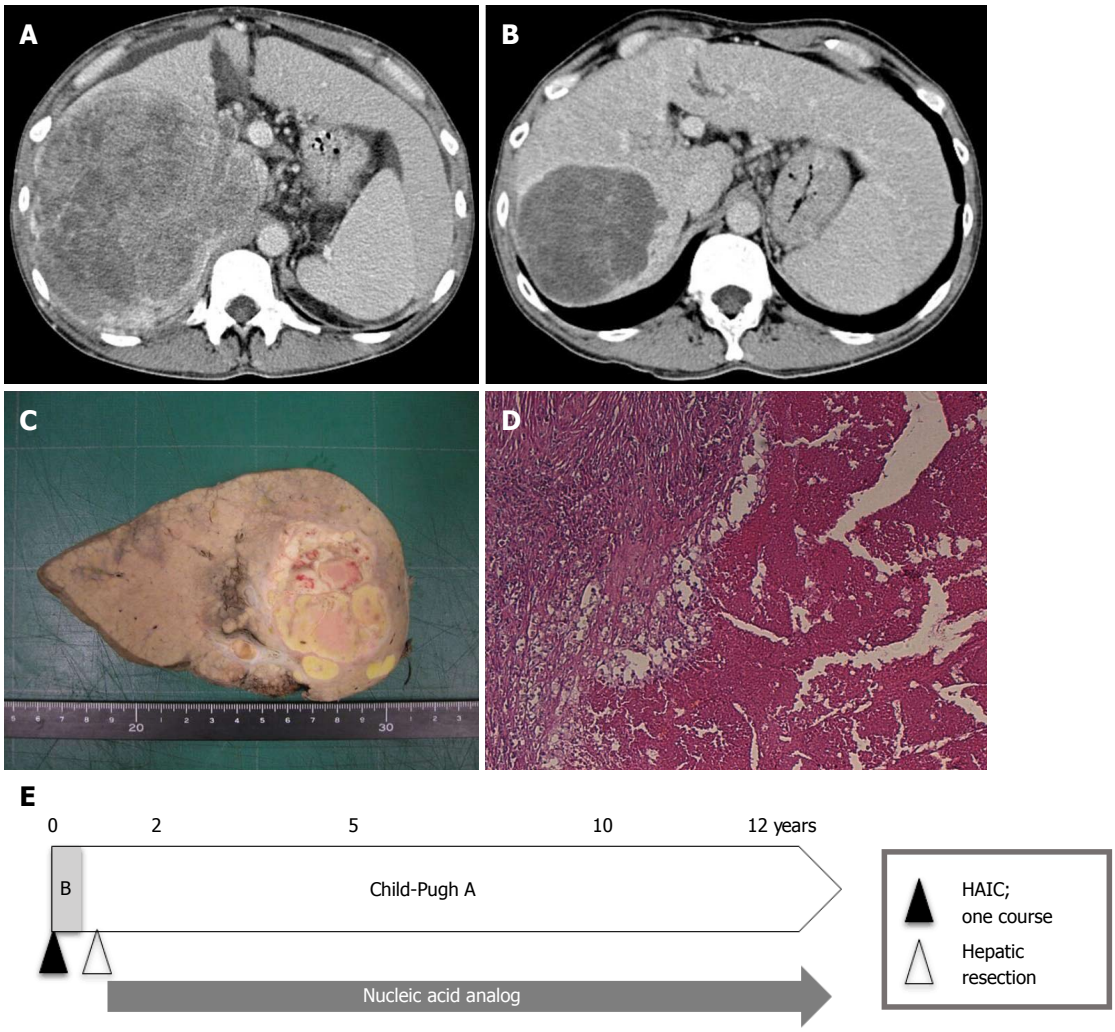

Figure 2 Patient with complete response treated with hepatic arterial infusion chemotherapy using low-dose cisplatin combined with a 5-fluorouracil (low-dose FP)-based regimen. A: This 44-year-old man had massive hepatocellular carcinoma (HCC) (16 cm in diameter) with tumor thrombosis in the right portal vein (Vp3) and the inferior vena cava (Vv3) on dynamic computed tomography; B: After one course of hepatic arterial infusion chemotherapy (HAIC), the liver tumor markedly decreased; however, as slight tumor vascularity remained, the patient was assessed as having partial response at that time; C, D: Three tumor markers [alpha-fetoprotein (AFP), des-γ-carboxyprothrombin (DCP), and AFP L3] decreased after HAIC (AFP from 7145 ng/mL to 12.7 ng/mL, DCP from 233460 mAU/mL to 51 mAU/mL, AFP L3 from 58.1% to 3.1%). The patient's Child-Pugh classification improved from B (8 points) to A (5 points). Thus, hepatic resection was performed, and histological findings showed no viable tumor cells (C, D). Finally, the patient was considered to have a complete response; E: The patient has been treated with nucleic acid analogs after the operation, and Child-Pugh A has been maintained. The patient is alive without HCC recurrence 148 mo after HAIC treatment.

required to compare long-term survival rates between sorafenib and HAIC.

Finally, we present a draft proposal of a treatment strategy for advanced HCC (Figure 3): (1) For advanced HCC patients without macroscopic vascular invasion and Child-Pugh A, the first-line treatment should be sorafenib, and second-line treatments should be either regorafenib[21] or HAIC; (2) For advanced HCC patients with macroscopic vascular invasion and Child-Pugh A, the first-line treatment should be HAIC, and the second-line treatments should be either sorafenib or experimental treatment in clinical trials; (3) For advanced HCC patients with Child-Pugh B, the first-line treatment should be HAIC, and the second-line treatment should be clinical trials. Miyaki *et al*[79] reported that additional therapy with sorafenib improved the prognosis of HAIC refractory patients compared with that of patients not treated with sorafenib therapy in a retrospective cohort study. Nonetheless, there have been no effective treatments for HAIC non-responders with deteriorated liver function (Child-Pugh B). We have shown the efficacy of an intra-arterial infusion therapy using the iron chelator deferoxamine for advanced HCC patients with deteriorated liver function[78,80], and clinical trials are now ongoing[81]. Because the best second-line treatment for HAIC non-responders with Child-Pugh B is to enroll in clinical trials, this remains an issue for future research.

CONCLUSION

We reviewed the current status and predictive biomarkers regarding the administration of sorafenib and HAIC for advanced HCC, and we have proposed a treatment strategy for patients with advanced HCC. The success

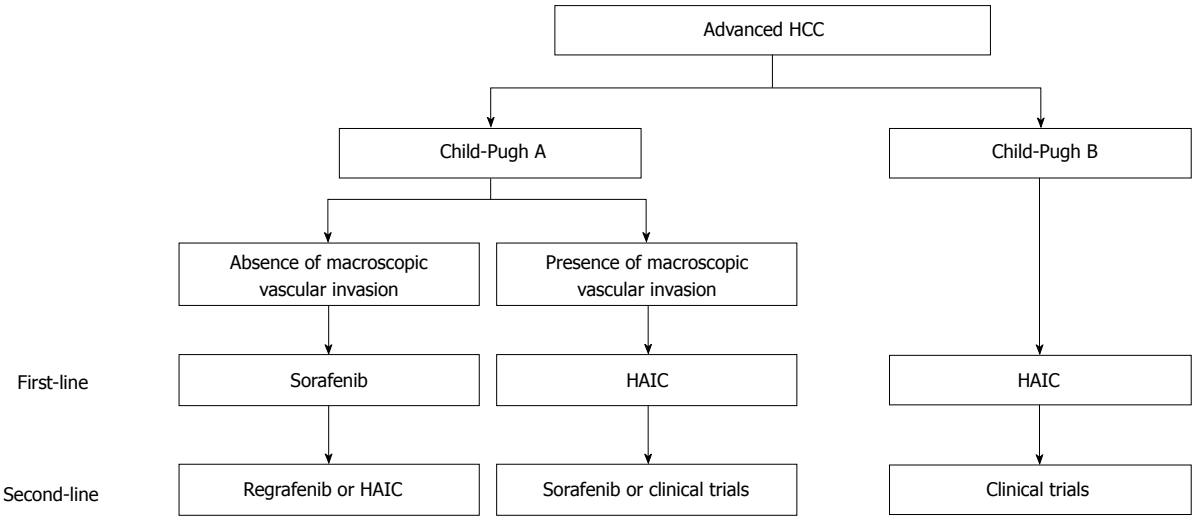

Figure 3 Draft proposal of a treatment strategy for advanced hepatocellular carcinoma. (1) For advanced hepatocellular carcinoma (HCC) patients without macroscopic vascular invasion and Child-Pugh A, the first-line treatment should be sorafenib, while second-line treatments should be either regorafenib or hepatic arterial infusion chemotherapy (HAIC); (2) For advanced HCC patients with macroscopic vascular invasion and Child-Pugh A, the first-line treatment should be HAIC, and the second-line treatments should be either sorafenib or experimental treatment in clinical trials; (3) For advanced HCC patients with Child-Pugh B, the first-line treatment should be HAIC, and the second-line treatment should be clinical trials.

of sorafenib, regorafenib, and lenvatinib in treating advanced HCC has shifted the treatment paradigm to molecular-targeted therapies. Furthermore, several immune-oncologic agents have been identified with potential for the treatment of advanced HCC[82,83]. Thus, the chemotherapeutic interventions for advanced HCC have been kept up-to-date through several advances. However, alternative therapies will be required because of the high cost and ineffectiveness of these molecular agents for patients with deteriorated liver function.

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MINIREVIEWS

Current evidence on the management of hepatitis B in pregnancy

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Abstract

Hepatitis B virus (HBV) infection is one of the main public health problems across the globe, since almost one third of the world population presents serological markers of contact with the virus. A profound impact on the epidemiology has been exerted by universal vaccination programmes in many countries, nevertheless the infection is still widespread also in its active form. In the areas of high endemicity (prevalence of hepatitis B surface antigen positivity > 7%), mother-to-child transmission represents the main modality of infection spread. That makes the correct management of HBV in pregnancy a matter of utmost importance. Furthermore, the infection in pregnancy needs to be carefully assessed and handled not only with respect to the risk of vertical transmission but also with respect to gravid women health. Each therapeutic or preventive choice deserves to be weighed upon attentively. On many aspects evidence is scarce or controversial. This review will highlight the latest insights into the paramount steps in managing HBV in pregnancy, with particular attention to recommendations from recent guidelines and data from up-do-date research syntheses.

Key words: Pregnancy; Hepatitis B immunoglobulin; Hepatitis B; Therapy; Immunoprophylaxis; Antiviral prophylaxis

Core tip: Hepatitis B is still a matter of concern worldwide. Particularly challenging is the correct management of infection during pregnancy. Two aspects have to be taken into account: The potential need to treat the mothers and, at once, the necessity to prevent

the vertical transmission of the virus to the infants. This review will discuss the most up-to-date evidence on therapeutic and preventive interventions in several scenarios characterizing the course of hepatitis B in pregnancy.

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INTRODUCTION

Despite the availability of effective preventive measures, particularly active immunization through vaccination[1], hepatitis B virus (HBV) infection is still today a major public health issue worldwide. According to the most recent and robust estimates, 3.61% of the global population is chronically infected, as expressed by the prevalence of hepatitis B surface antigen (HBsAg)-positivity[2]. Of course, there is a relevant heterogeneity both across and within continents and states as far as endemicity is concerned: Western Pacific Region and Africa are the world areas with the highest prevalence[2].

Even larger is the number of people having serologic markers of previous contact with HBV: About 2 billion subjects worldwide[3]. These individuals showing markers of resolved/occult HBV disease deserve special attention in case they undergo immunosuppressive treatment[4]. Nevertheless, and not surprisingly, the major disease burden is related to chronic infection. In 2013, HBV was responsible for nearly 686000 deaths globally, a figure that places the virus in the top 20 causes of mortality among humans[5]. Of note, different from other major communicable diseases, the burden of viral hepatitis, mainly driven by HBV and hepatitis C virus (HCV), has increased in terms of morbidity and mortality between 1990 and 2013[6].

Chronic HBV infection may evolve to cirrhosis, a condition characterized by profound alteration of liver architecture and function, in about 20% of subjects and may result in hepatocellular carcinoma (HCC), as a consequence of cirrhosis itself or of viral pro-oncogenic properties[7]. In turn, cirrhosis may be responsible for a vast array of complications: Infections[8], mainly spontaneous bacterial peritonitis[9] and bloodstream infections[10], ascites[11], hepatorenal syndrome[12], variceal bleeding[13].

Despite the remarkable efforts during the last decades aimed at implementing effective vaccination strategies worldwide[14], 50 million new cases of hepatitis B are still diagnosed each year, most due to mother-to-child transmission (MTCT)[15].

As a matter of fact, the transmission routes differ according to the entity of HBV endemicity: In areas of high prevalence (> 7%), vertical transmission prevails, whereas in low endemic regions (prevalence < 2%), sexual transmission is the major culprit[16]. The way and the timing of transmission are crucial factors influencing the probability of developing chronic HBV infection: Indeed, this likelihood is higher in subjects infected perinatally (up to 90%) when compared with rate of chronicity in adults after the acute phase (< 1%)[17]. Around 15%-40% of individuals suffering from chronic hepatitis develops cirrhosis[18].

All these figures underpin the necessity of correctly managing pregnant women with HBV infection, in order to reduce the burden of disease. Attention must be paid not only in developing countries, but also in regions such as Australia, United States, and Western Europe, where immigration from areas of high HBV endemicity may represent a challenge for physicians not accustomed to manage HBV infection in particular settings[15]. Of course, HBV infection in pregnancy is not only a problem for infants, but also for women's health (Figure 1). In this review, the current state of the art regarding the best management of HBV in pregnancy, both for the mother and child, will be discussed.

HBV INFECTION IN PREGNANCY: FROM THE PERSPECTIVE OF GRAVID WOMEN

How HBV impacts the health of pregnant subjects

The relationship between liver diseases and pregnancy is proteiform, and three categories of pathological conditions can be described: The ones representing underlying status, pre-existing to the moment of conception; the ones coincidental with maternity; and eventually, the ones specific of pregnancy (for example, pre-eclampsia)[19]. Viral hepatitis falls into the first two categories[19].

As far as acute hepatitis B is concerned, its occurrence during pregnancy is not associated with higher mortality, and the related clinical picture is not distinguishable from that in the general population[20]. This notion was further confirmed by a case-control study run in China, comparing 22 pregnant patients and 87 matched non-gravid women, all suffering from acute hepatitis B: No difference with regard to mortality and incidence of fulminant hepatitis was detected[21]. Of note, the HBsAg loss and seroconversion rates were lower in the first group, suggesting that pregnancy might act as a risk factor for chronicity[21].

An interesting and recent systematic review has assessed the impact of inactive HBV carriage on gravid women health, showing that this condition is not associated with complications in pregnancy, so this condition does not need any particular therapeutic measure[22]. When it comes to chronic (active) HBV infection already established before conception, the immunological modifications that occur in pregnancy may raise the level of HBV viremia, whereas alanine

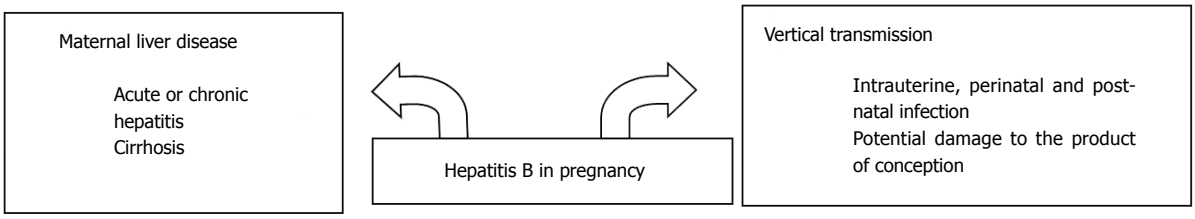

Figure 1 Hepatitis B in pregnancy: The two side of the problem.

aminotransferase (ALT) levels are normal or just above the upper limit of normal (ULN)[23].

A more relevant exacerbation of chronic hepatitis might happen after delivery in a notable percentage of women (up to 45%), as observed in a small retrospective cohort involving 38 pregnancies in 31 subjects, in which the flare was defined as a three times increase in ALT levels within 6 mo post-partum[24]. Authors suggested that this phenomenon, also found in the subgroup of women (8/13, 62%) who had undergone a course of lamivudine (LAM) during the third trimester, was attributable to the restoration after delivery of the immune system, whose functions were previously altered to prevent foetus rejection[24]. The topic has been further elucidated by a subsequent prospective study recruiting 126 women: Post-partum flares, defined as ALT levels twice the ULN or the baseline, were described in 27 (25%) individuals, usually asymptomatic and with spontaneous resolution[25]. At multivariate analysis, HBeAg positivity turned out to be the most relevant predictor of post-partum flares, although just barely not reaching the statistical significance (P = 0.051)[25]. Another study, a multicentre retrospective cohort involving 101 women and 113 pregnancies, did not identify clear risk factors for exacerbation of chronic hepatitis B after delivery[26].

With regard to maternal complications, chronic HBV infection does not seem a risk factor for many of them, as derived by research syntheses. A meta-analysis collecting data on 9088 placenta previa cases and 15571 placental abruption cases failed to demonstrate an association with HBV, implicated as a driver of an inflammation state able to induce dysfunction of trophoblasts: Odds ratio (OR) equal to 0.98 with 95%CI equal to 0.60-1.62 and OR = 1.42 with 95%CI: 0.93-2.15, respectively[27]. A further meta-analysis involving 439514 subjects showed that HBV was not associated with increased risk of gestational diabetes mellitus (adjusted OR = 1.11, 95%CI: 0.96-1.28), a link suggested by the potential role played by the virus in inducing insulin resistance[28]. Another research synthesis of observational studies did not detect a statistical significance between chronic HBV infection and preterm labour (OR = 1.12, 95%CI: 0.94-1.33)[29]. More recently, a meta-analysis including 11566 women has, quite surprisingly, highlighted a negative association between chronic HBV infection and preeclampsia (OR = 0.77, 95%CI: 0.65-0.90, P = 0.002): Actually, the protective effect, probably due to impaired immune response and/or increased immune-tolerance caused by the virus (preeclampsia is linked with exaggerated activation of immune system), was apparent only in an Asian population, as derived from the subgroup analysis[30].

Another aspect to be considered is how the most advanced stage of chronic liver disease, cirrhosis, impact the health of pregnant women, in the particular setting of HBV infection. Cirrhosis is, fortunately, an infrequent occurrence in pregnancy due to two factors: The development of end-stage liver disease requires time and more often takes place when women have gone beyond their reproductive age; moreover, hypothalamic-pituitary dysfunction related to cirrhosis[31] may ensue in anovulation and amenorrhea[32]. However, when present, cirrhosis is a relevant health issue for pregnant women. In a large population-based retrospective study in the United States on gravid women, comparing 339 cirrhotic cases with 6625 matched-controls, maternal mortality and complications of pregnancy (*e.g.*, uterovaginal haemorrhage, pre-eclampsia, peri-partum infections) were higher among individuals suffering from liver disease: For example, the maternal death rate was 1.8% *vs* 0% (P < 0.0001)[33]. Mortality among cirrhotic pregnant women was higher in cases of viral aetiology (HBV as well as HCV)[34]. The high burden of liver cirrhosis in pregnancy has been confirmed in a more recent prospective study, matching 176 cirrhotic gravid women with 2179 pregnant non-cirrhotic women and 1034 cirrhotic but not pregnant female subjects[34]. Maternal mortality rate was superior in the study group (7.8%) than in the first (0.2%) and second control group (2.5%; P = 0.001); variceal haemorrhage during vaginal delivery was the most frequent reason of maternal death[34]. Indeed, the rupture of oesophageal varices represents probably the most important complications among the ones directly related to cirrhosis in pregnant women, especially in the advanced phase of pregnancy or during labour[35]. An important predictor of liver-related complications during pregnancy is a model for end-stage liver disease (MELD) score $\geqslant$ 10[36].

Treatment criteria for acute and chronic hepatitis B in pregnancy

The paramount issue is which pregnant women with HBV infection should be treated[37].

In the case of acute hepatitis B, the main goal of the treatment should be the prevention of acute liver failure[38]. The quality of current evidence regarding pharmacological interventions in this setting is unfortunately very low[39]. Nevertheless, antiviral therapy is rarely necessary, since the large majority of adult patients (> 95%) have a full and spontaneous recovery[38], and, as mentioned above, the clinical course of this entity does not differ between pregnant and non-pregnant women. The problem is the management of cases suffering from severe acute HBV infection[40]. First, in case of serious hepatitis affecting gravid women, differential diagnosis is essential to rule out, for example, diseases unique to pregnancy, such as acute fatty liver of pregnancy (AFLP) as well as haemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome, representing two hepatic emergencies in the third trimester[40]. Once the viral aetiology is established, borrowing the recommendations applying to the general population, the treatment should rely on nucleos(t)ide analogue (NA) agents and the patients should be considered, as extrema ratio, for liver transplantation[38]. Therapy with NAs can prevent acute liver failure and the related mortality[38], but needs to be started early in the course of severe acute hepatitis, otherwise the protective effect does not display[41]. Due to the lack of high-quality data[39], there is high uncertainty regarding the best therapeutic options; recommendations for the general population support the use of tenofovir disoproxil fumarate (TDF), entecavir (ETV), and lamivudine (LAM)[38]. To date, there is no information about the use in severe acute hepatitis B of tenofovir alafenamide (TAF), the prodrug of tenofovir developed to ameliorate the safety and tolerability profile of TDF[42]. Among the above-mentioned NAs, LAM and TDF are preferable in pregnancy, in particular the latter one, owing to its high resistance barrier that is fundamental in case of prolongation of therapy for chronicity[43]. In extreme circumstances, liver transplantation might be a therapeutic option even during pregnancy if hepatic decompensation exceeds the point of no return: The difficulty of the task is doubled, as it involves two organisms (the mother and the foetus), but successful cases have been described, also related to fulminant hepatitis B[44].

At any rate, the management of severe acute hepatitis B in pregnancy needs more robust data to draw firm conclusions. Today, a case-by-case approach is needed. Chronic HBV infection is surely a more common scenario in pregnancy than fulminant hepatitis B. In the general population, according to the most authoritative guidelines endorsed by societies from all over the world, the main criteria for treatment are based on serum HBV DNA levels, serum ALT levels, and severity of liver disease[38,45,46]. Despite some discrepancies (for example, the locution "inactive carriers", discouraged by Asian and European guidelines but kept in the American ones), there is a substantial consensus upon the following items, concerning situations wherein antiviral therapy is recommended: Cirrhosis; absence of cirrhosis, but viraemia > 20000 International Units (IU)/mL and ALT levels > 2 × ULN; no cirrhosis, viraemia > 2000 IU/mL, ALT 1-2 × ULN but at least moderate to severe inflammation on liver biopsy[38,45,46]. In the general population, there are two therapeutic options: Interferon-alfa (IFNα) and NAs[16]. The rationale underpinning the choice of one strategy over another one is different. IFNα is administered to provide long-term immunological control through a finite duration treatment, attempting to achieve the so-called "functional cure" by HBsAg loss, but it is burdened by several side effects. NAs have a definitely better safety and tolerability profile, provide a very good virological control (persistent inhibition of HBV replication), but the duration of therapy is indefinite[47].

At any rate, in pregnant women, IFNα is contraindicated; therefore the therapeutic armamentarium is limited to NAs[48]. Among this category, currently TDF (alternatively TAF) and ETV are considered the first-line drugs when starting a new therapy for chronic HBV infection, combining an excellent safety profile with a genetic barrier of resistance higher than earlier available agents, such as LAM and telbivudine (LdT)[38,45]. Nevertheless, according to the 5-class labelling used by the Food and Drug Administration (FDA) until 2015 as for safety in pregnancy, ETV has been classified as a "category C" agent, differently from LdT and TDF, labelled as "category B" drugs: That means the absence of teratogenic effects in animal studies[49]. As a matter of fact, TDF is the drug of choice for pregnant females with chronic HBV infection requiring antiviral therapy (*i.e.*, advanced fibrosis and cirrhosis), also in light of the huge amount of data from the setting of gravid women under treatment against the human immunodeficiency virus (HIV), in which TDF is administered safely in combination with other antiretroviral agents throughout the pregnancy, since the first trimester[50].

A delicate issue is how to handle cases of women who become pregnant when already on anti-HBV treatment[51]. As a rule, appropriateness of therapy should be re-evaluated, striking a balance between benefits for the mother and the safety of the foetus[50]. Whatever the diseases severity, IFNα must be immediately stopped; therapy with a NA can be continued in case of advanced fibrosis or cirrhosis, switching to TDF if therapy was started before conception with another drug (for instance, ETV)[51]. In case of mild disease (*e.g.*, no advanced fibrosis, normal ALT levels, viraemia between 2000 UI/mL and 20000 IU/mL), discontinuation of therapy until delivery might be a viable option, as long as an adequate monitoring is carried out to re-start immediately treatment if necessary[51]. Eventually, another matter of concern is the management of HBV resistance cases: In pregnant women there is limited experience, nonetheless, in gravid subjects experiencing treatment failure (HBV DNA rebound) under LAM or LdT, switching to TDF appears a safe and effective option[52]. In Figure 2 the current knowledge regarding the treatment of HBV infection in pregnant women is summarized.

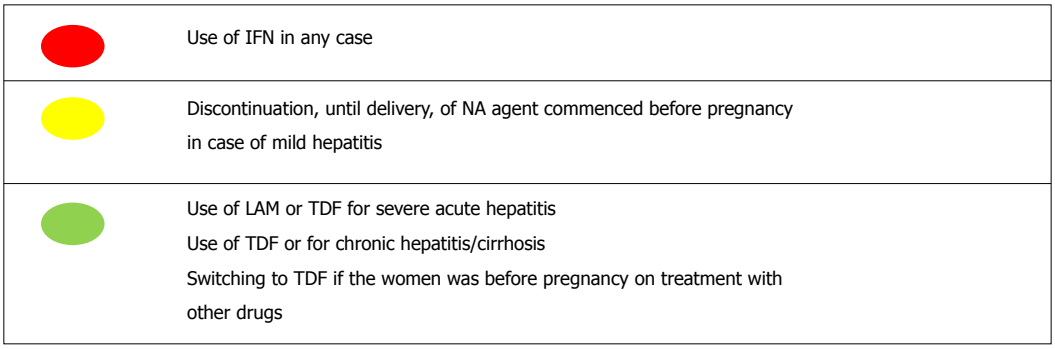

Figure 2 Treatment of acute and chronic hepatitis B in pregnancy. The column marked by the red circle refers to interventions that are not allowed. The column marked by the yellow circle refers to interventions that are backed up by low-quality or conflicting evidence. The column marked by the green circle refers to the best practice according to current evidence. IFN: Interferon; NA: Nucleos(t)ide analogue; LAM: Lamivudine; TDF: Tenofovir disoproxil fumarate.

HBV INFECTION IN PREGNANCY: FROM THE PERSPECTIVE OF FOETUSES AND NEWBORNS

Does HBV damage the product of conception?

Besides the "long-term" risks of HBV MTCT, such as chronic hepatitis, cirrhosis, and HCC[53], physicians caring for pregnant women with HBV infection have to take into account the "short-term" potential consequences for the product of conception: For example, small for gestational age (SGA), foetal distress, preterm birth (PTB), low birth weight (LBW), congenital anomalies, and neonatal jaundice[54].

PTB, defined as a birth occurring earlier than 37 completed weeks of gestation, represents one of the most feared complications, being worldwide the main cause of death in children under 5 years of age[55]. A very large population-based cohort study run in China, involving 489965 women who had singleton livebirths (of whom 20827, the 4.3%, with HBV infection diagnosed before pregnancy), showed that, adjusting for several covariates, in comparison with gravid subjects without HBV infection, HBsAg positive and HBeAg negative pregnant women had a 26% higher risk of PTB, whereas in women who were both HBsAg and HBeAg positive this percentage was equal to 20%[56]. Higher risks were observed also for early PTB (before 34 wk of gestation); unfortunately, data on viral load were not available. At any rate, the results of this recent study advocates proper medical intervention against HBV in pregnancy to improve neonatal outcomes[56].

Another large population-based study (sample size over 2000000 people), conducted in the United States and focused on neurological complications at birth, demonstrated that women with HBV, compared with gravid subjects without HBV, had a higher likelihood to generate infants who suffered from brachial plexus injury, even after adjusting for several confounders (OR = 2.04, 95%CI: 1.15-3.60)[57]. In a prospective cohort study in China, investigating 21004 pregnant women, of which were 513 HBV-positive and 20491 HBV-negative, no differences between the two groups were detected with regard to the rate of stillbirth, SGA and LBW, but the proportion of miscarriage was higher among gravid subjects with HBV (adjusted OR = 1.71, 95%CI: 1.23-2.38), but also in this study data on viraemia were lacking[58].

Criteria and options for antiviral prophylaxis against HBV MTCT

The viral load is just the key factor to determine the risk of HBV MTCT[59], that, in absence of any preventive measure, ranges from 10% to 40% when mothers are HBeAg-negative and from 70% to 90% in HBeAg-positive mothers[60], much higher rates in comparison with the other hepatotropic virus, HCV (0%-30%)[61].

The modalities of vertical transmission are: Intrauterine, peripartum and post-natal infection[59]. The transmission in utero is the most insidious route, since it is represents the most important cause of passive-active immunoprophylaxis failure[62]. There is no consensus to define correctly this occurrence (many criteria have been proposed, such as, among the many, persistent serum anti-HBc IgM positive after birth or Pre-S1 protein positivity in umbilical blood); supposed mechanisms are the passage of serum/body fluid through damaged placenta, the transmission of infected germ cells and the transfer of infected placenta or peripheral blood mononuclear cells[62]. HBeAg-positivity is a notable driver of intrauterine transmission: The antigen can cross the placenta barrier through leakages or through infected cells, and it is linked with higher levels of HBV replication[35]. Natal transmission during delivery represents the most impactful modality, being offspring exposed to blood or other maternal body fluids while passing the genital tract[62]. Finally, there is postnatal infection, which encompasses all the cases in which the transmission occurs after delivery, because of contacts with maternal fluids such breast milk or blood[62].

All guidelines, in line with the recommendations of the World Health Organization, support the administration, within 12 h of birth, of active (first dose of anti-HBV vaccine) and passive immunization through

hepatitis B immunoglobulin (HBIG) to the offspring born to HBsAg-positive mothers, a measure able to abate the rate of HBV MTC to > 90% to < 10%[38,45,46] and supported by high-quality evidence[63]. The paramount issue is how to avoid the failure of passive-active immunoprophylaxis, mainly ascribable to intrauterine transmission[64]. The most recent international guidelines recommend, for gravid women not already on NAs treatment, the use of antiviral prophylaxis from week 24-28 of gestation (third trimester) if viral load > 200000 IU/mL and/or serum HBsAg levels > 4 $\log_{10}$ IU/mL[38,45]. The viraemia threshold was first set based on a retrospective study involving 869 mother-newborns pairs who had received proper immunoprophylaxis: Failures occurred only in infants born to HBeAg-positive women with viral load > 200000 IU/mL (maternal viraemia levels, along with detectable HBV DNA in the cord blood, was the main risk factor at multivariate analysis)[65]. Subsequently, serum HBsAg levels emerged as a surrogate marker for viral load as well as predictive variable of HBV MTCT[66,67].

The benefit of antiviral prophylaxis as an additional measure to HBIG and vaccination was clear in a meta-analysis of 26 studies involving 3622 pregnant women, with a risk ratio equal to 0.3; the use of LdT, LAM, and TDF turned out to be safe[68]. There is no randomized controlled trial (RCT) directly comparing NAs as for HBV MTCT prevention: A Bayesian network meta-analysis (NMA) in 2016 demonstrated greater efficacy of LdT over LAM, but TDF was not taken into account[69]. A more recent NMA failed to demonstrate a superiority of TDF *vs* LAM[70]. At any rate, TDF is the favourite choice because of its superior barrier to resistance[38,45]. Indeed, TDF was the drug of choice for a non-randomized trial and two RTCs *vs* placebo published during the last 3 years[71-73]. The first two studies demonstrated that TDF (administered throughout the last trimester of pregnancy until 1 mo post-partum) decreased significantly the rate of HBV vertical transmission in comparison with placebo in women HBeAg-positive having high viral load[71,72]. On the contrary, the last and more recent RCT, not considered by guidelines due to publishing timing reasons, failed to detect a significant difference between the TDF group and the placebo arm (no events out of 147 newborns *vs* three infection out of 147 infants, respectively, $P = 0.29$)[73]. The study protocol contemplated the administration of TDF or placebo from 28 wk of gestation to 2 mo post-delivery in HBeAg-positive women, the large majority of them having viraemia > 20000 UI/mL, and its sample size was as large as the ones of the previous studies taken altogether[73]. Therefore, the results of this negative trial brings into question the usefulness of NAs, specifically TDF, as additional preventive measure during the last period of pregnancy and will need to be considered and put in the right perspective by the next research syntheses and guidelines. One of the possible explanation is the very early administration of HBV vaccination (the median time was just 1.2 h after birth)[74].

Pending new compelling evidence, the combination of HBIG and vaccine at birth is the mainstay of HBV MTCT prevention in newborns; antiviral prophylaxis in late pregnancy may be considered for HBeAg-positive gravid women with high viral load[75]. Furthermore, the absence of harm, weighing risks and benefits, might tip the scale in favour of NAs administration during the last trimester. The alarm raised by a case-control study (74 TDF-exposed and 69 TDF-unexposed infants) about the risk of lower neonatal bone mineral content (difference equal to 12% at 1 mo of birth) because of TDF during late pregnancy[76] has been refuted by subsequent work (conducted by the same study group) on 509 children: At 2 years of age TDF was not linked with lower length or head circumference[77]. This is in accordance with evidence from research synthesis confirming the safety, both for mothers and their offspring, of TDF use in pregnancy[78]. If administered, there is no consensus about when to stop prophylaxis. Some guidelines support its prolongation until 12 wk after delivery[38], others until 4 wk post-partum[45]: The protocol of main trials about TDF provided for the use of drug for 4[71,72] or 8[73] wk after delivery. The point is to strike a balance between the potential risk of interfering with breastfeeding and the benefit on possible post-partum hepatitis flares[35]. More conservative recommendations[45] rely on a prospective study recruiting 91 women (101 pregnancies), showing no advantages in terms of hepatitis flare rate for gravid subjects who extended antiviral prophylaxis with TDF beyond 4 wk after delivery[79]. Nevertheless, prolongation of antiviral prophylaxis[38] might be useful at least for women with elevated ALT during pregnancy, since they present a higher risk of post-partum hepatitis flare, as showed by a Chinese study wherein mothers were administered LdT[80]. With regard to other preventive strategies, unfortunately there is high uncertainty, also due to very low available evidence, upon the potential benefits of the antenatal administration of HBIG, to exploit the maternofoetal diffusion through the placenta, which reaches its peak during the third trimester[81].

Prevention of HBV MTCT: Beyond pharmacological options

The last issues involve the following topics: Delivery modalities, invasive procedures during pregnancy and breastfeeding[38,45]. There is a huge debate about the efficacy of caesarean section (C-section) as a preventive measure. Guidelines do not back its elective implementation[45], although meta-analyses reveal that C-section, compared with vaginal delivery, significantly decrease the risk of HBV vertical transmission[82,83]. The problem is the high heterogeneity of the studies whose results have been retrieved and analysed by these research syntheses, one collecting data from 10 studies[82], and the most recent from only Chinese

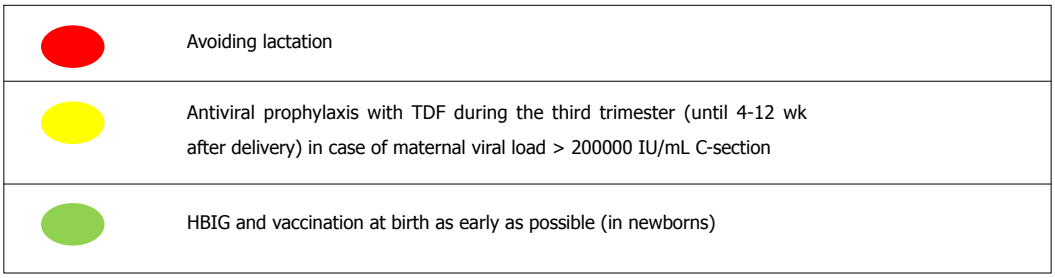

Figure 3 Prevention of hepatitis B virus vertical transmission. The column marked by the red circle refers to interventions that are not allowed. The column marked by the yellow circle refers to interventions that are backed up by low-quality or conflicting evidence. The column marked by the green circle refers to the best practice according to current evidence. TDF: Tenofovir disoproxil fumarate; IU: International units; C-section: Caesarean section; HBIG: Hepatitis B immunoglobulin.

datasets[83]: These relevant limitations advocate well designed studies to be performed in order to shed light on this matter.

Unfortunately, there is scarce evidence regarding the best practice when invasive procedures are carried out. As far as amniocentesis is concerned, a quite recent matched case-control study (63 infants whose HBsAg-positive mothers had underwent the procedure and 198 newborns whose HBsAg-positive mothers had not underwent amniocentesis) found that HBV MTCT was more frequent among cases (6.35% *vs* 2.53%; $P = 0.226$); notably, the difference was apparent when maternal viral load was taken into account, especially above the threshold of 200000 IU/mL (50% *vs* 4.5%, $P = 0.006$)[84]. Neither cases nor controls were born to mothers who were administered antiviral prophylaxis during pregnancy[84]. No strong recommendations can be drawn on this basis; therefore, while waiting for studies that will investigate the potential role of antiviral prophylaxis in women with high viraemia undergoing amniocentesis, guidelines suggest that a careful assessment of harms and benefits of the invasive procedure is necessary[45].

The last topic is breastfeeding. On one hand, lactating is allowed as long as the standard measures of passive/active prophylaxis are taken[38,45,46]. On the other hand, there are some concerns about the safety of NAs, particularly TDF, during breastfeeding[38,45,46]. Experiences in the HIV field indicate that antivirals are well tolerated[85] and in particular TDF appears to be safe as to infant outcomes[86]. In Figure 3, a summary of the current knowledge regarding the HBV MTCT prevention is depicted.

CONCLUSION

When facing HBV in pregnancy, there are two different problems to address: The first is represented by the maternal liver disease, the second by the risk of MTCT. The two issues are actually strictly inter-connected, but choices regarding potential antiviral use can profoundly differ, especially as for timing. Unfortunately, to date many questions present answers backed up by low-quality evidence, a not rare occurrence when pregnancy is involved. For instance, it is not simple to set up large and multicentre RCTs in this setting. Moreover, there is a constant need to take carefully into account benefits and harms of each intervention, potentially impacting not only one but two lives. Regarding the first issue, in essence the indications for treatment of general population also apply to pregnant women. The drug of choice is represented by TDF; in case the gravid subjects are already on treatment with another NA, a switch is advised. IFN is absolutely contraindicated.

As to the second issue, the only mandatory measure, underpinned by incontrovertible evidence, is represented by providing passive and active immunoprophylaxis to the newborns, starting the schedule as early as possible at birth. The use of antivirals as a preventive weapon, for women not falling in the categories that require treatment, is recommended during the last trimester (until 4-12 wk after delivery) just in case of high viraemia (> 200000 UI/mL), but evidence collected so far is not solid. The drug of choice also in this case is TDF, although there is no direct or indirect proof of superiority over other NAs allowed in pregnancy; nevertheless, among them it shows the highest barrier to resistance. To date there are no data regarding the use of TAF in pregnancy, which could represent an important option, combining the same efficacy with a better safety profile.

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MINIREVIEWS

Hepatocellular carcinoma occurrence in DAA-treated hepatitis C virus patients: Correlated or incidental? A brief review

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Abstract

Hepatitis C virus (HCV) chronic infection induces liver fibrosis and cirrhosis but is also responsible for a significant portion of hepatocellular carcinoma (HCC) occurrence. Since it was recognized as a causative factor of chronic hepatitis, there have been multiple efforts towards viral eradication, leading to the first-generation HCV treatment that was based on interferon (IFN)-α and its analogs, mainly PEGylated interferon-α (PEG IFNα). Sustained virological response (SVR), defined as the absence of detectable RNA of HCV in blood serum for at least 24 wk after discontinuing the treatment, was accepted as a marker of viral clearance and was achieved in approximately one-half of patients treated with PEG IFNα regimens. Further research on the molecular biology of HCV gave rise to a new generation of drugs, the so-called direct antiviral agents (DAAs). DAA regimens, as implied by their name, interfere with the HCV genome or its products and have high SVR rates, over 90%, after just 12 wk of per os treatment. Although there are no questions about their efficacy or their universality, as they lack the contraindication for advanced liver disease that marks PEG IFNα, some reports of undesired oncologic outcomes after DAA treatment raised suspicions about possible interference of this treatment in HCC development. The purpose of the present review is to investigate the validity of these concerns based on recent clinical studies, summarize the mechanisms of action of DAAs and survey the updated data on HCV-induced liver carcinogenesis.

Key words: Hepatocellular carcinoma; Hepatitis C virus infection; Direct antiviral agents; Liver carcinogenesis; advanced fibrosis; Hepatitis C virus-induced cancer sequence; Sustained virological response

Core tip: Inability to reach sustained virological response (SVR) and cirrhosis are independent prognostic factors for developing hepatocellular carcinoma (HCC) in hepatitis C virus (HCV) patients from the interferon (IFN) era. DAAs offer significantly better SVR rates. The first data regarding HCC occurrence after direct antiviral agent (DAA) treatment are similar to the data from patients who achieved SVR under IFN treatment. Some reports on early HCC occurrence or recurrence after DAA treatment are probably due to selection bias, as they were not reproduced in large comparative studies. DAAs can eradicate HCV, but they cannot terminate HCV-induced premalignant processes once triggered.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for approximately 5.6% of all cancers[1], making it the fifth most common cancer worldwide. There is an increasing trend in HCC incidence over the past two decades[2], so today, HCC is considered the second leading cause of cancer-related death[3]. One of the most well-known predisposing factors for HCC is chronic infection with hepatitis C virus (HCV), which is associated with a 15- to 20- fold increased risk for HCC development[4]. HCV was first recognized as a distinct clinical entity in 1989 and has since become one of the most rapidly evolving fields of hepatology. Multiple studies have reported progression of chronic HCV infection to severe fibrosis and cirrhosis in 5%-20% of the patients in a period of 5-20 years[2,3,5]. Once HCV-induced cirrhosis has been established, there is an estimated annual risk of 3%-7% of developing HCC[6]. The advent of the first anti-HCV drugs, predominantly PEGylated interferon-α (PEG IFNα), was a major breakthrough in the management of HCV infection and consequently in HCC prevention, as there was no other specific treatment at this time. Sustained virological response (SVR) with combined regimens, consisting of ribavirin and PEG IFN, can be achieved in approximately 55%[7] of patients, with some issues of tolerability and the limitation of being contraindicated in patients with decompensated cirrhosis. A meticulous study on the molecular biology and pathophysiology of HCV infection led to the invention of newer direct antiviral agents (DAAs), dramatically changing the landscape of HCV infection such that viral hepatitis C should be considered a highly curable disease. Although the eradication of HCV infection with DAAs seems to be feasible in the majority of patients, there are a measurable number of patients who will progress to HCC despite viral clearance. There are even some reports[8,9] that imply a correlation of HCC development with the use of newer oral regimes. In the present review, we attempt to clarify this puzzling topic based on recent reports from everyday clinical life, as well as reports on the pharmacological properties of the drugs used and the HCV-induced carcinogenesis sequence.

CURRENT DATA ON HCC OCCURRENCE IN HCV-TREATED PATIENTS

Chronic viral hepatitis, from either hepatitis B Virus (HBV) or HCV is the most significant predisposing factor for HCC. Forecasting models predict that without treatment, 14.4% of all HCV patients will develop HCC[10]. If we keep in mind that globally, 150 million individuals are estimated to have HCV infection[11], it is obvious that HCV eradication should be a major priority to decrease the incidence of HCC. SVR is currently accepted as the best tool to confirm viral eradication, and indeed, SVR has been shown to significantly reduce liver-related mortality, including the risk of HCC[3,4,12].

The IFN experience

Interferon (IFN)-α was the first drug to gain approval for HCV infection, in the late 1980s. The immunomodifying, antiviral and antitumoral properties of IFN-α raised hopes that it would succeed both in HCV eradication and in HCC prevention, as it also inhibits liver cancer growth[13]. The low SVR rates, low compliance and high toxicity reported with IFN led to the modification of the IFN molecule and the circulation of PEG-IFN in the 2000s. This modified form of recombinant human IFN-α has better absorption, prolonged half-life and better compliance, so it came into wider use in the treatment of HCV for about a decade, mainly in combination with the antiviral drug ribavirin.

Several studies[2,5,14] have focused on the SVR rates with IFN-based regimens and the impact the SVR has on HCC occurrence. Achievement of viral clearance was associated with reduced incidence of HCC and diminishment of other liver-related events (0 in SVR patients *vs* 1.88 in non-SVR per 100 person-years of follow-up), as shown in an Italian multicenter study[14]. In that paper, 920 patients with histologically proven cirrhosis were treated with monotherapy with conventional IFN-α. Although the SVR rate in that era was extremely low (124/920 or 13.5%), there was still

an obvious reduction in HCC occurrence in responders (5.6% *vs* 16%, $P < 0.01$). In the well-structured study of Van der Meer and colleagues, they compared long-term treatment outcomes among IFN monotherapy, IFN + ribavirin and PEG IFN + ribavirin in 5 hepatologic centers across Europe and Canada[15]. Recruited patients had advanced fibrosis (Ishak score > 4) and were followed up for a mean period of 8.4 years. SVR was achieved in 36% of the patients, and these patients had significantly lower all-cause mortality (8.9% *vs* 26.1%, $P < 0.001$) and liver-specific mortality (0.23 *vs* 3.20/100 pts, $P < 0.001$). The 10-year cumulative HCC incidence rate was 5.1% (95%CI: 1.3%-8.9%) for responders (SVR) *vs* 21.8% (95%CI: 16.6%-27.0%) for non-SVR patients ($P < 0.001$). The relatively low SVR rates shown in that study may be attributed to the advanced fibrosis of the patients, as well as the high percentage of patients (47%) with anti-hepatitis B core antigen positivity. Ogawa *et al*[16] conducted a prospective national multicenter study on the effect of PEG IFN + ribavirin treatment on chronic HCV infection, focusing on the oncologic outcomes, specifically on the incidence of HCC. The 1013-patient sample included cirrhotic (150) and non-cirrhotic (863) individuals, with 47 patients (4.6%) developing HCC during a mean follow-up of 3.6 years. As SVR rate of 55% was found, and not surprisingly, non-cirrhotic treatment responders had a better prognosis regarding HCC occurrence: 5-year cumulative incidence rates of HCC were 1.7% in the SVR group and 7.6% in the non-SVR group ($P = 0.003$), but this effect was attenuated in the cirrhotic group (18.9% *vs* 39.4%, $P = 0.03$). The authors identified platelet count < 150000/mL (HR = 4.04), failure of SVR (HR = 3.72), cirrhosis (HR = 3.22), male sex (HR = 2.98), age ⩾ 60 years (HR = 2.81) and α-fetoprotein (αFP) above 10 ng/mL (HR = 2.50) as independent risk factors for HCC development. Similar results were shown in a retrospective study from Japan[17], where reaching SVR after IFN-based treatment significantly reduced the 5-year HCC incidence (2.6% *vs* 8.2%, $P < 0.001$).

Hepatoma development after HCV treatment with IFN-based regimens was the main question of a meta-analysis by Morgan *et al*[18] in 2013. The authors included in their analysis data from 30 observational studies, comprising in total 31528 patients from 17 countries. Most of the studies selected stratified the patients according to fibrosis level, while 8 studies referred only to patients with advanced fibrosis and/or cirrhosis. Mean SVR rates were found to be quite low, as only 10853 patients (34.4%) achieved an SVR to treatment, while 1742 patients (or 5.5% of all patients) developed HCC during follow-up. When they adjusted HCC incidence according to the follow-up period and viral clearance, they found that hepatoma in HCV-treated patients developed at a rate of 0.33% per person-year (CI: 0.22% to 0.50%) in those who achieved SVR, significantly lower than the 1.67% (CI: 1.15% to 2.42%) of non-responders.

The brave new world of direct antiviral agents

The study and analysis of the HCV genome led to the production of pharmacologic agents targeting specific regions of its nucleic RNA. The first drugs to come into commercial use were telaprevir and boceprevir, both protease inhibitors of the non-structural viral protein 3-4A (NS3-4A)[19,20]. The addition of these agents to the "traditional" PEG IFN + ribavirin regimen increased SVR rates to approximately 70% and gained FDA approval for the treatment of HCV genotype 1 in 2011. A new target area (non-structural protein 5B -NS5B) was discovered, and sofosbuvir, an NS5B polymerase inhibitor[21], was approved in 2013. Addition of sofosbuvir to the PEG IFN + ribavirin combination achieved SVR rates above 90%, and most important of all, the scheme was active against genotypes 1 and 4, while the combination of sofosbuvir and ribavirin achieved similar SVR rates in genotypes 2 and 3[22]. The advent in late 2014 of NS5A inhibitors such as ledipasvir, in combination with sofosbuvir in a once-daily oral scheme, achieved SVR rates of 94%-99% in HCV genotype 1 patients in just 12 wk[23]. Since then, more agents and combinations have come into use, and today, there are a variety of treatment options for HCV eradication, as shown in Figure 1.

Reaching SVR significantly reduces the risk of developing hepatoma, as was already known from the IFN era, so one may have expected that with the newer DAAs, such a risk would be minimized to an occasional event, but first reports from the wider clinical applications of the new treatments were worrying. Conti *et al*[9] published in 2016 a regional multicenter study of 344 consecutive cirrhotic patients treated with DAAs, 285 of them with no previous history of HCC. Although DAA therapy achieved sustained virological response in 91% of patients, during the 24-wk follow-up, 9 of these 285 patients (3.16%, 95%CI: 1.45-5.90) developed HCC, much higher than previous reports on IFN responders. The authors noted though that the new IFN-free oral regimens lacked the tolerability and toxicity limitations of IFN therapy, so patients with advanced liver disease who were not candidates for treatment before were receiving therapy now. Furthermore, patients were examined solely by ultrasound before the initiation of treatment, increasing the possibility of small HCC nodules escaping notice. One other study, from Spain[8], that focused on early HCC recurrence in DAA-treated HCV patients expressed similar concerns, and a few months later, the announcement of preliminary findings by Kozbial *et al*[24] added to the cloud of suspicion. In a way, all these reports could be considered preliminary, keeping in mind that the new oral IFN-free regimens came into wide clinical use in 2015.

Larger studies were published last year, focusing

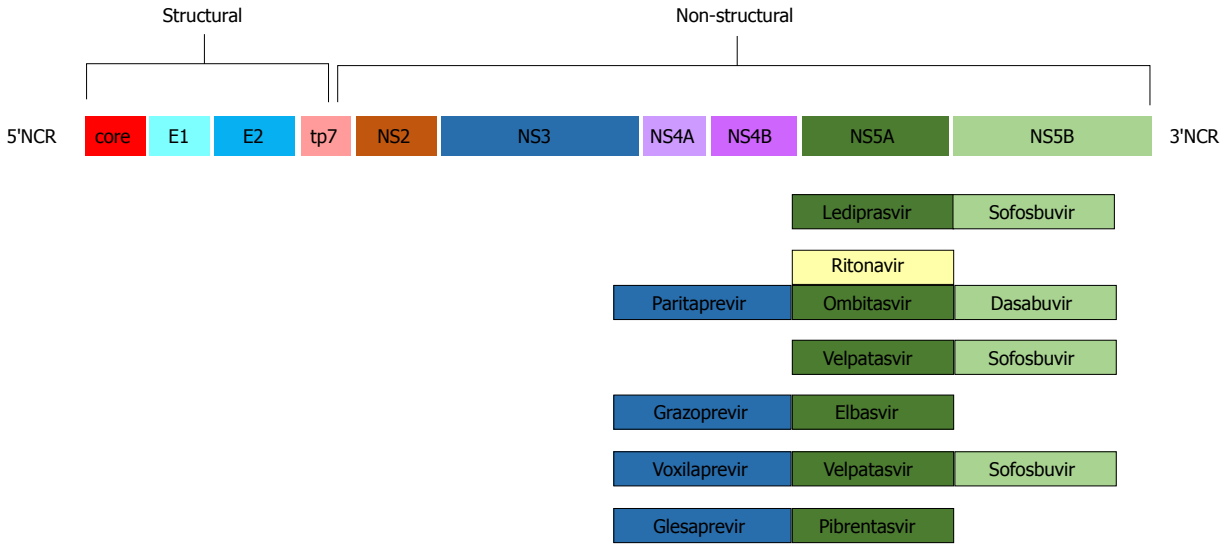

Figure 1 Current (March 2018) common direct antiviral agent regimes used in hepatitis C virus infection according to their target molecules in the hepatitis C virus genome.

on the concerns of carcinogenesis in DAA-treated patients. A big retrospective study from 129 Veteran Health Hospitals in the United States[25] reported an annual incidence of HCC of 0.9% in patients who accomplished viral clearance after treatment with DAAs. Approximately 40% of the patients included had cirrhosis at the initiation of treatment, and approximately one-half had significant comorbidities; that may be the reason that SVR rates were relatively low (86.7%). In subgroup analysis, however, non-cirrhotic patients who achieved SVR had an even lower annual incidence of HCC, at 0.34%, while the cirrhotic responders presented an annual incidence of 1.8%, comparable to that of non-responders irrespectively of cirrhosis status (3.45%).

The same team mentioned in the IFN era led by Dr. Ogawa[26] published a retrospective multicenter analysis in 2017 regarding early HCC development in patients who responded to DAA treatment. They reported an annual incidence of *de novo* hepatoma of 0.4% in non-cirrhotic patients, while in the cirrhotic ones HCC developed at an annual rate of 4.9%. End-of-treatment levels of αFP above 9.0 ng/mL in the individuals who were already cirrhotic before treatment, as well as low platelet count (< 150000/mL) and advanced fibrosis in the non-cirrhotic patients, were recognized as independent predictors of *de novo* HCC.

Recent comparative studies

As expected, research articles comparing the oncologic outcomes of IFN-based vs IFN-free regimens in HCV-infected persons were published in 2017. In the study of Nagata *et al*[17], 1897 patients treated for HCV over a 12-year period were analyzed, regardless of previous history of HCC. Although the IFN-free group of patients had worse baseline characteristics (advanced fibrosis/cirrhosis, older), the SVR rates were 65% for the IFN-based group and 96% for the IFN-free group ($P < 0.001$). The relatively high SVR rate for the IFN-based group can probably be attributed to the fact that 1/3 of the patients received DAAs in combination with PEG IFN. The 5-year incidence rates were 2.6% (SVR) *vs* 8.2% (non-SVR) for the IFN group ($P < 0.001$), and the 3-year incidence rates for the DAA-only group were 3.3% *vs* 5.9%, respectively ($P = 0.031$). In propensity score-matched analysis, no significant differences were found in HCC occurrence ($P = 0.49$). The authors reported that post-treatment αFP and *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA^+M2BP) could be used as independent prognostic factors of HCC development in patients achieving viral clearance.

Viral clearance by DAA treatment was associated with a 71% reduction in HCC risk in the large retrospective study by Ioannou *et al*[27]. The 62354 patients included were stratified in three subgroups according to HCV treatment: IFN only, IFN + DAA, and DAA only. Among patients in whom treatment succeeded (SVR), HCC developed in 2.5% of the IFN, 2.1% of the IFN+DAA group and 1.4% of the DAA group, but the follow-up period was shorter for the DAA-containing groups. Conversion to HCC incidence per patient-year showed a slight increase in hepatoma development in the DAA-only group, and this was due to the high prevalence of other risk factors that these patients had, such as cirrhosis, older age, diabetes and low serum albumin.

HCV patients with more advanced disease or other significant risk factors that were previously excluded for IFN therapy but now became candidates for treatment with DAAs probably create an unavoidable selection bias. To investigate this issue further, Waziry and colleagues[28] performed a meta-analysis of HCC

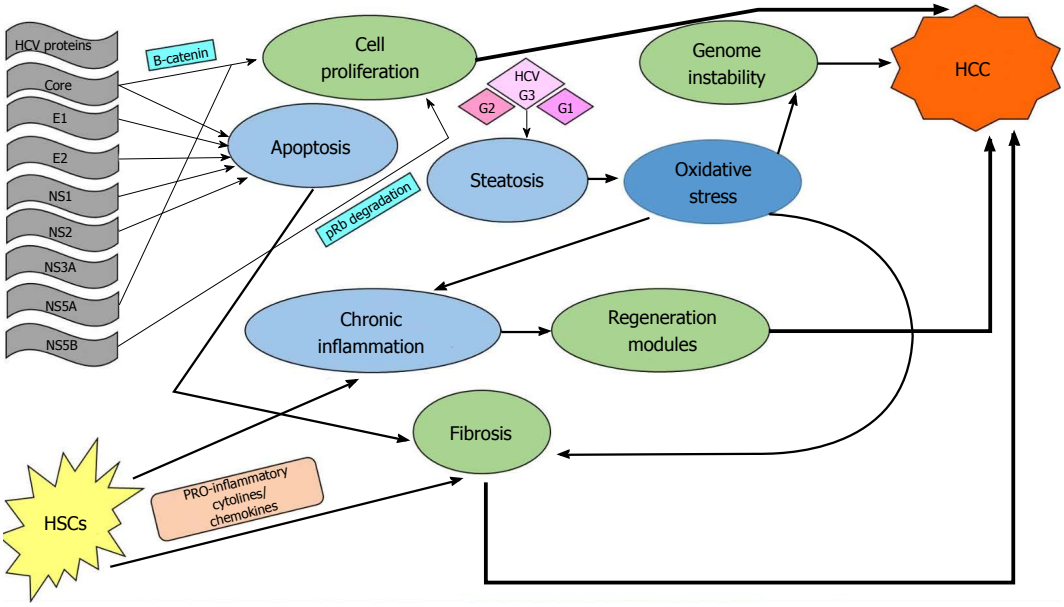

Figure 2 Schematic illustrations of identified pathways in hepatitis C virus-induced liver carcinogenesis. Viral DNA components promote host cell apoptosis deregulation, while certain viral proteins trigger proliferation signaling in the hepatic cell: Core and NS5A through the β-catenin pathway and NS5B by triggering tumor suppressor protein (pRb) degradation. Core, E1, E2, NS1 and NS2 proteins induce apoptosis, forcing a regeneration process, thereby promoting fibrosis. Oxidative stress due to inflammation also facilitates host cell genome instability and fibrosis. Inflammation activates hepatic stellate cells (HSCs) that in response secrete cytokines and chemokines, further promoting the inflammation, damage and regeneration cycle. HSCs also play a crucial role in fibrosis progression, as under chronic activation they switch their phenotype to matrix-secreting fibroblasts. Additionally, hepatitis C virus genotype 3 (as well as genotypes 1 and 2) induces steatosis, which further extends the oxidative stress, leading to earlier fibrosis. HCC: Hepatocellular carcinoma; HSCs: Hepatic stellate cells; HCV: Hepatitis C virus.

development following DAA therapy, including 17 studies with IFN treatment and 9 studies with DAA treatment. HCC occurrence in patient-years was estimated to be higher in DAA patients than in IFN-treated individuals because patients from the IFN-free group were followed for a shorter period and were older. Adjusted meta-regression analysis showed a relative risk of *de novo* HCC in DAA-treated patients vs IFN-treated of 0.67 (95%CI: 0.16-2.77, $P = 0.56$).

HCV INDUCED LIVER CARCINOGENESIS PATHWAYS

Relevant literature supports the concept that the occurrence of hepatocellular carcinoma is a multitasking process that occurs silently over years in patients with chronic HCV infection. Possible pathways include direct viral effects on the hepatic cells, immune-mediated genetic alterations, and stromal involvement via the fibrosis route. A schematic review of these pathways can be seen in Figure 2.

There is strong evidence that certain viral proteins trigger proliferating signaling in the hepatic cell, especially by the β-catenin pathway[29]. The Core and NS5A proteins are thought to play key roles in that interference, while NS5B has been found to trigger tumor suppressor protein degradation[30]. Additionally, viral proteins such as Core, E1, E2, NS1, and NS2 seem to induce the normal apoptotic pathway[31].

Immune-mediated effects of HCV infection comprise a complex pathway in liver carcinogenesis. In addition to the immediate effect of infection, which is the development of chronic inflammation and consequent regeneration[29], there is an accumulative effect on the host genome that progressively changes the hepatocellular phenotype[32]. Stromal involvement in the inflammatory process is mediated by activation of hepatic stellate cells (HSCs), which initially secrete cytokines and chemokines that further promote the inflammation/damage/regeneration cycle[30]. Activated HSCs become matrix-secreting myofibroblasts promoting liver fibrosis and therefore play a crucial role in cancer initiation[30,33]. Furthermore, certain HCV genotypes, mainly genotype 3 but also genotypes 1 and 2, induce viral steatohepatitis, probably through the action of viral Core protein[34]. Steatosis extends the oxidative stress induced by HCV infection itself[35], and once again, HSCs are activated, triggering collagen production and fibrosis[30].

From all the above, it becomes clear that there are distinct different pathways through which an HCV infection can induce HCC. It should be underlined, however, that some biologic cascades, once triggered, can go on independently of viral presence.

DISCUSSION

Nearly all studies regarding HCC incidence in HCV patients show 2 specific factors predicting unfavor-

able outcomes: cirrhosis and inability to reach a SVR. Which one of the two has the biggest impact on liver carcinogenesis may be suggested by the findings of Ioannou and colleagues[27]: The occurrence of hepatocellular carcinoma, regardless of treatment, was lowest in the non-cirrhotic responders (0.24), second lowest in non-cirrhotic non-responders (0.87), second highest among cirrhotic responders (1.97) and highest, as expected, in cirrhotic non-SVR patients (3.25). Based on this, one could rather safely suggest that presence of cirrhosis affects liver carcinogenesis more robustly than the HCV virus itself.

In the present review, we decided not to focus on recurrence of HCC in HCV patients treated with IFN-free regimens, for reasons associated with the oncogenic sequence mentioned above. To be more specific, the liver of a patient who has already developed a hepatoma due to HCV is probably in a diffuse precancerous state, meaning there are quite likely some other premalignant lesions, although they are not evident. This could be the reason that occurrence and recurrence of HCC after DAA therapy seem to occur quite early, presumably in the first year. Viral clearance may inhibit further direct oncogenic interference, but the fibrosis level as well as the regenerative process probably cannot be significantly reversed. Some authors believe that the sudden decrease in viral load achieved with DAAs causes immune distortion[36], deregulating the anti-tumor response and therefore releasing precancerous foci from immune surveillance. There are some data supporting this hypothesis, as DAA-induced SVR is associated with loss of intrahepatic immune activation by IFN[37], yet there is no evidence of direct or indirect oncogenic effects of direct antiviral regimens. On the other hand, Abdelaziz *et al*[38] conducted a retrospective analysis on HCC occurrence *vs* recurrence in DAA-treated patients. They found that *de novo* lesions had significantly better response rates to ablation. That could be interpreted as either that the recurrent tumors are less resistant or that DAA therapy somehow interferes with the malignant potential of *de novo* lesions.

IFN has shown antitumor properties[39], apart from its anti-inflammatory properties, that probably suppress-up to a point-the malignant growth in some patients. Whether there would be a benefit to prescribing IFN to patients who reached a sustained virologic response after IFN-free treatment, based on prognostic factors such as end-of-treatment αFP, low platelet count or low serum albumin, should be further investigated. Unfortunately, tolerability and contraindications limit the percentage of HCV patients who would benefit from such a tumor-preventive IFN maintenance.

CONCLUSION

Direct antiviral agents can radically change the landscape of HCV infection, as they achieve great SVR rates with excellent patient compliance. SVR is a well-recognized risk-reducing factor for HCC development, and early studies confirm this positive effect of DAA treatment. Although there are a few publications with unexpected and undesired oncologic outcomes after IFN-free treatment, it could be that they represent solitary incidental reports. More studies with longer follow-up are needed to investigate this topic. The new drugs seem to live up to their promise, so they deserve time to show their long-term effects in real life.

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MINIREVIEWS

Progression and status of antiviral monitoring in patients with chronic hepatitis B: From HBsAg to HBV RNA

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Abstract

As alternative indexes of hepatitis B virus (HBV), covalently closed circular DNA (cccDNA) transcriptional activity, hepatitis B surface antigen (HBsAg), hepatitis B core-related antigen (HBcrAg), and peripheral blood RNA known as pgRNA, have been advocated as novel serum markers for prediction of prognosis and treatment response in chronic hepatitis B (CHB). Since the availability of commercial quantitative assays of HBsAg in 2011, HBsAg has been widely used for predicting treatment response of patients with CHB. Patients who received interferon therapy have shown a sharper reduction of HBsAg level than those who received nucleoside drug (NAs) therapy. Upon peginterferon treatment, sustained responders have presented a larger reduction of HBsAg level than the non-responders. An absence of HBsAg decline, together with < 2log reduction in HBV DNA at week 12, can serve as a stopping rule in HBsAg-negative patients infected with genotype D HBV. A sharp reduction of HBsAg titer in the NAs therapy is a predictor of HBsAg clearance in long-term treatment. HBcrAg, which consists of three species of related proteins sharing an identical 149 amino acid sequence, including HbcAg, hepatitis B e antigen (HBeAg), and a truncated 22-kDa precore protein, is still detectable in situations where serum HBV DNA levels become undetectable or HBsAg loss is achieved. Therefore, HBcrAg remains a measurable serum marker to correlate with cccDNA in this situation. The decline in HBcrAg has been observed with NAs therapy and the pattern of decline might provide prognostic information on the risk of HBV post-treatment reactivation. Peripheral blood RNA, which is known as pgRNA, directly derives from cccDNA and reflects intrahepatic cccDNA level. Quantitative pgRNA has been suggested to be helpful in CHB management. However, commercial quantitative assays are lacking. Additionally, the use of simultaneous and continuous clearance of HBV RNA and HBV DNA in serum has been suggested to be a safe stopping rule of NAs therapy for patients with CHB. However, clinical studies of large sample sizes are needed to prove the feasibility and

significance of using serum HBV RNA as the assessment standard of antiviral therapy in CHB and the safety of the stopping rule in clinics.

Key words: Hepatitis B core-related antigen; Response prediction; Chronic hepatitis B; Interferon; Nucleos(t)ide analogs; Hepatitis B surface antigen; Progenome RNA

Core tip: As the surrogate biomarkers of intrahepatic viral replicative activity, hepatitis B surface antigen (HBsAg), hepatitis B core-related antigen (HbcrAg), and serum hepatitis B virus (HBV) RNA levels have been advocated as novel serum markers for treatment response in chronic hepatitis B. Currently, quantitative HBsAg has been widely used for predicting treatment response of chronic hepatitis B. HBcrAg can predict the risk of post-treatment reactivation of HBV as it is detectable in patients whose HBV DNA levels are undetectable or HBsAg loss is achieved. Serum HBV RNA may be useful in monitoring drug withdrawal, but clinical studies with large sample sizes remain necessary to further determine this capability.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is one of the threats to human health and has become a global health issue. Approximately 0.35 billion of the worldwide population is infected with HBV, and 75% of this number is in the Asia-Pacific region. Yearly, approximately 650 000 people die of hepatic failure, liver cirrhosis, and liver cancer related to HBV infection[1]. Antiviral drugs, which are currently approved for chronic hepatitis B (CHB) patients, include pegylated interferons (Peg-IFN-α)-2a and the following five oral polymerase inhibitors: Three nucleoside [lamivudine (LAM), telbivudine (LDT), and entecavir (ETV)] and two nucleotide [adefovir dipivoxil (ADV) and tenofovir (TDF)] drugs. Oral HBV polymerase inhibitors not only reduce the occurrence rate of corresponding complications by inhibiting HBV duplication for a long time but also increase the survival rates and living quality of patients with chronic HBV. However, these inhibitors cannot completely eliminate covalently closed circular DNA (cccDNA) molecules in hepatic cells, resulting in uncertain treatment periods. Furthermore, most patients may have to take medicines all their lives[2,3].

Long-term virus inhibition, which is manifested by hepatitis B envelope antigen (HBeAg) seroconversion, can induce virus immunity control of some patients. Moreover, hepatitis B surface antigen (HBsAg) clearance or seroconversion may occur in some patients. Therefore, seroconversion of HBeAg and HBsAg is widely accepted as the endpoint of therapy[2,3]. Spontaneous or therapy-induced seroconversion of HBeAg is viewed as the premise of HBsAg clearance or seroconversion, which implies the stable infection of HBV. Such seroconversion is presently acknowledged as the stopping rule[4,5]. However, indexes for predicting seroconversion of HBeAg in antiviral therapy are not completely certain. Indexes of disease activity, namely, tissue inflammation score and alanine aminotransferase (ALT) level, and viral indexes, such as HBV DNA and HBsAg, can be used to predict seroconversion of HBeAg[6-10]. Despite established markers, including serum HBV DNA levels and HBsAg titers, hepatitis B core-related antigen (HBcrAg) and HBV RNA are also considered serum markers of HBV infection. HBV RNA carries virus gene information, and its quantitative assay is not highly influenced by viral antigen and antibody immune compounds. Therefore, HBV RNA is important in the clinical diagnosis and response prediction. In particular, quantitative assay of serum HBV RNA level is superior to HBV DNA in terms of response prediction to HBV polymerase inhibitor based on therapy[11-13]. Serum HBV RNA level has a key role in the prediction of the stopping rule[14] and drug resistance[6]. HBcrAg, which contains three related proteins that share an identical 149 amino acid sequence [HBcAg, HBeAg, and a truncated 22-kDa precore protein (p22Cr)], is detectable in many patients with undetectable HBV DNA and HBsAg seroclearance. Along with the establishment of a fully automated detection method, HBcrAg may be extensively used in monitoring chronic HBV antiviral therapy in the future, particularly in situations where serum HBV DNA becomes undetectable. The major findings and potential clinical applications of HBcrAg in chronic HBV infection have been comprehensively described by Mak *et al*[15]. Therefore, this study mainly focuses on introducing the role of HBsAg titers and HBV RNA level in the antiviral therapy efficacy prediction of patients with CHB.

SIGNIFICANCE OF HBsAG LEVEL

HBsAg has been viewed as an important diagnosis index of HBV infection since its discovery by Blumberg *et al*[16] in 1965 and report in 1968. Recently, clinical significance of the HBsAg level has again attracted considerable attention since a corresponding quantitative assay was established and the correlation between HBsAg and cccDNA was confirmed[17,18].

Peg-IFN-α2a treatment

HBeAg-positive chronic hepatitis B: In HBeAg-

positive chronic hepatitis B serum, HBsAg level is closely related with intrahepatic cccDNA level and can reflect intrahepatic cccDNA contents. Reduction of HBsAg level implies a decrease in intrahepatic cccDNA[19-21]. Responses of HBeAg-positive patients to Peg-IFN-α2a therapy can be predicted according to HBsAg reduction. In an early study by Janssen *et al*[22], HBeAg-positive patients with sustained virological response (SVR) to Peg-IFN-α2a therapy showed a dramatic reduction of serum HBsAg. In addition, the HBsAg level in patients with SVR was lower than in non-responders at the end of therapy[22-24].

Seroconversion of HBeAg and SVR can be predicted from the baseline serum HBsAg level and its dynamic changes during Peg-IFN treatment. Early serological responses, which are defined as a low HBsAg level or a dramatic reduction of HBsAg during treatment, implies high seroconversion of HBeAg and HBV DNA suppression six months after treatment[24-26]. Specifically, patients who presented HBsAg < 300 IU/mL and HBeAg positive at week 24 during Peg-IFN-α2a treatment achieved an SVR of 62%, but the SVR of the remaining patients was only 11%[25]. Patients who had HBsAg reduction > 1log$_{10}$ IU/mL and the absolute HBsAg < 300 IU/mL at week 24 of the therapy achieved an SVR of 75% six months after treatment. However, SVR of those patients without this combined response was only 15% (P < 0.001). This combined HBsAg response generated positive predictive values (PPVs) of 75% and negative predictive values (NPVs) of 85% for achieving SVR in HBeAg-positive patients.

HBsAg level decline at weeks 12 and 24 during treatment is an alternative index to predict SVR in HBeAg-positive patients and identify non-responders. In the phase Ⅲ registration trial on Peg-IFN-α-2a, the PPV of HBsAg < 1500 IU/mL at week 12 and 24 on-treatment for achieving HBeAg seroconversion six months after treatment were 57% and 54% and NPV was 72% and 76%, respectively[26]. Sonneveld *et al*[27] discovered that SVR of patients without any decline of HBsAg at week 12 on-treatment was only 3%. Therefore, the absence of any decline in HBsAg at week 12 generated an NPV of 97% for response prediction six months after treatment.

HBeAg-negative chronic hepatitis B: Only a few studies have discussed the baseline response predictors for peginterferon-based therapy in HBeAg-negative patients. According to the existing data, low baseline HBsAg level is associated with SVR (defined as HBV DNA < 2000 IU/mL six mo after treatment)[28]. However, this finding has not been proven by other studies[29,30].

Dynamic monitoring of HBsAg levels in HBeAg-negative patients treated with peginterferon may complement the predicting value of HBV DNA alone[31-33]. Existing clinical data showed that SVR of HBeAg-negative patients to Peg-IFN-α2a can be predicted according to HBsAg reduction or the absolute level at week 12 or 24 during treatment. If HBsAg level decreased by 0.5log$_{10}$ IU/mL at week 12 and 1log$_{10}$ IU/mL at week 24, then the corresponding PPVs of SVR were 89% and 92% and NPVs were 90% and 97%, respectively[30]. Retrospective analysis of dynamic HBsAg level changes in 120 HBeAg-negative patients who were enrolled into the Peg-IFN-α-2a registration study found that patients with an HBsAg level decline of more than 10% from baseline at week 12 on-treatment achieved higher virus inhibition rates than those with declines of less than 10% (47% *vs* 16%, P < 0.01)[34]. However, HBsAg clearance occurred in a considerable proportion of patients who did not achieve more than 10% decline in HBsAg level.

Another study on Peg-IFN-α-2a therapy in HBeAg-negative patients predominantly infected with HBV genotype D indicated that dynamic monitoring of HBV DNA and HBsAg are superior to solely either marker in predicting therapeutic effects[29]. In this study, the absence of HBsAg level decline and HBV DNA reduction of less than 2 logs after 12 wk of PEG-IFN antiviral therapy were associated with no response (defined as HBV DNA > 10 000 copies/mL and ALT remains abnormal 6 mo after treatment). This finding is accepted as a stopping rule and is verified by some studies[29]. Nevertheless, such condition is hardly applicable to patients infected with other HBV genotypes. This result might be related with the changing influences of different genotypes on HBsAg during the treatment. Therefore, specific predictive values of different genotypes must be determined.

Additionally, a few studies have discussed the role of HBsAg level at the end of treatment in the prediction of follow-up SVR and subsequent HBsAg clearance[35]. In this study, 52% of 23 patients with HBsAg level < 10 IU/mL at the end of treatment achieved HBsAg clearance three years after treatment, and only 2% of the remaining patients achieved HBsAg clearance. Notably, HBsAg level at the end of treatment is more important than the HBV DNA level in predicting HBsAg clearance[34].

Conclusion: HBsAg reduction in HBeAg-positive patients at weeks 12 and 24 during Peg-IFN-α-2a therapy is conducive to the prediction of post-treatment SVR and effective identification of non-responders. Generally, HBsAg level identifies non-responders at week 12 and predicts SVR at week 24. The combined HBsAg and HBV DNA reduction in HBeAg-negative patients at week 12 can effectively recognize non-responders, especially patients infected with HBV genotype D.

NAs therapy

Variation trend of HBsAg level during NAs the-

rapy: NAs inhibits HBV replication by directly preventing HBV polymerase without affecting the synthesis of HBsAg. Selective virus gene mutation of NAs might result in changes in S open reading frame[36]. Although no direct evidence exists for influences of these genetic changes on serum HBsAg level, these changes can certainly cause retention of intrahepatic HBsAg and increase carcinogenic risks[37]. These factors affect the value of HBsAg quantification in predicting the antiviral efficacy of NAs. Consequently, a few studies on this topic are available, and numerous heterogeneities exist among these studies[38-43]. Overall, serum HBsAg reduction in NAs therapy is slower and less significant compared with that in the interferon therapy[40,41]. HBeAg-positive patients showed a larger reduction of HBsAg level than HBeAg-negative patients[43].

HBeAg-positive chronic hepatitis B: Wursthorn *et al*[42] conducted a three-year follow-up observation of 162 HBeAg-positive patients on LDT treatment. After two years of treatment, all patients showed HBV DNA ⩽ 60 IU/mL. Moreover, nine patients (6%) achieved HBsAg clearance. HBsAg clearance can be predicted from the sharp reduction of HBsAg (> 1log) after one year of treatment. This study confirmed the importance of quantitative HBsAg monitoring in the prediction of HBsAg clearance during NAs treatment. Similar results have been obtained in the follow-up TDF studies[44,45]. One small Chinese study disclosed that HBsAg < 100 IU/mL at the end of treatment was a sign of HBsAg seroconversion for two years after treatment[43].

HBeAg-negative chronic hepatitis B: Among ETV- and TDF-treated patients, HBeAg-negative patients achieved a smaller reduction of HBsAg level compared with HBeAg-positive patients[44,46]. A study in Hong Kong including 53 HBeAg-negative patients who had an average of 19 mo continuous LAM treatment and at least 12 mo post-treatment follow-up demonstrated that the end-of-treatment HBsAg titer was an independent predictor for 12 mo after treatment sustained viral suppression (HBV DNA ⩽ 200 IU/mL)[47]. All five patients with HBsAg ⩽ 100 IU/mL and reduction > 1log IU/mL (PPV 100%) and four of the eight patients with either HBsAg ⩽ 100 IU/mL or HBsAg reduction > 1log (PPV 50%) achieved 12 mo treatment sustained viral suppression. Moreover, other 40 patients with HBsAg reduction ⩽ 1log and the absolute level > 100 IU/mL were recognized as non-responders (NPV 100%) at 12 mo after treatment. HBsAg level at the end of treatment can also predict cumulative sustained response and HBsAg clearance at five years after stopping LAM.

Overall, quantitative assay of serum HBsAg in patients with CHB can provide some references regarding prediction of response to IFN and NAs therapies. However, HBsAg level cannot completely reflect intrahepatic cccDNA activity, which is related with HBsAg synthesis process, quantitative assay, and antiviral drug effects. Therefore, other indexes must be combined to predict the efficacy of HBV antiviral therapy.

HBV RNA LEVEL IN PERIPHERAL BLOOD

Quantitative detection of HBV RNA in peripheral blood

The replication cycle of HBV DNA starts from endonuclear cccDNA transcription of pre-genomic RNA (pgRNA). pgRNA is enveloped in the nucleocapsid during the formation of virus, and HBV DNA polymerase transcripts offspring DNA using pgRNA as the template. The offspring DNAs enter into the cell nucleus and facilitate virus circulation. Some offspring DNAs are assembled in the endoplasmic reticulum into complete virions and secreted from cells[48-51]. The replicative cycle of HBV DNA shows that HBV RNA exists in cells and detecting HBV RNA in serum is difficult. In the early 1990s, some studies reported the detection of HBV RNA in peripheral blood mononuclear cells of patients infected with HBV[51-54]. However, it was until 1996 that Köck *et al*[55] reported the detection of HBV RNA in peripheral blood virions of patients with chronic HBV infection using the reverse transcription PCR method. In 2001, Su *et al*[11] detected full-length RNA (fRNA) of HBV and truncated RNA (tRNA) in peripheral blood of patients with chronic HBV infection. They also proved that fRNA is correlated with HBeAg and HBV DNA, while trRNA is independent of HBeAg and is weakly related with HBV DNA. Subsequently, they further studied the various modes of DNA and RNA in peripheral blood in patients with CHB after a short-term LAM therapy. HBV RNA-carrying virions only account for 1% of the total virions in peripheral blood of treatment-naive HBV-infected individuals. However, HBV RNA-carrying virions began to take the dominant role in virions after the LAM therapy. Moreover, HBV DNA level decreased more than HBV RNA level during the LAM therapy[56,57]. Rokuhara *et al*[58] gained similar results from a follow-up of 24 patients with CHB on LAM treatment. They concluded that HBV RNA level in peripheral blood can reflect cccDNA level. Most recently, Huang *et al*[59] further examined the correlation between serum HBV RNA and intrahepatic cccDNA level. Their results indicated that serum HBV RNA reflects cccDNA activity in HBeAg-positive CHB, and total serum nucleic acids (HBV DNA plus RNA) can better reflect the activity of intrahepatic cccDNA compared with the serum HBV RNA or HBV DNA level.

Quantitative HBV RNA in peripheral blood during antiviral therapy

As an alternative index that directly reflects intrahepatic cccDNA level, HBV RNA level is one of the HBV antiviral therapy efficacy evaluation markers. HBV

RNA level can also be used to predict antiviral drug resistance and relapse after drug withdrawal[12-14,59-61].

Peginterferon treatment

HBeAg-positive chronic hepatitis B: Peginterferon can adjust immunity and directly inhibit virus. It can also inhibit replication of virus DNA, RNA, and cccDNA. Dynamic changes of virus RNA can demonstrate the effects of Peginterferon therapy in patients with CHB. Jansen *et al*[12] performed dynamic monitoring on virus RNA level in peripheral blood of 13 HBeAg-positive patients on 48 wk of interferon combined with ADV therapy and two years of follow-up visits. They discovered that the baseline HBV RNA level is unrelated with response to the therapy. HBV RNA decreases less compared with HBV DNA level at different time points. HBV RNA level in combined responders (defined as HBeAg clearance, HBV DNA $\leqslant$ 2000 IU/mL and normal ALT level at weeks 24 and 144 after treatment) is lower than that in non-responders at all-time points. Statistical differences exist between the two groups in terms of HBV RNA levels at all-time points after week 30 ($P < 0.001$). Therefore, responses of patients with CHB to the antiviral therapy can be predicted according to HBV RNA level. However, this study failed to disclose the threshold of prediction.

HBeAg-negative chronic hepatitis B: For HBeAg-negative patients, the HBV RNA levels of combined responders (HBV DNA $\leqslant$ 2000 IU/mL and persistent ALT normalization at 24 and 144 wk of treatment-free follow-up) are lower than those of non-responders before and during the treatment ($P < 0.001$). Some combined responders showed a considerable reduction of HBV RNA in the early period. During treatment, HBV RNA level of all combined responders at week six is lower than the minimum limit of detection. On the contrary, HBV RNA level of most non-responders is higher than the minimum limit of detection (1.8 ± 0.2 log10 copies/mL *vs* 3.7 ± 0.7 log10 copies/mL, $P = 0.028$) at week six. Therefore, baseline HBV RNA level is an independent predictor of responses to the therapy[12].

Nucleos(t)ide analog treatment: HBV RNA can be detected in HBeAg-positive or -negative patients with CHB before the treatment. During the NAs therapy, the reduction rate of HBV RNA is slower than that of HBV DNA level. At the end of the follow-up (week 120), HBV RNA level in HBeAg-negative patients is lower than that in HBeAg-positive patients[12]. HBV RNA levels of most patients are still higher than the minimum limit of detection when the HBV DNA levels are lower than the minimum limit of detection. However, HBV RNA levels in most HBeAg-negative patients are lower than the minimum limit of detection[12].

Prediction of antiviral drug resistance and relapse after drug withdrawal: Given that NAs cannot directly inhibit cccDNA, many patients easily suffer from relapse after drug withdrawal. They have to take medicines for a long time, even their entire lives[2,3]. Some patients may develop gene mutation related to drug resistance during the long-term antiviral therapy, thus resulting in the re-emergence of HBV DNA, hepatitis reflash, and even hepatic failure. Therefore, prediction of antiviral drug resistance and relapse after drug withdrawal is crucial. Existing evidence suggested that HBV RNA level can be a potential biomarker for monitoring gene mutation related to drug resistance and relapse after drug withdrawal[14,60].

Hatakeyama *et al*[60] detected HBV RNA levels in peripheral blood of 7 ETV-treated patients and 36 LAM-treated patients and found that the median serum HBV RNA levels were considerably higher in patients with YMDD mutations within one year of treatment (n =6, 1.788log copies/mL) than in those with YMDD mutations with more than one year of treatment (n = 12, 0.456log copies/mL, $P = 0.0125$) or in those without YMDD mutation (n = 18, 0.688log copies/mL, $P = 0.039$). The results indicated that high HBV RNA level in the early state is related with YMDD mutation.

In 2013, Tsuge *et al*[61] studied the correlation between HBV RNA and relapse of HBV DNA after drug withdrawal. Based on a 24-wk follow-up of 36 patients with CHB treated by NAs for at least 6 mo, 19 patients had HBV DNA relapse and 12 patients had ALT level rebound at 24 wk after discontinuation of NAs therapy. Serum total nucleic acids after three months of treatment were markedly correlated with HBV DNA rebound [odds ratio (OR) 9.474, 95% confidence interval (CI): 1.069-83.957, $P = 0.015$]. It is an independent predictor of virological recovery within 24 wk of NAs withdrawal. Wang *et al*[14] observed the performance of 33 patients at week 24 after NAs treatment for three or more years. All21 HBV RNA-positive patients experienced HBV DNA rebound at the end of treatment at week 24 after drug withdrawal, but only 3 (25%) of 12 end-of-treatment HBV RNA-negative patients had virological relapse at week 24 after drug withdrawal. According to the multivariate analysis, the end-of-treatment HBV RNA level is related to virological relapse at week 24 after drug withdrawal ($P = 0.001$). Summaries of the progression and status of antiviral monitor in patients with CHB are shown in Figure 1.

CONCLUSION

In summary, HBsAg comes from either cccDNA or integrated gene fragments[62]. HBsAg cannot completely represent the transcription activity of HBV cccDNA. HBV RNA, also known as pgRNA, only comes from cccDNA and can accurately reflect the cccDNA level. With the comprehensive knowledge on HBV RNA, the use of simultaneous continuous clearance of serum HBV DNA

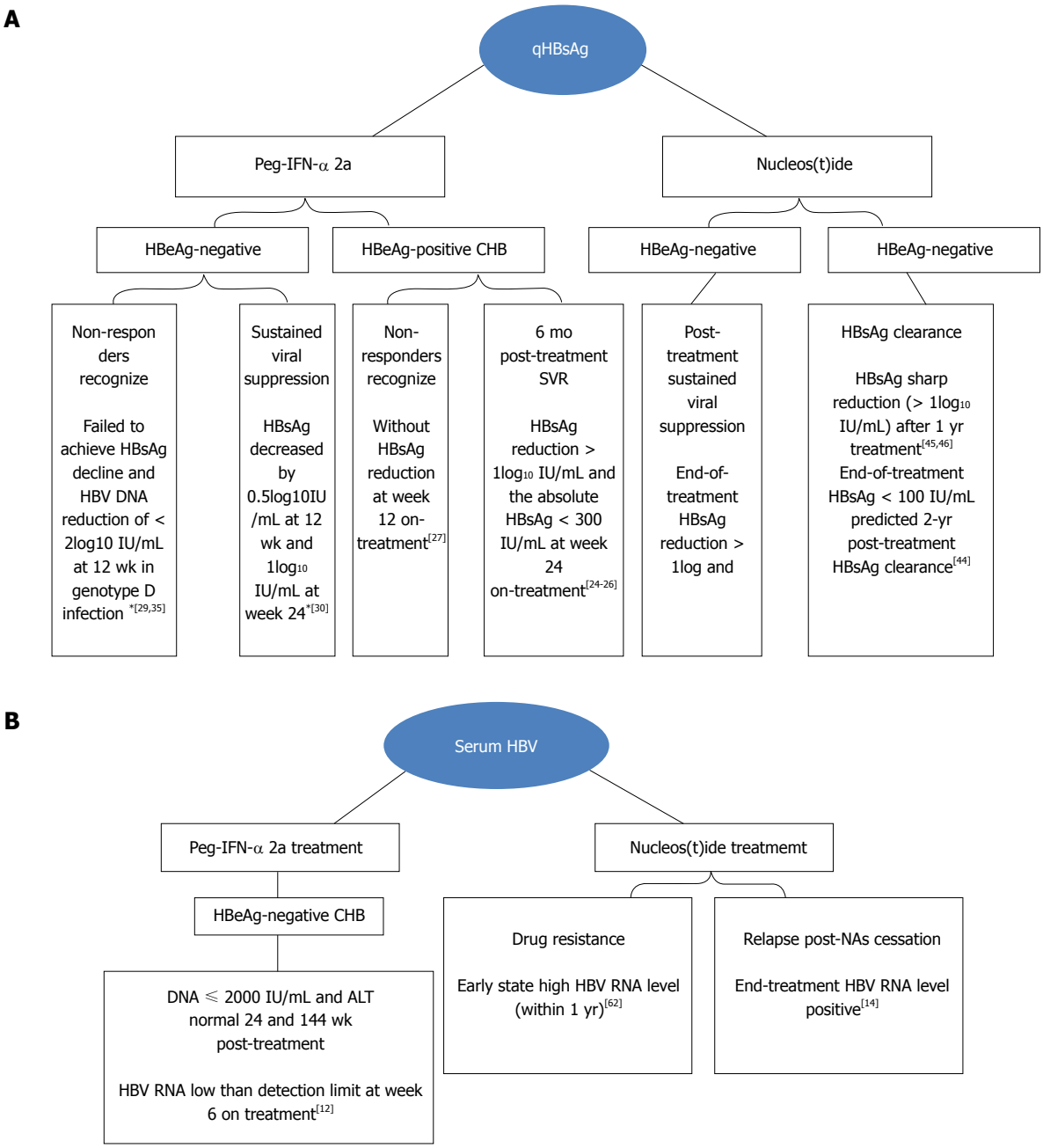

Figure 1 Progression and status of hepatitis B surface antigen (A) and serum hepatitis B virus (B) RNA in antiviral monitor in patients with chronic hepatitis B. HBsAg: Hepatitis B surface antigen; CHB: Chronic hepatitis B; HBV: Hepatitis B virus; Peg-IFN: Pegylated interferons; ALT: Alanine aminotransferase.

and HBV RNA is suggested as the safe stopping rule in patients with CHB on NAs treatment. However, clinical evidence based on a large sample size is needed to prove the feasibility and significance of this stopping rule.

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ORIGINAL ARTICLE

Retrospective Study

Metabolic syndrome does not affect sustained virologic response of direct-acting antivirals while hepatitis C clearance improves hemoglobin A1c

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Abstract

AIM

To determine whether successful treatment with direc-

tacting antivirals (DAA) is associated with improvements in hemoglobin A1c (HbA1c) and if type 2 diabetes mellitus (T2DM) or metabolic syndrome affects sustained virologic response (SVR).

METHODS

We performed a retrospective analysis of all hepatitis C virus (HCV) patients at the VA Greater Los Angeles Healthcare System treated with varying DAA therapy between 2014-2016. Separate multivariable logistic regression was performed to determine predictors of HbA1c decrease $\geqslant$ 0.5 after DAA treatment and predictors of SVR 12-wk post treatment (SVR12).

RESULTS

A total of 1068 patients were treated with DAA therapy between 2014-2016. The presence of T2DM or metabolic syndrome did not adversely affect SVR12. 106 patients had both HCV and T2DM. Within that cohort, patients who achieved SVR12 had lower mean HbA1c pre treatment (7.35 *vs* 8.60, P = 0.02), and lower mean HbA1c post-treatment compared to non-responders (6.55 *vs* 8.61, P = 0.01). The mean reduction in HbA1c after treatment was greater for those who achieved SVR12 than for non-responders (0.79 *vs* 0.01, P = 0.03). In adjusted models, patients that achieved SVR12 were more likely to have a HbA1c decrease of $\geqslant$ 0.5 than those that did not achieve SVR12 (adjusted OR = 7.24, 95%CI: 1.22-42.94).

CONCLUSION

In HCV patients with T2DM, successful treatment with DAA was associated with a significant reduction in HbA1c suggesting that DAA may have a role in improving insulin sensitivity. Furthermore, the presence of T2DM or metabolic syndrome does not adversely affect SVR12 rates in patients treated with DAA.

Key words: Hepatitis C virus; Hemoglobin A1c; Diabetes mellitus; Direct-acting antivirals; Metabolic syndrome

Core tip: The relationship of chronic hepatitis C virus (HCV) and type 2 diabetes mellitus is complex and lesser is known about its relationship to metabolic syndrome. While metabolic syndrome and type 2 diabetes may have had negative outcomes during the era of pegylated-interferon, research is being actively pursued to understand how direct acting antivirals (DAA) may affect these comorbidities. In this study, we show that unlike with pegylated-interferon, direct active antiviral success rates are not affected by the presence of metabolic syndrome. We further show that successful treatment of HCV with DAAs actually leads to better glycemic control 1-year post-treatment.

Dong TS, Aby ES, Benhammou JN, Kawamoto J, Han SH, May FP, Pisegna JR. Metabolic syndrome does not affect sustained virologic response of direct-acting antivirals while hepatitis C clearance improves hemoglobin A1c. *World J Hepatol* 2018; 10(9): 612-621 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i9/612.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i9.612

INTRODUCTION

Hepatitis C virus (HCV) infection is a major worldwide health problem. It is one of the most common blood-borne infections in the United States with an estimated 2.7 million people chronically infected in the United States[1]. HCV is also one of the leading causes of cirrhosis and liver transplantation[1,2]. There are increasing reports indicating an association between HCV and type 2 diabetes mellitus (T2DM). Individuals with HCV are more likely to have risk factors to develop T2DM and patients with T2DM have at least a 2-fold greater risk of developing HCV infection than the general population[3,4].

Prior studies have shown that chronic HCV infection is associated with a greater risk for the development of insulin resistance[5]. In a retrospective analysis of cirrhotic patients, those with HCV infection were 10 times more likely to have T2DM than those without HCV infection[5]. There is evidence that patients with chronic HCV infection and increased insulin resistance have a higher prevalence of hepatic fibrosis, hepatocellular carcinoma, and other extrahepatic manifestations[6-9]. While there are unclear mechanisms for increased insulin resistance among those with HCV, factors such as metabolic syndrome, increased hepatic iron, and serum tumor necrosis factor-α have been implicated[10,11]. Molecular studies have also shown that the mechanism of insulin resistance can differ by HCV genotype. In particular, HCV genotype 3 has been shown to be an independent risk factor for hepatic steatosis and its viral proteins can directly interfere with intracellular insulin signaling[12].

Previously standard therapy for HCV required the use of pegylated interferon-α (P-IFN) and ribavirin. However, these regimens had low sustained virologic response (SVR) rates and were poorly tolerated. During the era of P-IFN therapy, several studies showed that the presence of obesity and/or steatosis led to a reduction of SVR rate in HCV patients[13,14]. In patients with diabetes and HCV who were treated with IFN-based therapies, HCV clearance was associated with improved insulin resistance and beta cell function[15]. In 2013 and 2014, approval of newer direct acting antiviral agents (DAA) created IFN-free regimens with SVR rates greater than 90%, radically changing HCV treatment. Due to the novelty of DAA regimen, research is being actively pursued in a variety of patient populations. Within the Veterans Health Administration (VA), the incidence of chronic HCV is 2-3 times higher than the general public[16]. Additionally, patients that receive care in the

VA also have a higher prevalence of obesity and T2DM compared to the general population[17,18]. Thus, the VA presents an ideal population to evaluate the relationship between HCV and T2DM. We aim to determine if successful treatment with DAA is associated with improvements in hemoglobin A1c (HbA1c) and if the presence of T2DM or metabolic syndrome affects SVR rates.

MATERIALS AND METHODS

Study population and data collection

DAA were introduced to the VA Greater Los Angeles Healthcare System (VAGLAHS) at the beginning of April 2014. Therefore, we included all patients being treated with DAA between April 1st, 2014 and April 30th, 2016 for this study. We queried the Corporate Data Warehouse (CDW), a repository of all clinical data within the VA healthcare system, for all patients with an International Classification of Disease, Ninth Revision and/or Tenth Revision, Clinical Modification (ICD-9 CM/ICD-10 CM) diagnosis of HCV and T2DM (ICD-9: 250.00-250.93, ICD-10: E08-E13). Patients with HCV were also included if they had diabetic medications on their medication list during the study period. Patients were excluded from the cohort if they did not have SVR12 data or HbA1c one year after completion of DAA therapy. In addition to the CDW query, we manually extracted patient clinical and demographic data as well as confirm the diagnosis of T2DM from the VA electronic medical records, the Computerized Patient Record System (CPRS), of all patients screened to have both HCV and T2DM by ICD-9 CM or ICD-10 CM codes.

Outcome variables

The primary outcome was the change in HbA1c before and after DAA treatment. The secondary outcomes of interest were change in body mass index (BMI) over the same time period and if the presence of T2DM or other traits of metabolic syndrome such as hyperlipidemia (HDL), hypertension (HTN), or obesity affected SVR12. Serial BMI and HbA1c values were obtained from the year before and the year after HCV treatment and summarized as one-year pre-treatment and one-year post-treatment averages, respectively. A significant change of HbA1c was defined as a difference of 0.5 or greater, consistent with prior similar studies[19,20]. Through chart review, we also documented whether a patient had an increase or decrease in oral hypoglycemic dose and/or injectable insulin dose from one year before to one year after treatment with DAA. A change of greater than 10% from baseline was considered a significant change in medication, similar to prior studies[21]. Daily insulin was calculated as a total amount of basal and/or meal-time insulin over a 24-h period as documented in the patient's medication list. We also documented if there was a change in the overall number of diabetes medications from one year before to one year after treatment with DAA.

Predictor variables

Data extraction included patient demographic, comorbidity, laboratory, and medication data from CPRS. Demographic data included age, sex, BMI, race, and ethnicity. For race and ethnicity, we used one mutually-exclusive variable that combined concepts of race and ethnicity by including Hispanic as a primary race (non-Hispanic white, non-Hispanic black, Hispanic, Asian, other). Comorbidities included the presence of cirrhosis by chart review, elevated Fibrosis-4 (Fib-4) score (a measure of advanced fibrosis), hyperlipidemia (HLD), hypertension (HTN), HIV, and metabolic syndrome as identified by ICD9/ICD10 codes or by chart review. A cutoff value of 3.25 was used to determine a significant Fib-4 score as consistent with prior studies[22]. Within the cohort of patients with both HCV and T2DM, the presence of cirrhosis, ascites, and hepatic encephalopathy were confirmed by chart review of the patient's hepatology notes. If a patient had cirrhosis, clinical laboratory values were used to calculate a patient's Child-Turcotte-Pugh score and Child-TurcottePugh class (CTP). Using a modified World Health Organization definition for metabolic syndrome, we defined metabolic syndrome as having at least diabetes mellitus and at least two of the following baseline characteristics as determined by ICD-9 CM codes, ICD-10 CM codes, or by medication review: A BMI $\geqslant$ 30 kg/m^2, HTN, and HLD[23]. The presence of HLD was used as a surrogate for elevated triglyceride or reduced HDL cholesterol as we were unable to accurately obtain triglyceride or HDL levels for all patients before statin therapy. Furthermore, because many patients did not have urine albumin or urine creatine measurements, the presence of microalbuminuria was not analyzed. We also documented serological virologic clearance at 12-wk post treatment (SVR12), HCV genotype, and prior HCV treatment for all patients.

Statistical analysis

We performed proportions to summarize demographic and clinical characteristics and used χ^2 tests to examine differences between patients that did achieve SVR12 and those that did not. Medians were compared using the Wilcoxon rank-sum test, and means were compared using analysis of variance (ANOVA). All means are expressed with their respective standard error.

To determine predictors of SVR12, a multivariable logistic regression was performed. Due to the rare event of DAA failure, to examine the relationship between SVR12 and HbA1c, we performed univariable and multivariable penalized maximum likelihood logistic regression analyses similar to prior studies[24,25]. Predictors included age, sex, race/ethnicity, HCV genotype, treatment experienced or naïve, DAA used, and comorbid conditions (HTN, DM, HLD, HIV, elevated Fib-4, obesity, metabolic syndrome). The reference

group was female, white, treatment experienced, low Fib-4, without HTN/HLD/metabolic syndrome, genotype 1a, and treated with sofosbuvir/simeprevir. In addition to this model, we performed sub-analyses to determine associations between SVR12 and change in insulin dose required before and after DAA therapy. Statistical assistance was provided by the Institute for Digital Research and Education at UCLA. This study along with a waiver of informed consent was approved by the VA Institutional Review Board and the Research and Development Committee at VAGLAHS.

RESULTS

Cohort characteristics

A total of 1068 patients met inclusion criteria. Baseline characteristics are summarized in Table 1. In all patients treated with DAA, SVR12 rates differ by age, Fib-4 score, HCV genotype, DAA therapy, and DAA planned duration of treatment. Patients who achieved SVR12 were older (62.0 years *vs* 60.7 years, $P = 0.02$). Patients with a Fib-4 score < 3.25 had an SVR12 rate of 90.9% as compared to 80.2% for patients with a Fib-4 score ⩾ 3.25. SVR12 rates were varied by HCV genotype, with genotype 2 and 3 having lower SVR12 rates than genotype 1 ($P < 0.01$). SVR12 rates also were varied by treatment and duration of therapy. SVR12 rates were highest for patients treated with shorter duration (8 and 12 wk as compared to 16 and 24 wk) and with sofosbuvir/ledipasvir (93.7%) as compared to other regimens.

Of the 1068 patients treated with DAA, 106 patients concomitantly had T2DM and HCV and at least one HbA1c value 1-year post SVR12 (Table 2). The average age was 63.2 years (0.5). Within the cohort, 105 (99.1%) were male, 29 (27.4%) were white, 39 (36.8%) were African-American, and 27 (25.5%) were Hispanic. A total of 98 patients (92.5%) achieved SVR12 and 8 patients (7.5%) did not achieve SVR12. Fifty-seven patients (53.8%) had cirrhosis with a majority (82.5%) being compensated (*i.e.*, CTP A). Seventy-two patients (67.9%) were treatment-naïve while 34 patients (32.1%) had been treated with pegylated-interferon in the past. The majority of patients had genotype 1a ($n = 60$, 56.6%) or 1b ($n = 24$, 22.6%) disease. Only 16 patients (15.1%) had a normal BMI at the time of treatment (⩾ 18 and < 25) while the rest had a BMI ⩾ 25. Only 3 patients (2.8%) were co-infected with HIV. Ninety-five patients (89.6%) had HTN, 79 (74.5%) had HLD, and 85 (80.2%) had metabolic syndrome.

The average age for patients who achieved SVR12 was higher than those who did not (63.5 years *vs* 59.6 years, respectively, $P = 0.04$). There were no significant differences between patients who achieved SVR12 and those who did not in regards to sex, race/ethnicity, presence of cirrhosis, prior treatment experience, HCV genotype, treatment regimen, BMI, HIV status, HTN, HLD, or metabolic syndrome (Table 2).

Changes in HbA1c and BMI

Overall, average HbA1c was significantly lower after DAA therapy: 7.44% *vs* 6.71%, $P = 0.01$. For the subgroup of patients that achieved SVR12, the average HbA1c before treatment was significantly higher than the average after treatment (7.35% *vs* 6.55%, $P < 0.01$). When SVR12 was not achieved, however, HbA1c was not significantly different before and after treatment: 8.60% *vs* 8.61%, $P = 0.99$ (Table 3). This relationship was preserved across all genotypes with the greatest difference occurring in genotype 3 (Table 3). However, there was no difference in the change of HbA1c between genotypes or treatment regimens (Table 4).

Forty-six patients were on insulin before treatment and 43 patients were on insulin after treatment. Of those patients who were on insulin, the average daily insulin requirement before treatment was 55.1 IU (5.7) and 49.7 IU (6.2) after treatment ($P = 0.50$). For patients on insulin who achieved SVR12, the average daily insulin requirement before treatment was 55.0 IU (5.85) and the average daily insulin requirement after treatment was 48.2 IU (6.30) ($P = 0.42$). Insulin requirement also did not change significantly for patients who did not achieve SVR12 [55.5 IU (20.4) *vs* 58.1 IU (21.8), $P = 0.93$]. No patients analyzed were on any non-insulin injectable diabetes medications. There was no difference between the number of diabetes medications per patient before or after DAA therapy (1.23 *vs* 1.26, $P = 0.43$).

The study included 44 patients (41.5%) defined as overweight (BMI ⩾ 25), 30 (28.3%) that were defined as obese (BMI ⩾ 30), and 12 (10.4%) with severe obesity (BMI ⩾ 40). The average BMI for all patients before treatment was 30.1 kg/m^2 (0.53), and the average BMI for all patients after treatment was 30.2 kg/m^2 (0.54) ($P = 0.92$). For patients who achieved SVR12, the average BMI before and after treatment were not statistically different: 30.3 kg/m^2 (0.56) *vs* 30.3 kg/m^2 (0.57), $P = 0.96$. Similarly, the average BMI was not different before and after treatment for the patients that did not achieve SVR12: 28.8 kg/m^2 (1.4) *vs* 29.2 kg/m^2 (1.5), $P = 0.92$.

SVR12 is not affected by the components of metabolic syndrome

After adjusting for age, sex, race/ethnicity, cirrhosis, treatment experience, HCV genotype, treatment regimen, HIV status, and treatment duration, the individual components of metabolic syndrome (obesity, HTN, HLD, and T2DM) and the presence of metabolic syndrome itself did not predict SVR12 (Table 5). For the full logistic regression for SVR12, please see supplemental Table 1.

SVR12 as a predictor of improved insulin resistance

When adjusting for age, sex, race/ethnicity, cirrhosis,

Table 1 Baseline characteristics of all patients treated with direct-acting antivirals *n* (%)

	All patients (*n* = 1068)	SVR12 not achieved (*n* = 138)	SVR12 achieved (*n* = 930)	*P*-value
Age (mean ± SE, yr)	61.8 ± 0.2	60.7 ± 0.5	62.0 ± 0.2	0.02
Sex				
Male	97.5 (1041)	12.8 (133)	87.2 (908)	0.78
Female	2.5 (27)	18.500 (5)	81.50 (22)	
Race/ethnicity				
White	37.5 (400)	11.00 (44)	89.0 (356)	0.14
African American	37.5 (401)	14.70 (59)	85.3 (342)	
Hispanic	15.1 (161)	16.80 (27)	83.2 (134)	
Asian	0.70 (7)	14.30 (1)	85.700 (6)	
Other	9.3 (99)	7.10 (7)	92.90 (92)	
Fib4 < 3.25	64.6 (690)	9.1 (63)	90.9 (627)	< 0.01
Fib4 ⩾ 3.25	35.4 (378)	19.80 (75)	80.2 (303)	
Treatment naïve	79.5 (849)	16.4 (102)	83.6 (747)	0.09
Treatment experienced	20.5 (219)	16.40 (36)	83.6 (183)	
HCV genotype				
HCV genotype 1a	58.0 (619)	12.10 (75)	87.9 (544)	< 0.01
HCV genotype 1b	25.9 (277)	10.10 (28)	89.9 (249)	
HCV genotype 2	7.9 (84)	21.40 (18)	78.60 (66)	
HCV genotype 3	6.9 (74)	23.00 (17)	77.00 (57)	
HCV genotype 4	1.2 (13)	0.00 (0)	100.0 (13)	
HCV genotype 6	0.10 (1)	0.00 (0)	100.0 (1)	
Treatment				
PrOD	4.5 (48)	8.30 (4)	91.70 (44)	0.01
PrOD/RBV	17.6 (188)	14.40 (27)	85.6 (161)	
Sofosbuvir/Ledipasvir	34.4 (367)	6.3 (23)	93.7 (344)	
Sofosbuvir/Ledipasvir/RBV	13.4 (143)	13.30 (19)	86.7 (124)	
Sofosbuvir/RBV	9.8 (105)	23.80 (25)	76.20 (80)	
Sofosbuvir/Simeprevir	17.5 (187)	19.80 (37)	80.2 (150)	
Other regimens	2.9 (30)	10.0 (3)	90.00 (27)	
No HIV	96.7 (1033)	13.1 (135)	86.9 (898)	0.44
HIV	3.3 (35)	8.60 (3)	91.40 (32)	
Duration of treatment				
8 wk	19.9 (213)	6.6 (14)	93.4 (199)	< 0.01
12 wk	73.1 (781)	13.4 (105)	86.6 (676)	
16 wk	2.8 (30)	40.00 (12)	60.00 (18)	
24 wk	4.1 (44)	15.90 (7)	84.10 (37)	
BMI < 30	66.5 (710)	12.50 (89)	87.5 (621)	0.60
BMI > 30	33.5 (358)	13.70 (49)	86.3 (309)	
No HTN	43.7 (467)	13.30 (62)	86.7 (405)	0.76
HTN	56.3 (601)	12.60 (76)	87.5 (525)	
No T2DM	85.6 (914)	12.4 (113)	87.6 (801)	0.15
T2DM	14.4 (154)	16.20 (25)	83.8 (129)	
No HLD	58.1 (620)	14.50 (90)	85.5 (530)	0.70
HLD	41.9 (448)	10.70 (48)	89.3 (400)	
No metabolic syndrome	87.1 (930)	13.0 (121)	87.0 (809)	0.84
Metabolic syndrome	12.9 (138)	12.30 (17)	87.7 (121)	

PrODL: Ombitasvir/Paritaprevir/Ritonavir/Dasabuvir; Fib4: Fibrosis 4 score; RBV: Ribavirin; BMI: Body mass index; HTN: Hypertension; T2DM: Type 2 diabetes mellitus; HLD: Hyperlipidemia; SVR: Sustained virologic response; HCV: Hepatitis C virus.

treatment experience, HCV genotype, treatment regimen, obesity, HIV status, HTN, HLD, and metabolic syndrome, SVR12 significantly predicted a 0.5-unit improvement in HbA1c with treatment in patients with both T2DM and HCV: Adjusted OR (aOR) = 7.24 (95%CI: 1.22-42.94) (Table 6). Patients who achieved SVR12 were also significantly less likely to require an increase in insulin after HCV therapy than those who did not achieve SVR12: aOR = 0.166 (95%CI: 0.03-0.74). A decrease of HbA1c was not associated with an increase in oral hypoglycemic dose (aOR = 1.07, 95%CI: 0.48-1.35) or injectable insulin dose (aOR = 0.6, 95%CI: 0.57-1.87) after DAA therapy.

DISCUSSION

This study demonstrates that HCV clearance with DAAs is associated with a clinically significant decrease in HbA1c independent of other demographic and clinical factors such as metabolic syndrome and BMI. This data corroborates recent studies that showed similar outcomes with DAA therapy[26]. While Hum *et al*[26] attempted to control for DM medication use by looking at the percent of patients taking any DM medication or by the number of DM medication class, being unable to look at dosage change or changes in medication for an individual patient was a limitation of their study.

Table 2 Baseline characteristics of patients with hepatitis C virus and type 2 diabetes mellitus

	All patients (n = 106)	SVR12 not achieved (n = 8)	SVR12 achieved (n = 98)	P-value
Age (mean ± SD, yr)	63.2 ± 0.5	59.6 ± 1.4	63.5 ± 0.5	0.04
Sex				
Male	99.1 (105)	7.60 (8)	92.40 (97)	0.74
Female	0.90 (1)	0.00 (0)	100.0 (1)	
Race/ethnicity				
White	27.40 (29)	6.90 (2)	93.20 (27)	0.15
African American	36.80 (39)	5.10 (2)	94.90 (37)	
Hispanic	25.50 (27)	11.1 (3)	88.90 (24)	
Asian	1.90 (2)	50.0 (1)	50.00 (1)	
Other	8.50 (9)	0.00 (0)	100.0 (9)	
Cirrhosis				
Yes	53.80 (57)	8.80 (5)	91.20 (52)	0.61
No	46.20 (49)	6.10 (3)	93.90 (46)	
CTP class				
CTP A	82.50 (47)	6.40 (3)	93.60 (44)	0.21
CTP B	14.00 (8)	25.0 (2)	75.0 (6)	
CTP C	3.50 (2)	0.00 (0)	100.0 (2)	
Treatment status				
Naive	67.90 (72)	5.60 (4)	94.40 (68)	0.26
Experienced	32.10 (34)	11.8 (4)	88.20 (30)	
HCV genotype				
Genotype 1a	56.60 (60)	10.0 (6)	90.00 (54)	0.67
Genotype 1b	22.60 (24)	4.20 (1)	95.80 (23)	
Genotype 2	12.30 (13)	0.00 (0)	100.0 (13)	
Genotype 3	7.60 (8)	12.5 (1)	87.50 (7)	
Genotype 4	0.90 (1)	0.00 (0)	100.0 (1)	
Treatment regimen				
Sofosbuvir/Simeprevir	39.60 (42)	7.10 (3)	92.90 (39)	0.96
Sofosbuvir/Ribavirin	17.00 (18)	5.60 (1)	94.40 (17)	
PrOD	18.90 (20)	10.0 (2)	90.00 (18)	
Sofosbuvir/Ledipasvir	24.50 (26)	7.70 (2)	92.30 (24)	
Body mass index				
Normal	15.10 (16)	6.20 (1)	93.80 (15)	0.78
Overweight	41.50 (44)	9.10 (4)	90.90 (40)	
Obese	28.30 (30)	6.70 (2)	93.30 (28)	
Severely obese	10.40 (12)	8.30 (1)	91.70 (11)	
Very severely obese	4.70 (4)	0.00 (0)	100.0 (4)	
Co-HIV infection	2.80 (3)	100.0 (3)	0.0 (0)	0.62
HTN	89.60 (95)	8.40 (8)	91.60 (87)	0.32
HLD	74.50 (79)	6.30 (5)	93.70 (74)	0.42
Metabolic syndrome	80.20 (85)	7.10 (6)	92.90 (79)	0.71

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HTN: Hypertension; HLD: Hyperlipidemia; PrOD: Ombitasvir/Paritaprevir/Ritonavir/Dasabuvir; SVR: Sustained virologic response; CTP: Child-Turcotte-Pugh.

By analyzing each individual patient and examining both changes in the number of DM medication and the dosage, we find that the change of HbA1c was not due to an increase in oral hypoglycemic or insulin treatment. The findings imply that the change in HbA1c was most likely due to a change in host insulin resistance due to HCV clearance. This is in line with a recent study showing that HCV clearance with DAA reverses insulin resistance[27]. Our finding, however, is contrary to what Chaudhury *et al*[28] described in their recent paper published in December of 2017. While their study showed no change in HbA1c with DAA therapy, only 17% of patients had diabetes and their average baseline HbA1c was 5.75%. A reduction in HbA1c in a non-diabetic patient is hard to achieve. Therefore, it is reasonable to suggest that if they only examined patients with T2DM with a baseline HbA1c $\geqslant$ 6.5% they would have found similar conclusions. Our data is further corroborated by data showing that successful treatment with DAA is also associated with a decreased likelihood of requiring higher insulin doses for DM management. This is contrary to a paper by Stine *et al*[29] showing that a third of patients required an increase in their DM medication after DAA therapy. However, they only examined patients up to the time of SVR12. It is possible that if they examined data one year after SVR12 they would have had similar results. Our study also shows that changes in HbA1c did not vary between HCV genotype and DAA treatment regimen, implying that HCV clearance itself plays an important role in insulin resistance. Even though the baseline HbA1c was different in those who did achieve SVR12 compared to those that did not, our study shows that metabolic syndrome or its individual components

Table 3 Mean hemoglobin A1c before and after hepatitis C virus treatment by hepatitis C virus genotype and sustained virologic response 12-wk status

Genotype	Before DAA	SE	After DAA	SE	*P*-value
Mean HbA1c of patients who did achieve SVR12					
1a	7.5	0.19	6.68	0.14	0.001
1b	7.3	0.22	6.59	0.21	0.03
2	7.09	0.35	6.29	0.21	0.06
3	7.12	0.37	6.10	0.39	0.08
4	5.5	NA	5.30	NA	NA
Overall	7.35	0.13	6.55	0.11	< 0.01
Mean HbA1c of patients who did not achieve SVR12					
1a	8.98	1.14	8.95	1.41	0.98
1b	6.4	NA	6.50	NA	NA
2	No observations				
3	8.5	NA	8.70	NA	NA
4	No observations				
Overall	8.6	0.89	8.61	1.08	0.99

HbA1c: Hemoglobin A1c; SVR12: Sustained virologic response 12 wk after treatment; DAA: Direct acting antiviral; SE: Standard error.

Table 4 Mean difference in hemoglobin A1c by hepatitis C virus genotype and direct acting antiviral treatment

	Average change HbA1c (SE)	*P*-value
HCV genotype		
1a	-0.73 (0.13)	0.97
1b	-0.69 (0.20)	
2	-0.79 (0.27)	
3	-0.88 (0.51)	
4	-0.20 (NA)	
Treatment		
Sofosbuvir/Simeprevir	-0.71 (0.16)	0.97
Sofosbuvir/Ribavirin	-0.77 (0.27)	
PrOD	-0.66 (0.15)	
Ledipasvir/Sofosbuvir	-0.80 (0.24)	

HbA1c: Hemoglobin A1c; HCV: Hepatitis C virus; PrOD: Ombitasvir/ Paritaprevir/Ritonavir/ Dasabuvir

Table 5 Components of metabolic syndrome on Sustained virologic response 12 wk after treatment

	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Obese	0.90 (0.62-1.31)	1.25 (0.82-1.90)
HTN	1.06 (0.74-1.52)	0.81 (0.51-1.28)
HLD	1.42 (0.97-2.10)	1.31 (0.87-1.97)
T2DM	0.72 (0.48-1.10)	0.82 (0.55-1.09)
Metabolic syndrome	1.04 (0.68-1.60)	1.81 (0.75-4.37)

HTN: Hypertension; HLD: Hyperlipidemia; T2DM: Type 2 diabetes mellitus.

(HTN, T2DM, Obesity, HLD) does not negatively affect SVR12 rates.

Prior studies have demonstrated an association between HCV infection and host metabolism. It has been shown that the hepatitis C virus depends on host lipids for entry and replication in hepatocytes[30-32]. HCV infection, in return, leads to a disruption of host metabolism, which can result in insulin resistance, lipid dysregulation, and hepatic steatosis[33].

The data represented here are consistent with data during the pegylated-interferon era where SVR clearance was associated with decreased insulin resistance and improved beta-cell function[10,15,34,35]. Kawaguchi *et al*[15], for example, demonstrated a three-fold increase in insulin receptor substrate 1 and 2 expressed in hepatocytes in 29 biopsy-proven HCV infection who maintained SVR after pegylated-interferon therapy. They also demonstrated a correlation between serum ferritin and homeostatic model assessment (HOMA) of β-cell function. Their data supports prior data that shows hepatic iron-induced oxidative stress may be related to insulin resistance[36]. Knobler *et al*[11] proposed an alternative mechanism in which HCV infection may mediate diabetes. In a comparison of 23 patients with DM and HCV to 28 patients with HCV alone, they showed that serum TNF-α levels were more prominent in those with concurrent DM. This is similar to prior data that shows that TNF-α signaling may be related to obesity and insulin resistance, thus suggesting a role of inflammation in creating a "diabetogenic" state[37].

During the era of pegylated-interferon, Bressler *et al*[14] showed that obesity was an independent risk factor for reduced SVR12. Because pegylated-interferon is primarily taken up in the lymphatic system and because obese patients have poor lymphatic circulation, they proposed that the difference they described was due to dissimilar pharmacokinetic properties of obese and non-obese patients. While that may have been true for pegylated-interferon, our study shows that obesity, HTN, T2DM, or HLD does not significantly affect SVR12 rates of patients on DAA therapy. Unsurprisingly, SVR12 rates were reduced in patients with an elevated Fib-4 score, in patients with genotype 3, and in patients treated with suboptimal regimens such as sofosbuvir/ribavirin or sofosbuvir/simeprevir. This is similar to prior studies showing reduced SVR12 in similar treated groups[38-40]. We further hypothesized that patients who were more ill were more likely to be treated for a longer duration thus explaining the discrepancy between SVR rates and treatment duration.

While this study demonstrates the interplay between host metabolisms with HCV infection, there are certain limitations. The study implies that HCV clearance is associated with a reduction in insulin resistance, however, we were unable to measure insulin resistance directly before and after treatment. With primary care providers preferentially using HbA1c over fasting blood glucose, we were unable to use HOMA-IR (insulin resistance), HOMA-β (β-cell function), or other indices of insulin resistance. Nonetheless, HbA1c is a well-accepted and commonly utilized method to evaluate for insulin sensitivity, and we feel that using HbA1c as an outcome is consistent with clinical diagnosis, management, and prior studies[18,20]. Second,

Table 6 Odds ratio for a decrease of hemoglobin A1c > 0.5 among type 2 diabetes mellitus patients treated with direct acting antiviral

Predictor	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
Age	1.03 (0.95-1.11)	1.07 (0.96-1.20)
Race/ethnicity		
White[1]	1.58 (0.64-3.91)	
African American	0.89 (0.32-2.43)	0.52 (0.13-2.06)
Hispanic	0.53 (0.18-1.57)	0.65 (0.16-2.66)
Asian	0.50 (0.02-8.85)	0.8 (0.04-10.01)
Other	0.83 (0.16-4.21)	0.98 (0.12-7.90)
Cirrhosis (by chart review)	0.62 (0.28-1.37)	0.59 (0.20-1.69)
Treatment naïve	0.84 (0.87-4.59)	2.61 (0.92-7.29)
HCV genotype		
1a[1]	0.77 (0.35-1.71)	
1b	1.33 (0.49-3.60)	0.79 (0.22-2.86)
2	1.50 (0.41-5.42)	6.75 (0.26-176.51)
3	0.66 (0.15-2.92)	3.81 (0.29-50.00)
Treatment regimen		
Sofosbuvir/Simeprevir[1]	1.23 (0.55-2.75)	
Sofosbuvir/Ribavirin	0.75 (0.27-2.09)	0.07 (0.003-1.50)
PrOD	1.60 (0.56-4.56)	0.71 (0.17-2.86)
Ledipasvir/Sofosbuvir	0.66 (0.27-1.62)	0.35 (0.09-1.36)
Overweight	1.36 (0.42-4.35)	0.89 (0.20-1.69)
Obese	1.55 (0.44-5.40)	1.10 (0.20-5.93)
Severely obese	1.08 (0.24-4.94)	0.41 (0.05-3.07)
Very severely obese	0.26 (0.21-3.06)	0.08 (0.003-2.07)
HTN	1.36 (0.38-4.80)	1.15 (0.15-8.86)
HLD	1.69 (0.69-4.09)	1.45 (0.29-7.26)
Metabolic syndrome	1.58 (0.60-4.15)	0.96 (0.10-9.19)
SVR12[a]	10.67 (1.76-64.7)	7.24 (1.22-42.94)

[1]Reference group; [a]$P < 0.05$. HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HTN: Hypertension; HLD: Hyperlipidemia; SVR12: Sustained virologic response 12 wk post-treatment; PrOD: Ombitasvir/Paritaprevir/Ritonavir/ Dasabuvir.

our study was a single center study conducted in the VA. VA patients are predominantly male and have different socioeconomic factors that may affect health as compared to the general population. Nonetheless, GLAVA is one of the largest VA medical center that includes an integrated network of 12 sites serving a racially and ethnically diverse population in Southern California.

In conclusion, this study of a diverse VA patient population demonstrates that HCV clearance with DAAs is associated with a clinically significant decrease in HbA1c. This change was consistent across all genotypes and treatment regimens. The change in HbA1c was independent of changes in BMI and DM medication requirements and so may represent a decrease in host insulin resistance or increased insulin sensitivity. This study substantiates similar studies during the pegylated-interferon era by showing that HCV clearance irrespective of treatment regimen and genotype leads to improved DM outcomes. However, unlike prior studies during the period of pegylated-interferon, we show that HTN, HLD, T2DM, obesity, and metabolic syndrome do not negatively affect SVR12 rates for DAA. Future prospective studies analyzing patient fasting blood glucose and serum insulin should be performed to validate these findings and to help elucidate the relationship between HCV, insulin sensitivity, and longer-term DM outcomes such as cardiovascular events.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) infection is a major worldwide health problem. There are increasing reports indicating an association between HCV and type 2 diabetes mellitus (T2DM). Individuals with HCV are more likely to have risk factors to develop T2DM and patients with T2DM have at least a 2-fold greater risk of developing HCV infection than the general population. Previously standard therapy for HCV required the use of pegylated interferon-α (P-IFN) and ribavirin. However, these regimens had low sustained virologic response (SVR) rates and were poorly tolerated. During the era of P-IFN therapy, several studies showed that the presence of obesity and/or steatosis led to a reduction of SVR rate in HCV patients. In patients with diabetes and HCV who were treated with IFN-based therapies, HCV clearance was associated with improved insulin resistance and beta cell function.

Research motivation

In 2013 and 2014, approval of newer direct acting antiviral agents (DAA) created IFN-free regimens with SVR rates greater than 90%, radically changing HCV treatment. Due to the novelty of DAA regimen, research is being actively pursued in a variety of patient populations.

Research ojectives

We aim to determine if successful treatment with DAA is associated with improvements in hemoglobin A1c (HbA1c) and if the presence of T2DM or metabolic syndrome affects SVR rates.

Research methods

DAA were introduced to the VA Greater Los Angeles Healthcare System (VAGLAHS) at the beginning of April 2014. Therefore, we included all patients being treated with DAA between April 1st, 2014 and April 30th, 2016 for this study.

We performed a retrospective analysis of all HCV patients at the VA Greater Los Angeles Healthcare System treated with DAA therapy between 2014-2016. Separate multivariable logistic regression was performed to determine predictors of HbA1c decrease $\geqslant$ 0.5 after DAA treatment and predictors of SVR 12-wk post treatment (SVR12). Patients with HCV were also included if they had diabetic medications on their medication list during the study period. Patients were excluded from the cohort if they did not have SVR12 data or a HbA1c one year after completion of DAA therapy.

Research results

A total of 1068 patients were treated with DAA therapy between 2014-2016. The presence of T2DM or metabolic syndrome did not adversely affect SVR12. 106 patients had both HCV and T2DM. Within that cohort, patients who achieved SVR12 had lower mean HbA1c pre-treatment (7.35 *vs* 8.60, P = 0.02), and lower mean HbA1c post-treatment compared to non-responders (6.55 *vs* 8.61, P = 0.01). The mean reduction in HbA1c after treatment was greater for those who achieved SVR12 than for non-responders (0.79 *vs* 0.01, P = 0.03). In adjusted models, patients that achieved SVR12 were more likely to have a HbA1c decrease of > 0.5 than those that did not achieve SVR12 (adjusted OR = 7.24, 95%CI: 1.22-42.94).

Research conclusions

In conclusion, this study of a diverse VA patient population demonstrates that HCV clearance with DAAs is associated with a clinically significant decrease in HbA1c. This change was consistent across all genotypes and treatment regimens. The change in HbA1c was independent of changes in BMI and DM medication requirements and so may represent a decrease in host insulin resistance or increased insulin sensitivity. This study substantiates similar studies during the pegylated-interferon era by showing that HCV clearance irrespective of treatment regimen and genotype leads to improved DM outcomes. However, unlike prior studies during the period of pegylated-interferon, we show that hypertension, hyperlipidemia, T2DM, obesity, and metabolic syndrome do not negatively affect SVR12 rates for DAA.

Research perspectives

Future prospective studies analyzing patient fasting blood glucose and serum insulin should be performed to validate these findings and to help elucidate the relationship between HCV, insulin sensitivity, and longer-term DM outcomes such as cardiovascular events.

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ORIGINAL ARTICLE

Observational Study

Chronic hepatitis B virus monoinfection at a university hospital in Zambia

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Abstract

AIM

To characterize antiviral therapy eligibility among hepatitis B virus (HBV)-infected adults at a university hospital in Zambia.

METHODS

Hepatitis B surface antigen-positive adults (n = 160) who were HIV-negative and referred to the hospital after a routine or clinically-driven HBV test were enrolled. Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), platelet count, hepatitis B e-antigen, and HBV DNA were measured. Liver fibrosis/cirrhosis was assessed by physical examination, AST-to-platelet ratio index, and transient elastography. In antiviral therapy-naïve individuals, we described HBV stages and antiviral therapy eligibility per World Health Organization (WHO) and by HBV test (routine *vs* clinical). Elevated ALT was > 19 in women and > 30 U/L in men. Among treatment-experienced individuals, we described medication side effects, adherence, and viral suppression.

RESULTS

The median age was 33 years, 71.9% were men, and 30.9% were diagnosed with HBV through a clinically-driven test with the remainder identified *via* routine testing (at the blood bank, community events, *etc.*). Among 120 treatment-naïve individuals, 2.5% were categorized as immune tolerant, 11.7% were immune active, 35.6% were inactive carriers, and 46.7% had an indeterminate phenotype. Per WHO guidelines, 13 (10.8%) were eligible for immediate antiviral therapy. The odds of eligibility were eight times higher for those diagnosed at clinical *vs* routine settings (adjusted odds ratio, 8.33; 95%CI: 2.26-29.41). Among 40 treatment-experienced HBV patients, virtually all took tenofovir, and a history of mild side effects was reported in 20%. Though reported adherence was good, 12 of 29 (41.4%) had HBV DNA > 20 IU/mL.

CONCLUSION

Approximately one in ten HBV-monoinfected Zambians were eligible for antivirals. Many had indeterminate phenotype and needed clinical follow-up.

Key words: Hepatitis B virus; Liver fibrosis; Treatment; Tenofovir; Africa

Core tip: Data to inform the scale-up of hepatitis B testing and treatment in Africa are badly lacking. Among 120 recently diagnosed hepatitis B surface antigen-positive and HIV negative adults in Zambia, Southern Africa, 10% met the WHO's criteria for immediate antiviral therapy and an additional 40% had an "indeterminate" hepatitis B virus (HBV) phenotype with either elevated alanine aminotransferase or HBV DNA > 2000 IU/mL. Among 40 additional patients who were antiviral therapy-experienced (primarily with tenofovir), tolerability and adherence were good; however, nearly half had incomplete HBV DNA suppression. Effective approaches to retain antiviral-ineligible HBV patients in care will be important in Zambia.

Vinikoor MJ, Sinkala E, Kanunga A, Muchimba M, Nsokolo B, Chilengi R, Wandeler G, Mulenga J, Chisenga T, Bhattacharya D, Saag MS, Foster G, Fried MW, Kelly P. Chronic hepatitis B virus monoinfection at a university hospital in Zambia. *World J Hepatol* 2018; 10(9): 622-628 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i9/622.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i9.622

INTRODUCTION

In Africa, approximately 60 million individuals are chronically infected with hepatitis B virus (HBV). Informed by Asian and European data, international recommendations were developed to guide policymakers and implementers on diagnosis and treatment of chronic HBV in resource-constrained settings[1-3]. In settings with hepatitis B surface antigen (HBsAg) positivity > 2%, both general population testing and targeted testing of populations with higher disease burden are recommended; however, limited real world data from Africa are available. In addition, understanding the proportion of patients diagnosed with chronic HBV infection who would benefit from antiviral therapy in African settings is not well-established.

Several recent studies have informed scale-up of HBV treatment in Africa. In Zambia, integration of HBsAg testing into a population-based HIV survey revealed 5.6% of adults and 1.3% of children to be HBsAg-positive (Zambian Ministry of Health, 2016 #599). A research program in Gambia demonstrated the feasibility of community screening, linkage to a comprehensive liver evaluation, and initiation of antiviral treatment[4]. Among potential key populations (sex workers and men who have sex with men) in West Africa and adults in Ethiopia, approximately 10% met criteria for antiviral therapy but another 50% had markers requiring longitudinal follow-

up[5]. Building on these and other studies, we established an observational cohort study at a referral hospital in Lusaka, Zambia. We described where Zambians are currently being tested for HBV and among those with no prior HBV treatment, we categorized patients into the classical stages of HBV using biochemical, virological, and non-invasive markers of liver fibrosis. We also estimated patient eligibility for antivirals according to international guidelines. Among patients already taking antiviral therapy, we described medication side effects, adherence, and viral suppression.

MATERIALS AND METHODS

Study setting and participant recruitment

In Zambia, 5.6% of adults and 1.3% of children are estimated to be HBsAg-positive[6], and HBV genotypes A1 and E are present at nearly equal proportions[7]. HBsAg testing is part of the evaluation of signs or symptoms of liver disease, and routine testing in Zambia occurs at several settings, including at the blood bank, during community screening events (sometimes integrated with HIV testing), and as part of medical check-ups to obtain a driving license, enroll in college, start a new job, *etc*. Following an HBsAg-positive diagnosis, some Lusaka area residents are referred to University Teaching Hospital (UTH), a public tertiary care and academic medical facility with > 1000 inpatient beds located in Zambia's capital of Lusaka. At UTH, the Department of Internal Medicine hosts a weekly liver clinic that is staffed by three gastroenterology-trained physicians and provides specialty care for patients with acute and chronic liver disease, hepatic lesions, and portal hypertension.

From August 23, 2016 to August 18, 2017, after obtaining the required ethical and government approvals, 160 HBsAg-positive HIV-negative adults (18+ years old) were enrolled in a cohort study at the UTH liver clinic. HIV-HBV coinfected individuals identified during recruitment for the cohort were referred for immediate linkage to and initiation of antiretroviral therapy according to national guidelines[8]. We excluded patients from provinces outside of Lusaka to reduce losses to follow-up. Participants provided written informed consent to be followed for up to five years.

Study measures

Although specific HBV management guidelines are limited in Zambia, international standards of care are followed. At cohort enrollment, we performed a complete physical examination, including vital signs and body mass index (BMI), and extracted data from participants' medical records related to HBV testing, treatment, laboratory and imaging tests, and liver biopsy results when available. A standardized questionnaire was used to document sociodemographic information, comorbid conditions, and to screen for and quantify alcohol consumption using the alcohol use disorders identification test-consumption (AUDIT-C)[9]. Among those already on antiviral drugs for HBV at cohort enrollment, we assessed current or prior drug side effects and self-reported medication adherence in the past 7 d.

Blood was collected for measurement of serum transaminases, hemoglobin, platelet count, hepatitis B e-antigen (HBeAg), and HBV viral load, which was determined using either the Roche COBAS AmpliPrep/COBAS Taqman platform (Pleasanton, CA, United States) or an in-house real time PCR assay[10]. We measured liver stiffness non-invasively with the Aspartate Aminotransferase (AST)-to-platelet ratio index[11] and with transient elastography (TE; Fibroscan 402, Echosens, Paris, France). TE was performed by a nurse or physician trained according to manufacturer guidelines and with experience performing > 500 tests. Testing for hepatitis C and hepatitis delta was not routinely performed as they are rare in Zambia[12,13].

Statistical analysis

Among treatment-naïve patients (*i.e.*, no prior history of antiviral therapy), we described demographics and clinical features stratified by type of HBsAg test (clinical or routine). We compared baseline characteristics measured on categorical scale between clinical and routine diagnosis using Fisher's exact test. For comparison of median between the two groups, we used Wilcoxon rank sum test. Obesity was BMI > 30 and unhealthy alcohol consumption was AUDIT-C of 4+ for men and 3+ for women. Among antiviral naïve participants, we described HBV viral loads, HBeAg, serum transaminase levels, and the proportion of patients with cirrhosis. In our primary analysis, we defined elevated ALT as > 30 U/L for men and > 20 U/L for women per WHO recommendations[3]. We defined persistently elevated ALT as any degree of baseline ALT elevation plus an elevated measurement at > 60 d prior to baseline. Significant fibrosis (equivalent to Metavir fibrosis stages F2-4) was defined as liver stiffness measurement (LSM) of 7.9-9.5 kPa based on a validation study in West Africa[14]. We defined cirrhosis as having at least one of the following: APRI > 2.0[11], LSM > 9.5 kPa[14], or decompensated cirrhosis on physical examination defined by the presence of ascites.

Using ALT, HBeAg, and HBV DNA at enrollment, we categorized treatment-naïve participants into one of the classical stages of chronic HBV infection. Immune tolerant stage was defined as HBeAg-positivity, normal ALT, and HBV DNA > 20000 IU/mL. Immune active HBV was defined as either HBeAg-positive, elevated ALT, and HBV DNA > 20000 IU/mL or HBeAg-negative/unknown with elevated ALT and HBV DNA > 2000 IU/mL. Inactive carriers were those with HBeAg-negative/unknown status, normal ALT, and HBV DNA < 2000 IU/mL. Participants not categorized into one of these three stages were considered to have an indeterminate phenotype[15]. We repeated this categorization using the "conventional" ALT upper limit of normal (*i.e.*, 40 IU/mL). We compared unhealthy alcohol use and obesity

between indeterminate patients with HBV DNA < 2000 and ALT elevation and inactive carriers using a χ^2 test.

We determined eligibility for antiviral therapy if patients met one of these criteria based on WHO guidelines: (1) decompensated cirrhosis; (2) APRI > 2.0; or (3) HBV DNA > 20000 IU/mL with ALT elevation and age > 30 years. We also assessed treatment eligibility per European Association for Study of the Liver (EASL) criteria[2] as follows: (1) cirrhosis by physical examination (with or without decompensation) and detectable HBV DNA; (2) HBeAg-positive and age > 30 years; (3) LSM > 7.9 and HBV DNA > 2000 IU/mL, or (4) ALT > 80 U/L and HBV DNA > 20000 IU/mL. We compared treatment eligibility, per WHO criteria, between those who were diagnosed with a clinically-driven *vs* routine HBsAg test using logistic regression and adjusting for age and sex.

Among treatment experienced patients, we described antiviral therapies received, time on therapy, history of side effects, and self-reported adherence. We reported the proportion with viral suppression (VS) defined as HBV DNA < 20 IU/mL among those on therapy for 2+ years. Analyses were performed using Stata version 14 (Statacorp, College Station, TX, United States). The statistical review of the study was performed by a biomedical statistician. The cohort was approved by the ethics committees at University of Zambia (Lusaka, Zambia) and University of Alabama at Birmingham (Birmingham, AL, United States).

RESULTS

Median age was 33 years (IQR, 26-42), 115 (71.9%) were men, and the majority (n = 84, 52.6%) were recently diagnosed with HBV. The majority were diagnosed at routine HBsAg testing during community/routine medical check-ups (n = 58; 36.2%) or at the blood bank, while 49 (30.6%) were tested due to signs/symptoms of possible liver disease (*i.e.*, clinical test). Current alcohol consumption at "unhealthy levels" was reported by 19 (12.2%) participants. At enrollment, 120 (75.0%) were antiviral therapy naïve. Serum transaminases were available for 145 (90.6%) and 104 (65.0%) had a prior ALT measurement available at a median of 308 d (IQR, 62-469) before enrollment. HBeAg testing was performed for 143 (89.4%), 149 (93.1%) had an HBV DNA measurement, and 97 (60.6%) underwent TE. A description of treatment naïve patients by HBsAg test type (routine *vs* clinical) is shown in Table 1.

Among 120 treatment naïve patients, 5 (4.2%) had decompensated cirrhosis (*i.e.*, ascites) by physical examination. Among the 62 with sufficient data, median APRI was 0.29 (IQR, 0.18-0.51) and 4 (6.4%) had APRI > 2.0. Among the 69 that underwent TE, median LSM was 6.3 kPa (IQR, 4.8-8.3), 6 (8.7%) had LSM suggestive of significant fibrosis, and 14 (20.3%) had LSM suggestive of cirrhosis. A cumulative 17 (14.2%) patients had cirrhosis by either physical examination, APRI, or TE. Median ALT was 23 (IQR, 17-36) and 44 (40.7%) had an elevated ALT at enrollment based on WHO-recommended thresholds. Among the 66 with serial ALT levels, 30 (45.4%) had persistently normal ALT, 20 (30.3%) had intermittently elevated ALT, and 16 (24.2%) had persistently elevated ALT. Median HBV DNA level was 232 IU/mL (IQR, 23-3495) and viral loads were low (*i.e.*, < 2000 IU/mL) for 77 patients (69.4%), moderate (2000-20000 IU/mL) for 16 (14.4%), and high (> 20000 IU/mL) for 18 (16.2%). HBeAg-positivity was present in 18 (18.9%), and among HBeAg-positives, 9 (50.0%) had high and 5 (27.8%) had moderate HBV DNA levels with 4 at HBV DNA < 2000 IU/mL.

Using baseline ALT, HBV DNA, and HBeAg, we categorized 3 (2.5%) treatment-naïve patients as immune tolerant, 14 (11.7%) as immune active, 47 (35.6%) as inactive carriers, and 56 (46.7%) as having indeterminate stage. While 18 of 56 were considered indeterminate due to a missing ALT, HBV DNA, or HBeAg, 29 had HBV DNA < 20000 IU/mL with elevated ALT and 7 had HBV DNA > 20000 with normal ALT. Elevated ALT in patients with indeterminate stage could not be attributed to overweight/obesity or unhealthy alcohol use, as rates of these were similar to those of inactive carriers (data not shown). After applying the conventional ALT threshold (*i.e.*, 40 IU/mL), there were 6 (5.0%) immune active patients, 10 (8.3%) immune active patients, 65 (54.2%) inactive carriers, and 39 (32.5%) with an indeterminate phenotype.

According to WHO guidelines, 13 (10.8%) treatment naïve patients were deemed to be eligible for antiviral therapy at cohort enrollment (Table 2). Of these, five became eligible for decompensated cirrhosis, two for APRI > 2.0, and six for HBV DNA > 20000 IU/mL with ALT elevation and age > 30 years. Among patients diagnosed during a routine HBsAg-test, 4 (4.6%) met WHO treatment criteria *vs* 9 (29.0%) of those with clinically-driven HBsAg testing. After adjusting for age and sex, there was 8 times higher odds of treatment eligibility for clinically *vs* routinely-diagnosed patients (adjusted odds ratio, 8.33; 95%CI: 2.26-29.41). By EASL guidelines, 21 (17.5%) were eligible for antiviral therapy.

Among 40 treatment-experienced patients, 31 were currently taking antivirals at enrollment with the majority (85%) on fixed dose combination TDF plus 3TC. Median time on therapy was 12.2 mo (IQR, 5.0-24.3). Although complete medical records were not available for all patients, many treatment-experienced patients had a history of significant fibrosis/cirrhosis at time of initiation. A minority of patients (n = 6; 20.0%) reported at least one side effect in the past/present attributed to antivirals, including nausea, diarrhea, skin rash, itchiness, dizziness, drowsiness, or mild headache. Among those on therapy at enrollment, 17 of 29 (58.6%) had complete HBV DNA suppression including 5 of 9 with 2+ years on treatment. Among the four individuals with HBV DNA non-suppression during long-term

Table 1 Baseline characteristics of treatment-naïve hepatitis B monoinfected adults referred to a university hospital in Zambia according to setting of hepatitis B virus diagnosis

	Clinical diagnosis (n = 31)	Routine diagnosis (n = 89)	P value
Median age, in years	37 (29-42)	33 (26-41)	0.15
Male sex	22 (71.0)	63 (70.8)	0.99
Education level completed			
None to 6th grade	5 (16.1)	3 (3.4)	0.05[a]
7th to 12th grade	14 (45.2)	49 (55.1)	
College	12 (38.7)	37 (41.6)	
Lifetime alcohol abstinence	13 (41.9)	36 (40.9)	0.92
Herbal medicine use, past month	7 (31.8)	14 (16.3)	0.10
Body mass index			
< 25	23 (76.7)	56 (62.9)	0.35
25-30	5 (16.7)	20 (22.5)	
> 30	2 (6.7)	13 (14.6)	
HBV DNA level, IU/mL			
< 20	4 (15.4)	21 (24.7)	0.12
20-1999	10 (38.5)	42 (49.4)	
2000-19199	4 (15.4)	12 (14.1)	
⩾ 20000	8 (30.8)	10 (11.8)	
HBeAg positive	11 (37.9)	11 (14.5)	0.008[1]
Median ALT, in U/L (IQR)	22 (17-28)	23 (17-36)	0.82
Elevated ALT	8 (32.0)	36 (43.4)	0.31
AST-to-platelet ratio index ⩾ 2.0	4 (21.1)	0	0.002[1]
Liver stiffness measurement in kPa			
< 7.9	4 (25.0)	45 (84.9)	< 0.001[1]
7.9-9.5	2 (12.5)	4 (7.6)	
> 9.5	10 (62.5)	4 (7.6)	

[1]Data with statistical significance. HBV: Hepatitis B virus; ALT: Alanine aminotransferase. All values are n (%) or median (IQR).

therapy, all HBV DNA levels were < 2000 IU/mL. Among those currently on antivirals, median adherence was 100% but 12.5% reported missing at least one dose in the prior 7 d.

DISCUSSION

Approximately 1 in 10 Zambian adults with HBV monoinfection met international guidelines for immediate antiviral therapy, and an additional half had either elevated ALT or HBV DNA > 2000 IU/mL, suggesting the need for further follow-up. Participants diagnosed on suspicion of having HBV were more likely to require therapy compared to those HBsAg tested at routine settings. Antiviral therapy was well tolerated among treatment-experienced participants; however, a subset had HBV DNA non-suppression. These data are some of the first data on modern HBV treatment in Southern Africa and provide insights around the scale-up of testing for and treatment of chronic HBV infection in Africa.

While many chronic HBV patients in Zambia had elevated ALT, relatively few qualified for immediate antiviral therapy when we applied international guidelines. By WHO guidelines around 10% of treatment-naïve patients met criteria and by EASL criteria this was closer to 20%. These results are supported by Gambian data where 4.4% in the community and 9.7% of blood donors met EASL criteria[4]. In a smaller study of female sex workers, men who have sex with men, and inmates in West Africa, 10.0% were also treatment-eligible based on modified WHO criteria[5]. Taken together these research data suggest that the WHO criteria are very stringent and might be missing some patients who could benefit from antiviral therapy. Although not surprising, we also observed that symptomatic patients were more likely to meet treatment eligibility compared to those tested in routine settings. Further data are needed to understand the most efficient way to identify HBV patients who would benefit from antiviral therapy in Zambia and similar settings.

Based on their enrollment values, 35% of our participants were classified as inactive carriers and half had indeterminate HBV phenotypes. Identification of inactive carriers, a group at low risk to progress to cirrhosis or develop HCC[16], is useful in programmatic settings in Africa. For example, at an HBV treatment program in Ethiopia, inactive carriers (also representing around one-third of patients) will be discharged from care after one year in order to focus resources on those more likely to benefit from therapy[17]. Unfortunately, our cohort and others have reported that a substantial percentage of patients cannot be initially categorized as needing or not needing therapy (*i.e.*, indeterminate phenotype). In Senegal, 53% did not qualify for antivirals but had either significant fibrosis, raised ALT, or significant HBV DNA levels[5]. In the HBV Research Network (HBRN) in North America, 38% had indeterminate phenotype at baseline and will be followed longitudinally[15]. Similar to the HBRN, most patients with indeterminate disease stage

Table 2 Hepatitis B virus stage and eligibility for immediate antiviral therapy among treatment-naïve Zambian adults with chronic hepatitis B virus infection *n* (%)

		Overall (*n* = 120)	Clinical diagnosis (*n* = 31)	Routine diagnosis[1] (*n* = 89)	*P*[2]
HBV stage	Immune tolerant	3 (2.5)	2 (6.4)	1 (1.1)	0.16
	Immune active	14 (11.7)	4 (12.9)	10 (11.2)	0.8
	Inactive carrier	47 (35.6)	7 (20.0)	40 (41.2)	0.02[3]
	Indeterminate	56 (46.7)	18 (58.1)	38 (42.7)	0.14
Eligibility for immediate therapy per guidelines	WHO 2015 guidelines	13 (10.8)	9 (29.0)	4 (4.5)	< 0.01[3]
	EASL 2017 guidelines	21 (17.5)	12 (38.7)	9 (10.1)	< 0.01[3]

[1]Routine diagnosis was defined as being tested for hepatitis B surface antigen at the blood bank, antenatal care, a community screening event, or as part of a routine medical check-up; [2]A χ^2 test was used to compare clinically and routinely HBV-diagnosed participants; [3]Data with statistical significance. HBV: Hepatitis B virus; WHO: World Health Organization; EASL: European Association for Study of the Liver.

in Zambia had elevated ALT with HBV DNA < 20000 IU/mL, suggesting non-HBV reasons for ALT elevation. Although we did not find that they were correlated with having indeterminate stage, fatty liver and hazardous alcohol use are potential causes of elevated ALT in HBV patients. Longitudinal follow-up to ascertain whether patients of indeterminate stage develop treatment indications or become inactive carriers is needed in Africa to guide HBV policy[5].

Our data from treatment-experienced Zambians with chronic HBV monoinfection support the feasibility of longitudinal treatment with antiviral therapy in African settings. TDF + XTC was the most common regimen as this fixed-dose combination drug is found in large supply in Zambia to treat and prevent HIV/AIDS. We documented HBV DNA non-suppression among the small group on long-term therapy which requires further follow-up. We presumed suboptimal adherence as the mechanism, although most of our patients self-reported good adherence in the prior week. Our data are contrasted by the PROLIFICA study in the Gambia where 43 of 47 (91.5%) tenofovir-treated HBV monoinfected individuals achieved viral suppression at one year[4]. Lower HBV DNA suppression in our cohort may reflect differences in study design (clinical trial *vs* hospital cohort) or could reflect HBV genotypic differences as Gambia has predominantly HBV genotype E and Zambia has both E and A1, a genotype that rapidly developed lamivudine-resistance in Malawi[18].

This study has several limitations. Most importantly, our cohort is based at one site in Zambia and may not represent other HBV-infected populations. Our cohort is hospital-based and enriched for sicker patients evidenced by the fact that participants referred from clinical settings were eight times more likely to be treatment-eligible compared to other HBsAg-positives. To offset this, we also characterized a subset of participants diagnosed at routine settings such as the blood bank and a population-based survey. Finally, we had incomplete laboratory and clinical data that led to incomplete evaluation of some patients and inflated our estimate of the proportion with indeterminate HBV. We believe the missing data occurred at random and would not have introduced bias into the distribution of HBV stages.

In summary, an HBV monoinfection cohort was established at a referral hospital in Zambia to begin to answer a number of clinical and operational questions around HBV treatment in Africa. We observed that 1 in 10 patients who underwent comprehensive assessment met the WHO criteria for therapy, although the number was higher among those with signs/symptoms and lower among those diagnosed during routine HBsAg testing. These data support scale-up of HBV testing and treatment in Africa, but further operational and clinical research is needed to define the most effective and efficient way to reduce HBV-related mortality and morbidity.

ARTICLE HIGHLIGHTS

Research background

Africa has 60 million individuals living with chronic hepatitis B virus (HBV) infection yet limited data to inform how to identify, link to care, and treat them to reduce the burden of cirrhosis and liver cancer.

Research motivation

Not all HBV patients need antivirals. Estimates on how many do will guide policy implementation. Also, few data are available on patient adherence, retention, and viral suppression with current antiviral drugs.

Research objectives

Our objective was to perform a comprehensive clinical assessment on chronic HBV-infected adults in Zambia and apply international criteria to learn what percentage may need antiviral drugs. In those already on antivirals, we measured the viral control.

Research methods

At a university hospital in Zambia, a cross-sectional assessment of adults (18+ years old) who were hepatitis B surface antigen positive and HIV negative was undertaken during 2016-2017. We used tests available in upper-income settings such as HBV DNA testing and transient elastography to assess HBV in these patients.

Research results

One hundred and sixty enrolled in the study, including 120 who were recently diagnosed with HBV and 40 already on antiviral drugs. The average age was 33, and 72% were men. We found that 1 in 10 met the World Health Organization guidelines to start antivirals; however, nearly 1 in 2 had at least one finding that would need clinical follow-up. Patients diagnosed because of signs or symptoms of HBV were slightly more likely to need antivirals compared to those diagnosed *via* routine testing (such as at the blood bank). Among those

already on the antivirals, few had side effects; however, 41% did not completely control their viral load.

Research conclusions

This is the first Southern African study to apply international HBV criteria.

Research perspectives

Additional data are needed on whether those with high ALT or viral loads at baseline will later need antivirals. HBV testing that focuses on symptomatic individuals could be more efficient (than routine testing for all) to find those needing treatment but more information is needed.

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SYSTEMATIC REVIEWS

Acute liver failure secondary to severe systemic disease from fatal hemophagocytic lymphohistiocytosis: Case report and systematic literature review

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Author contributions: Cappell MS and Hader I are equal primary authors of this work; Hader I wrote drafts of the case report and results sections; and provided medical care for this patient; Cappell MS as mentor edited the case report section, and wrote most of the other sections; Amin M performed all the microscopic pathology, and wrote the pathologic sections of the paper.

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Abstract

AIM

To systematically review liver disease associated with hemophagocytic lymphohistiocytosis (HLH), propose reasonable contraindications for liver transplantation for liver failure in HLH, and report an illustrative case.

METHODS

Systematic review according to PRISMA guidelines of hepatic manifestations of HLH using computerized

literature search *via* PubMed of articles published since 1980 with keywords ("hemophagocytic lymphohistiocytosis" or "HLH") AND ("liver" or "hepatic"). Two authors independently performed literature search and incorporated articles into this review by consensus. Illustrative case report presented based on review of medical chart, and expert re-review of endoscopic photographs, radiologic images, and pathologic slides.

RESULTS

A 47-year-old Caucasian male, was hospitalized with high-grade pyrexia, rash, total bilirubin = 45 g/dL, moderately elevated hepatic transaminases, ferritin of 3300 ng/dL, leukopenia, and profound neutropenia (absolute neutrophil count < 100 cells/mm^3). Viral serologies for hepatitis A, B, and C were negative. Abdominal computed tomography scan and magnetic resonance imaging revealed no hepatic or biliary abnormalities. Pathologic analysis of liver biopsy revealed relatively well-preserved hepatic parenchyma without lymphocytic infiltrates or macrophage invasion, except for sparse, focal hepatocyte necrosis. Bone marrow biopsy and aspirate revealed foamy macrophages engulfing mature and precursor erythrocytes, consistent with HLH. Interleukin-2 receptor (CD25) was highly elevated, confirming diagnosis of HLH according to Histiocytic Society criteria. Patient initially improved after high-dose prednisone therapy. Patient was judged not to be a liver transplant candidate despite model for end stage liver disease (MELD) score = 33 because liver failure was secondary to severe systemic disease from HLH, including septic shock, focal centrilobular hepatocyte necrosis from hypotension, bone marrow failure, and explosive immune activation from HLH. The patient eventually succumbed to overwhelming sepsis, progressive liver failure, and disseminated intravascular coagulopathy. Systematic review reveals liver injury is very common in HLH, and liver failure can sometimes occur. Data on liver transplantation for patients with HLH are very limited, and so far the results have shown a generally much worse prognosis than for other liver transplant indications. Liver transplantation should not be guided solely by MELD score, but should include liver biopsy results and determination whether liver failure is from intrinsic liver injury *vs* multisystem (extrahepatic) organ failure from HLH.

CONCLUSION

This case report illustrates that liver transplantation may not be warranted when liver failure associated with HLH is primarily from multisystem failure from HLH. Liver biopsy may be very helpful in determining the severity and pathophysiology of the liver disease.

Key words: Hemophagocytic lymphohistiocytosis; Acute liver failure; Liver injury; Liver transplantation; Acquired immune hyperactivation; Pancytopenia

Core tip: This work systematically reviews liver disease in hemophagocytic lymphohistiocytosis (HLH), proposes contraindications for liver-transplantation in such patients, and reports an illustrative case. A 47-year-old-man was hospitalized with high-grade pyrexia, total-bilirubin = 45 g/dL, and profound neutropenia. Bone marrow biopsy/aspirate revealed hemophagocytic histiocytes. The patient had HLH, satisfying 6 Histiocytic Society criteria. Liver-biopsy histopathology revealed relatively well-preserved hepatic parenchyma, except for sparse hepatocytic necrosis. The patient was not a liver-transplant-candidate, despite model for end stage liver disease-score = 33, due to multi-organ failure from HLH. He expired from multi-organ failure despite receiving corticosteroids. This case and systematic review reveals patients with HLH may not be liver transplant candidates because of liver failure from multisystem failure.

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INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a syndrome of aggressive immune hyperactivation from hypercytokinemia that frequently causes liver injury and sometimes causes acute liver failure (ALF) which can contribute to the high syndromic mortality. Systematic literature review revealed hundreds of reported cases of HLH in adults, but provides insufficient data on indications and contraindications for liver transplantation for ALF associated with HLH[1]. Liver transplantation for ALF associated with HLH is currently controversial due to prominent systemic morbidities from HLH, the generally poor condition of patients suffering from both ALF and HLH, potential curability of ALF from HLH with HLH-specific therapy alone, and risk of recurrent HLH after liver transplantation[2,3]. A case is reported of fatal HLH in an adult presenting prominently with ALF, with the liver injury primarily due to severe systemic disease from HLH, as documented by liver biopsy and clinical evaluation, and liver transplantation was refused on this basis. This case report is important in illustrating potential pitfalls in liver transplantation for ALF associated with HLH.

MATERIALS AND METHODS

This case was thoroughly reviewed based on medical chart, including re-review of original endoscopic photographs by an expert endoscopist, radiologic images by an expert radiologist, and pathologic slides by an

expert pathologist. The IRB at William Beaumont Hospital, Royal Oak exempted/approved this case report on November 15, 2017.

Literature on hepatic manifestations of HLH was systematically reviewed searching PubMed for articles with the following medical subject headings/keywords ("hemophagocytic lymphohistiocytosis" or "HLH") AND ("liver" or hepatic"), and by reviewing sections on HLH in standard pathology textbooks/monographs. Articles before 1980 were selectively excluded because clinical evaluations before 1980 often lacked currently required clinical tests. Large clinical trials, meta-analyses, systematic reviews, and controlled trials were assigned higher priority than review articles or small clinical series, and individual case reports were assigned the lowest priority. Two authors independently performed a literature search, and decided on which articles to incorporate in this review according to article priority based on consensus. Dr. Cappell has considerable experience in conducting systematic reviews, with 4 published systematic reviews in peer-reviewed journals indexed in PubMed during the last 2 years, and with a PhD in neurophysiology that involved 5 years of training and research in biomedical statistics.

Illustrative case report

A 47-year-old, non-alcoholic, non-obese, Caucasian man with type Ⅱ diabetes mellitus treated with oral glipizide and metformin for 4 years, and no prior liver disease presented with a pruritic, maculopapular rash over his entire body associated with progressive fatigue, pyrexia, and profound jaundice for 7 d that began just after completing a 10-d course of oral trimethoprim-sulfamethoxazole therapy for an upper respiratory infection. Physical examination on admission revealed temperature = 39.5 ℃, blood pressure = 89/48 mmHg, profound jaundice, no abdominal tenderness, no hepatosplenomegaly, no peripheral stigmata of chronic liver disease, and no peripheral lymphadenopathy. Laboratory values included: Leukocyte count = 700/μL (lab normal: 3500-10100/μL), and platelets = 283000/μL (normal: 150000-400000/μL). The sodium = 116 mmol/L (normal: 135-145 mmol/L), and creatinine = 1.67 mg/dL (normal: 0.60-1.40 mg/dL). Total bilirubin = 45.1 mg/dL (normal: 0.3-1.2 mg/dL), direct bilirubin = 26.4 mg/dL (normal: 0.0-0.3 mg/dL), alkaline phosphatase = 490 U/L (normal: 30-110 U/L), aspartate aminotransferase (AST) = 70 U/L (normal: 10-37 U/L), alanine aminoranferase (ALT) = 167 U/L (normal: 9-47 U/L), gamma glutamyl transferase = 730 U/L (normal: 13-60 U/L), albumin = 2.4 g/dL (normal: 3.5-5.1 g/dL), international normalized ratio (INR) = 1.9 (normal: 0.9-1.1), and lipase = 9 U/L (normal: 7-60 U/L). The cholesterol = 661 mg/dL (normal: 70-199 mg/dL), triglyceride = 184 mg/dL (normal: 30-149 mg/dL), and HDL cholesterol = 5 mg/dL (normal: 40-90 mg/dL). Serum IgG level was normal.

The acetaminophen level was 0. Acute viral hepatitis panel was negative for hepatitis A, B, C, and E. Anti-smooth muscle and anti-mitochondrial antibodies were absent in serum. Genotyping for hemochromatosis was negative for C282Y, and was heterozygous for H63D mutation. Abdominal ultrasound and computerized tomographic scans revealed normal hepatobiliary findings. Magnetic resonance cholangiopancreatography revealed a normal biliary tree, and mild, diffuse, hepatosplenic iron deposition. Histopathologic examination of a transjugular liver biopsy revealed relatively well-preserved hepatic parenchyma without lymphocytic infiltrates or macrophages in nearly all the cores (Figure 1A and B), except for sparsely distributed, focal, and small centrilobular hepatocellular necrosis (Figure 1D and E). Although M30 and M65 serum levels were not determined as assays for apoptosis, the patient only had aggregates of nonviable hepatocytes on liver biopsy examination which strongly favors the diagnosis of hepatocellular necrosis over that of apoptosis. Immunohistochemical staining for CD-68, a marker for Kupffer cells and tissue macrophages, revealed only normal-appearing CD-68-positive Kupffer cells (dark brown staining, black arrows) with characteristic slender, elongated morphology in non-ischemic areas (Figure 1C); but revealed an additional population of plump, CD-68 positive macrophages (blue arrows) within areas of focal hepatic necrosis (Figure 1F). CD-71, a marker for nucleated erythrocytes, showed no nucleated erythrocytes (no hemophagocytosis) within the Kupffer cells in the non-ischemic area or within macrophages in the ischemic areas (negative immunostains not shown). Due to his employment as a sewer inspector, leptospirosis was excluded by absence of leptospiral antibodies. He was treated with intravenous (IV) aztreonam and vancomycin after isolating Pseudomonas and group A streptococcus from blood cultures.

Histopathologic examination of a bone marrow biopsy and aspiration smears revealed hypocellular bone marrow due to marked granulocytic hypoplasia and prominent histiocytic hemophagocytosis (Figure 2). Plasma cells present in the marrow showed no light chain restriction, and appeared to be reactive. Concomitant flow cytometry showed no evidence of Hodgkin's lymphoma or leukemia. The patient was diagnosed with HLH by satisfying the following 6 of 8 diagnostic criteria established by the Histiocyte Society (minimum 5 criteria required for diagnosis of HLH): (1) temperature = 39.5℃ (qualifying-criterion: temperature > 38.5℃); (2) cytopenia of 2 blood cell lineages: hemoglobin = 8.6 g/dL (qualifying-criterion: hemoglobin < 9.0 g/dL, lab normal: 13.5-17.0 g/dL), and absolute neutrophil count < 100/μL (qualifying-criterion: < 1000/μL, lab-normal: 1600-7200 cells/μL); (3) fibrinogen = 135 mg/dL (qualifying criterion < 150 mg/dL; lab-normal: 175-375 mg/dL); (4) hemophagocytosis in bone marrow biopsy (qualifying-criterion: present); (5) ferritin = 3100 ng/mL (qualifying criterion: > 500 ng/mL, lab-normal: 14-338 ng/mL); and (6) elevated soluble interleukin 2

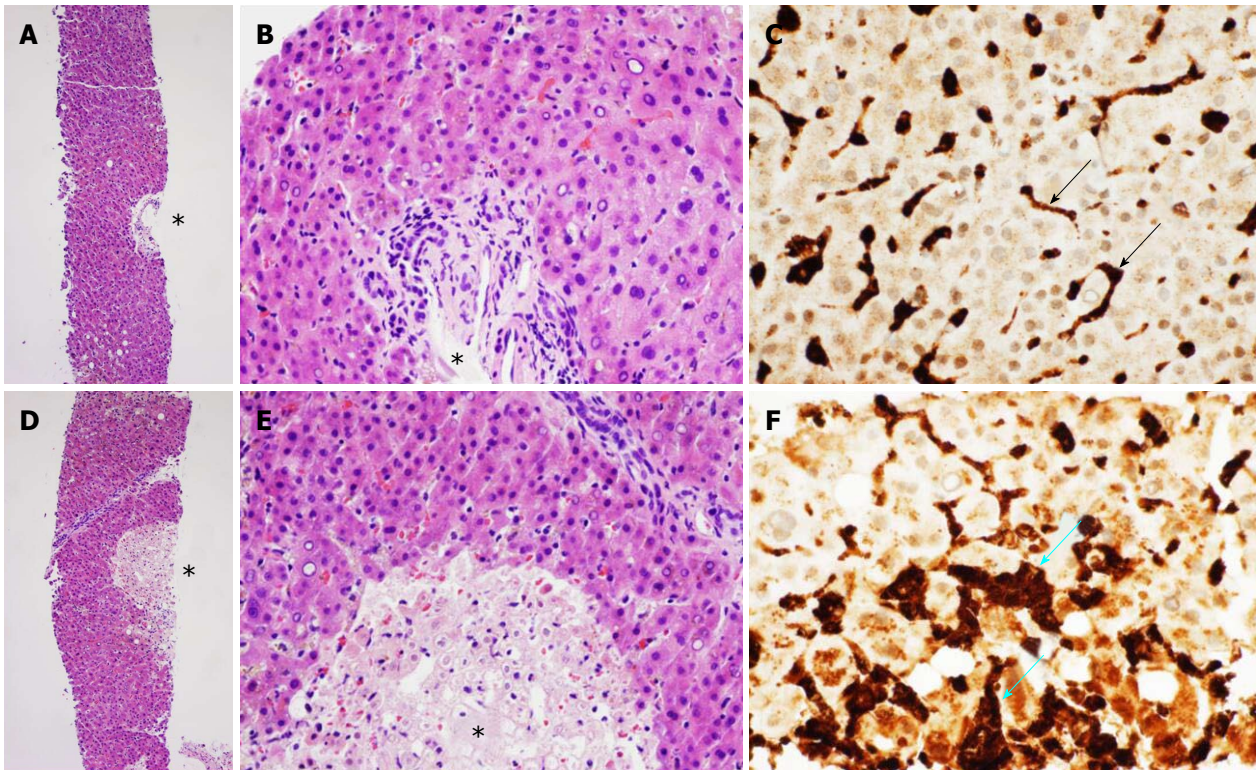

Figure 1 Liver biopsy. A, B: Low-power (A) and high-power (B) photomicrographs of a representative area of hematoxylin and eosin-stained section of the transjugular liver needle biopsy shows a normal portal triad (asterisk) and viable and relatively normal appearing hepatocytes with characteristically deeply eosinophilic cytoplasm and without evidence of hepatocyte necrosis, lymphocytic infiltrates, macrophages or hemophagocytic macrophages/histiocytes; C: Medium power photomicrograph with immunohistochemistry for CD-68, a molecular marker for Kupffer cells and tissue macrophages, shows CD-68 positive (brown staining) cells with elongated and slender morphology characteristic of Kupffer cells (arrows) within hepatic sinusoids; D, E: Low-power (D) and high-power (E) photomicrographs of hematoxylin and eosin-stained section of liver biopsy exhibiting focal centrilobular necrosis (asterisk), seen as a pale, circumscribed area containing pyknotic nuclei in liver biopsy specimen. Despite the liver failure, the areas of necrosis were sparsely distributed, and the overwhelming majority of the liver biopsy specimen contained viable and relatively well-preserved hepatocytes except for the cholestasis, demonstrated by a faint yellowish-brown tint in hepatocytes. No lymphocytic infiltrates or hemophagocytic macrophages/histiocytes are detected in this H and E stain; F: Medium-power photomicrograph with immunohistochemistry for CD-68, a molecular marker for Kupffer cells and macrophages, shows CD-68-positive (brown staining) plump cells with irregular contours characteristic of macrophages (blue arrows). The macrophages have been recruited to phagocytose the dying hepatocytes in the focally ischemic centrilobular area.

(IL-2)-receptor alpha = 9910 pg/mL (normal < 1033 pg/mL) (additionally, Histiocytic Society criterion #2-not satisfied, and criterion #6-not tested). Patient then received prednisone 100 mg orally daily. Liver transplant evaluation concluded that the patient was not a liver transplant candidate despite MELD score = 33 because of severe systemic illnesses. The patient and family refused experimental therapy with artificial hepatic assist devices, such as liver dialysis machines. Patient was not treated with porous CytoSorb polymer beads (CytoSorbents Corporation, Monmouth Junction, New Jersey, United States) to adsorb toxic molecules from septic shock because of no institutional experience with this treatment.

Patient then bled at the prior liver biopsy site because of disseminated intravascular coagulopathy. He became hypotensive, and was endotracheally intubated. Hemostasis was achieved by embolizing two major hepatic artery branches during two arteriogram sessions. He was transfused 13 units of packed erythrocytes, 6 units of fresh-frozen-plasma, 4 units of cryoprecipitate, and 4 units of platelets during this bleeding episode.

During the ensuing 10 d his ferritin increased > 33000 ng/mL, and the bilirubin and transaminase levels modestly declined. Despite temporary clinical stabilization after endotracheal intubation, numerous antibiotic therapeutic regimens, Ⅳ fluids, and Ⅳ vasopressors, the patient succumbed to overwhelming sepsis, disseminated intravascular coagulopathy, and liver failure 42-d after admission.

RESULTS

HLH has no racial or sexual predilection[4]. It most commonly affects infants < 18 mo old, but it can occur in older children and rarely occur in adults. It often presents with nonspecific symptoms and signs[4-6].

HLH has primary (genetic) etiologies, which usually manifest in infants with genetic immunologic mutations[7], and secondary (acquired) etiologies which typically manifest in older children or adults. Secondary etiologies include infection, especially with Epstein-Barr virus[8,9] or herpes simplex virus-1[10]; malignancy; or autoimmune diseases (called macrophage activation syndrome when HLH is associated with autoimmune diseases)[11]. The common denominator in all acquired etiologies is disrupted immune homeostasis.

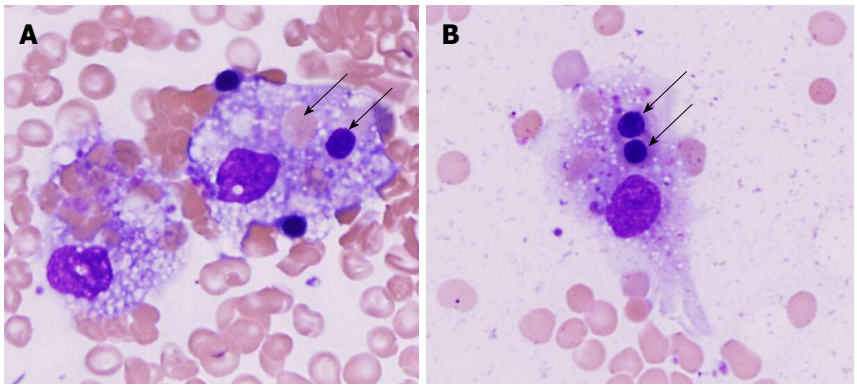

Figure 2 Hemophagocytosis in bone marrow. A: High-power photomicrograph of Diff Quick stain of bone marrow smear showing two large macrophages with foamy cytoplasm within a background of mature erythrocytes. The macrophage on the right has phagocytosed a mature erythrocyte (pink cell with no nucleus, higher arrow) and a nucleated erythrocyte precursor (pink cell with deeply purple nucleus, lower arrow; B: high-power photomicrograph of another large macrophage with foamy cytoplasm containing two phagocytosed nucleated erythrocyte precursor cells (purple nucleus, two arrows).

Prompt diagnosis is critical for patient survival, but the diagnosis is often delayed or overlooked, which contributes to its high mortality. HLH occurs in about 1% of hematologic malignancies[12,13]; sometimes from chemotherapy for these malignancies[14]. Patients with malignancy have extremely high mortality, partly due to delayed diagnosis of HLH[15,16]. Suggested mechanisms of HLH associated with malignancy include: profound inflammation from immune activation, persistent antigen stimulation by cancer cells, and deranged immune response secondary to chemotherapy.

The Histiocyte Society criteria, which are the generally accepted diagnostic criteria, are very strict and can, therefore, miss some cases of HLH (false negatives). HLH should be clinically considered in patients satisfying 3 or 4 criteria[4]. Moreover, the Histiocyte Society incorporates two tests with limited clinical availability: Level of α chain of soluble interleukin-2 receptor (sIL-2r)[17], and 51-Cr release assay reflecting NK-cell activity; unavailability of these tests militate against early diagnosis and therapy. Ferritin levels provide a reliable prognostic indicator and monitor of treatment efficacy[18].

Treatment focuses on controlling the triggering event, such as underlying viral infection, when identified, and on 8-wk-induction-therapy with immunosuppresants, including corticosteroids, etoposide, and cyclosporine, to suppress exaggerated immune responses in HLH. The precipitating event in the current patient was likely the antecedent upper respiratory infection, but the specific (presumably viral) infectious agent was not identified despite extensive (viral) work-up. This patient was treated with high-dose prednisone, but not etoposide or cyclosporine because of the profound hyperbilirubinemia. Allogeneic hematopoietic stem cell transplantation (allo-HSCT), can sometimes achieve complete remission when the patient presents with profound HLH without life-threatening liver failure[19-21].

Patients with established HLH commonly demonstrate markedly elevated biochemical parameters of liver function. For example, among 30 adult patients with HLH, about 60% had AST > 2 times the upper limit of normal[22,23]. Likewise, about 60% of patients had lactate dehydrogenase (LDH) > 2 times the upper limit of normal[22,24]. About 80% of patients present with jaundice[23], which is often profound[25]. The hyperbilirubinemia is predominantly direct. In a study of 28 patients, 9 had moderately severe hypertriglyceridemia, with levels > 265 mg/dL, from cholestasis[26]. Hypoalbuminemia is common, reflecting decreased liver synthetic function[27]. Up to 90% of patients with advanced HLH have severe coagulopathies or disseminated intravascular coagulopathy[22,23,28]. Liver injury is associated with overproduction of cytokines[17]. Severely elevated ferritin levels, and iron overload, detected by hepatic magnetic resonance imaging (MRI), reflect severe hepatic injury.

Lymphocytes commonly infiltrate the liver from immune activation from HLH. For example, in a study of 27 autopsies of infants or children with congenital HLH, 22 (81%) had lymphocytic infiltrates, including 13 (48%) with dense lymphocytic infiltrates[27]. Lymphocytic infiltrates are more commonly periportal than centrilobular[28]. Treatment with corticosteroids or other immunosuppresants may decrease the density and distribution of intrahepatic lymphocytic infiltrates[27]. Macrophages are occasionally present in the liver, but hemophagocytic macrophages are detected in only about 10% of liver biopsies, whereas hemophagocytic macrophages are commonly detected in spleen or bone marrow biopsies[27].

ALF is currently reported secondary to HLH which was diagnosed based on satisfying 6 of 8 criteria for HLH. Despite biochemical evidence of severe hepatic injury, this patient had only focal hepatic necrosis, and relatively well-preserved hepatocytes in the rest of the biopsy sample, without intrahepatic lymphocytic infiltration or hemophagocytic macrophages. This patient had not received corticosteroids or other immunosuppresants prior to liver biopsy, which could have attenuated intrahepatic lymphocytic infiltration. These relatively bland liver biopsy findings in the setting of ALF strongly suggest that liver failure in this patient

with HLH is not necessarily from intrinsic liver disease, say from viral infection or drug toxicity, but may be secondary to multi-organ, systemic, and extrahepatic injury from HLH.

Liver transplantation has been offered to patients who present predominantly with ALF, from severe hepatic inflammation and necrosis, in association with potentially reversible HLH[1-3]. In a literature review of 7 individual case reports, only 2 (28%) of 7 patients undergoing liver transplantation for ALF with HLH survived > 6 mo[2,3]. In a small clinical series, 9 pediatric patients underwent liver transplantation for life-threatening ALF associated with secondary HLH[3]. These patients typically had extremely abnormal liver function tests: ALT = 2512 ± 1158 U/L, (range: 1135-4113 U/L), AST = 3165 ± 2276 U/L (range: 779-8789 U/L), conjugated bilirubin = 257 ± 108 micromol/L (range: 141-448 micromol/L, normal 0-2 micromol/L), and INR = 7.7 ± 1.7 units (range: 3.9- > 9 units). Liver biopsy in these patients generally revealed severe-to-massive (25%-95%) hepatocyte necrosis, lymphocytic infiltrates, numerous macrophages, and intrahepatic erythrophagocytosis[3]. These patients had extremely frequent medical and surgical complications after transplantation, including: severe or opportunistic infections-6, acute liver rejection-5, recurrent HLH-5, bile duct strictures-3, post-transplant lymphoproliferative disease of liver-2, bowel obstruction-1, and wound dehiscence-1. Three patients died at 1.5, 8, and 14 mo after liver transplantation. The other 6 pediatric patients were alive and well at a median of 24 mo after liver transplantation[3].

In contradistinction, the currently reported patient had much less evident liver injury, but severe clinical manifestations of HLH. The current work may suggest that liver transplantation is not indicated for HLH when: (1)-ALF is not present or imminent (MELD score < 20-22); (2)-the patient has a poor prognosis due to the combined effects of ALF and HLH; (3)-the liver injury by itself does not underlie this poor patient prognosis; or (4)-the HLH is advanced and highly likely irreversible. This case illustrates three of these factors that militated against liver transplantation; in the current case the ALF was most likely from septic shock with focal centrilobular ischemia from hypotension, bone marrow failure, and explosive immune activation from HLH.

This prior clinical data suggest caution in liver transplantation for HLH. The data are limited. Reported outcomes for liver transplantation with HLH are much worse than that for liver transplantation without HLH[3]. The current work suggests that high mortality from advanced and likely irreversible HLH may limit the benefits of liver transplantation. Liver biopsy may be very helpful to assess the liver injury and to determine whether ALF associated with HLH is due primarily to intrinsic liver injury *vs* secondary to extrahepatic injury from HLH to help determine whether liver transplantation is warranted.

About 25% of patients had a rash associated with HLH in one large study[28]. The rash is most commonly generalized, maculopapular, and erythematous[29]. Patients can also have petechiae and purpura from thrombocytopenia. This patient's rash started after administering trimethoprim-sulfamethoxazole and was most likely secondary to it.

DISCUSSION

HLH frequently involves the liver. It frequently causes AST levels > 2 times the upper limit of normal[22,23], frequently causes LDH levels > 2 times the upper limit of normal[22,24], and very frequently causes profound direct hyperbilirubinemia[23,25]. Patients may also commonly present with moderately severe hypertriglyceridemia from cholestasis[26], and hypoalbuminemia from decreased liver synthetic function[27]. The liver injury is associated with overproduction of cytokines[17], severe hepatic inflammation, and potentially hepatic necrosis.

When patients present with imminent or confirmed liver failure from predominantly liver involvement from HLH, liver transplant may be considered[1-3]. In such cases, the liver failure may be documented by a MELD score > 24-26, and the liver failure should contribute significantly to the poor patient prognosis. Liver biopsy should be performed to determine the extent of the liver injury and the relative roles of intrinsic liver injury *vs* systemic HLH in the patient's ALF. However, the current case illustrates that patients may be poor candidates for liver transplantation from ALF secondary to HLH, if the HLH is likely irreversible, or if the patient has major comorbidities (systemic complications) from the HLH that are highly lethal. For example, the currently reported patient had highly lethal, comorbidities of overwhelming sepsis, shock, and disseminated intravascular coagulopathy, that precluded liver transplantation despite having a MELD score of 33 that would normally qualify the patient for liver transplantation. This work illustrates that decisions on liver transplantation in patients with HLH depend upon the presence of imminent or present ALF, the potential reversibility of the HLH, and the absence of severe comorbidities associated with the HLH that are most likely fatal. Patients with high MELD scores in the setting of HLH should be evaluated for liver transplantation before these highly lethal complications supervene.

Study limitations include: (1) this is a single retrospectively reported case report; (2) the etiology of HLH was undetermined, as occurs in about 25% of adult cases[4,30]; (3) the microbiology of the antecedent upper respiratory infection, that likely stimulated the HLH, is unknown; (4) the reported pathologic findings at liver biopsy are subject to sample bias; and (5) the possible, but unlikely, role of trimethoprim-sulfamethoxazole in the ALF[31]. Study strengths include the well-established diagnosis of HLH, and the academic evaluation of this patient at a tertiary-care-university hospital with a liver

transplant center.

ARTICLE HIGHLIGHTS

Research background

Hemophagocytic lymphohistiocytosis (HLH) is a syndrome of aggressive immune hyperactivation from hypercytokinemia that frequently causes liver injury and sometimes causes acute liver failure (ALF) that can contribute to the high syndromic mortality. Systematic literature review revealed hundreds of reported cases of HLH in adults, but provided insufficient data on the patterns and pathophysiology of the liver injury. It is particularly important to clinically determine whether ALF associated with HLH is due primarily to intrinsic liver injury *vs* secondary to extrahepatic causes from the HLH, to help determine whether liver transplantation is potentially warranted. A case is reported of fatal HLH in an adult presenting prominently with ALF, with the liver injury secondary to severe systemic disease from HLH, as documented by liver biopsy and clinical evaluation, and liver transplantation refused on this basis.

Research motivation

It is clinically important to determine whether ALF associated with HLH is primarily from intrinsic liver injury *vs* secondarily from extrahepatic injury from HLH to help determine the potential efficacy of liver transplantation for ALF associated with HLH. ALF from direct liver injury from HLH might be treatable by liver transplantation, whereas liver injury secondary to extrahepatic damage from HLH would be unlikely to be successfully treated by liver transplantation.

Research objectives

To determine whether ALF associated with HLH is due primarily to intrinsic liver injury *vs* secondary to extrahepatic injury from HLH to help determine whether liver transplantation is potentially warranted. This study involves systematic review of the literature on liver injury associated with HLH. Additionally, a case report is presented to illustrate that a patient with ALF secondary to HLH may be an inappropriate candidate for liver transplantation, despite a high model for end stage liver disease (MELD) score, when the patient has other likely lethal complications of HLH, such as overwhelming sepsis, septic shock, or disseminated intravascular coagulation secondary to explosive immune activation from HLH.

Research methods

Literature on hepatic manifestations of HLH, including liver injury or ALF secondary to HLH, was systematically reviewed by computerized search using PubMed for articles with the following medical subject headings/keywords ("hemophagocytic lymphohistiocytosis" or "HLH") AND ("liver" or hepatic"), and by reviewing sections on HLH in standard pathology textbooks/monographs. Articles before 1980 were selectively excluded because clinical evaluations before 1980 often lacked currently required clinical tests of hepatic function, radiologic imaging of hepatic anatomy, and modern criteria for HLH. Large clinical trials, meta-analyses, systematic reviews, and controlled trials were assigned higher priority than review articles or small clinical series, and individual case reports were assigned the lowest priority. Two authors independently performed a literature search, and decided on which articles to incorporate into this review according to article priority based on consensus. Dr. Cappell has considerable experience in conducting systematic reviews, with 4 systematic reviews in peer-reviewed journals, indexed in PubMed, published during the last 2 years, and with a PhD in neurophysiology that involved 5 years of training and research in biomedical statistics. A case report is presented to illustrate that a patient with ALF secondary to HLH may be an inappropriate candidate for liver transplantation, despite a high MELD score, when the patient has other, likely lethal, complications of HLH. This case report was thoroughly analyzed based on the medical chart, including re-review of original endoscopic photographs by an expert endoscopist, radiologic images by an expert radiologist, and pathologic slides by an expert pathologist.

Research results

This systematic review shows that HLH frequently involves the liver. It frequently causes aspartate aminotransferase levels > 2 times the upper limit of normal, frequently causes LDH levels > 2 times the upper limit of normal, and very frequently causes profound direct hyperbilirubinemia. Likewise, patients commonly present with moderately severe hypertriglyceridemia from cholestasis, and hypoalbuminemia from decreased liver synthetic function. The liver injury is associated with overproduction of cytokines, severe hepatic inflammation, and potentially hepatic necrosis. When patients present with imminent or evident ALF predominantly from direct liver involvement from HLH, liver transplant may be potentially indicated. In such cases, the ALF may be documented by a MELD score > 24-26, and the ALF should contribute significantly to the poor prognosis. However, the currently reported case illustrates that patients may be poor candidates for liver transplantation from ALF secondary to HLH, when the HLH is likely irreversible, or if the patient has potentially lethal, major, comorbidities (systemic complications) from the HLH. For example, the currently reported patient had highly lethal, comorbidities of overwhelming sepsis, shock, and disseminated intravascular coagulopathy, that precluded liver transplantation despite having a MELD score of 33, which would normally have qualified the patient for liver transplantation. This work illustrates that decisions on liver transplantation in patients with HLH depend upon the presence of imminent or evident ALF, the potential reversibility of the HLH, and the absence of severe and most likely fatal comorbidities associated with HLH.

Research conclusions

HLH frequently involves the liver and can directly cause severe liver injury or ALF from hepatic inflammation and hepatic necrosis from explosive immune hyperactivation related to hypercytokinemia. HLH can also indirectly cause livery injury from extrahepatic disease secondary to HLH, such as overwhelming sepsis, septic shock, hypoxemia, and disseminated intravascular coagulation. It is important to determine the relative contributions of these two alternative mechanisms of liver injury when contemplating liver transplantation for HLH. Intrinsic liver disease may respond to liver transplantation, whereas liver injury secondary to extrahepatic causes, such as septic shock, is unlikely to respond to liver transplantation if the extrahepatic causes are irreversible.

Research perspectives

This work places in perspective that decisions on liver transplantation for ALF associated with HLH must consider not only the functional status of the liver, as indicated by the MELD score, but the etiology of the liver injury. When the liver injury is directly from the HLH (*e.g.*, hepatic inflammation and necrosis from hypercytokinemia) liver transplantation may be considered, whereas when the liver injury is secondary to extrahepatic (other organ) failure (*e.g.*, overwhelming sepsis, hyperimmune activation in other organs, disseminated intravascular coagulation, or respiratory failure) liver transplant is unlikely to be successful. This concept is illustrated by a case report wherein the ALF associated with HLH was secondary to extrahepatic causes and not amenable to liver transplantation, and by systematic review of liver injury from HLH. This work may prove useful to clinicians in decisions on whether to perform liver transplantation for ALF associated with HLH.

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LETTERS TO THE EDITOR

Protecting kidneys in liver transplant patients: A pathway to preventive interventions

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Abstract

Acute kidney injury (AKI) is a frequent postoperative complication after liver transplantation. The etiology is multifactorial, including perioperative renal status, surgery related events, and postoperative immunosuppression therapy. The role of renal hypoperfusion and hepatic ischemia-reperfusion injury as causes of early AKI are now being increasingly recognized. Further studies should focus on therapies that would attenuate this injury.

Key words: Acute kidney injury; Liver transplantation; Hepatic ischemia-reperfusion injury; Marginal grafts; Small interfering ribonucleic acid

Core tip: Acute kidney injury early post liver transplantation is a major cause of morbidity and mortality. The etiology is multifactorial. The renal hypoperfusion and hepatic ischemia-reperfusion injury are being increasingly recognized. Further studies need to focus on therapies that would attenuate this injury.

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TO THE EDITOR

Acute kidney injury (AKI) is a frequent and serious

postoperative complication in the early period after liver transplantation (LT) and is believed to be a major cause of morbidity and mortality. The incidence of postoperative AKI varies widely, from 17% to 95% of the recipients, with some requiring renal replacement therapy. The etiology is suggested to be multifactorial and related to almost all aspects of perioperative management. The factors that have been widely discussed in the literature concentrate on the recipient factors including their preoperative renal status, surgery-related events, including blood loss, hypotension, and postoperative immunosuppression therapy effects, such as calcineurin inhibitor–induced vasoconstriction. Based on these independent risk factors, many preventive interventions to reduce the risk of renal injury have been instituted.

Renal hypoperfusion during liver transplantation has been recognized as an important cause of AKI for a long time and studies have estimated, that ischemic and hypovolemic acute tubular necrosis are the cause for AKI in more than a third of liver transplant recipients[1]. Recent progress has been made in the field of open heart surgery, another surgical procedure, with a high incidence of ischemic and hypovolemic AKI. A small interfering ribonucleic acid (siRNA) targeting p53 developed by Quark Pharmaceuticals, has been developed for prevention of Delayed Graft Function following renal transplantation but we now have evolving evidence that use of this siRNA during cardiac surgery results in a 26% relative risk reduction in the incidence of AKI[2]. Consideration should be given to also study the use of this siRNA in liver transplantation.

Presently, the role of hepatic ischemia- reperfusion injury (IRI) in the pathogenesis of AKI early after liver transplantation is being increasingly recognized. IRI is defined as cellular damage after the reperfusion of the previously viable ischemic tissues, *i.e.,* liver allograft. Reperfusion of the liver allograft is associated with increased reactive oxygen species production and inflammation, resulting in renal tubular cell injury and death. The severity of the IRI and the postoperative systemic inflammatory response, which is the common pathway in organ dysfunction, including kidney injury, has been linked to the use of the marginal organs, including liver grafts from older donors, liver grafts with prolonged cold ischemia time, graft steatosis, split liver allografts, and donation after circulatory death (DCD). Leithead *et al*[3] demonstrated that in the immediate postoperative period DCD liver transplantation was associated with an increased incidence of AKI [DCD 53.4%; donor after brain death (DBD) 31.8%, P = 0.004]. In their study increased peak perioperative aspartate aminotransferase (AST), a surrogate marker of hepatic ischemia-reperfusion injury, was the only consistent predictor of renal dysfunction after DCD transplantation (OR 7.44, 95%CI: 2.78–19.88, P < 0.001). Rahman *et al*[4] showed that peak serum AST was the only consistent predictor of AKI in multivariate analysis (OR 1.001, 95%CI: 1.00-1.001, P = 0.02), suggesting that hepatic ischemia-reperfusion injury may play a critical role in the pathogenesis of post-transplant renal dysfunction. In various studies, AKI and IRI were associated with a longer time to extubation, increased length of intensive care unit stay and reduced patient and graft survival. AKI has also been shown to be a risk factor for chronic kidney disease[3,4].

Recently, the growing demand for organs, has necessitated the increased use of more marginal liver grafts, potentially leading to the increasing incidence of the IRI and AKI. Given the high incidence of post-LT AKI and its significant impact on recipient survival and complications, further research should be aimed at the attenuating hepatic ischemia-reperfusion injury, while investigating its effects on preventing post-LT AKI and eventually improving outcomes. Because preventive strategies are limited and the evidence is still lacking, further studies should focus on therapies, such as liver machine perfusion and pharmacologic therapies similar to siRNA approaches, that silence genes associated with ischemic AKI or ischemia-reperfusion injury, and participation in clinical trials would elucidate the translational research from the bench to the bedside.

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EDITORIAL

European Association for the Study of the Liver and French hepatitis C recent guidelines: The paradigm shift

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Abstract

The latest Association Française pour l'Etude du Foie - French Association for Study of the Liver (AFEF) and European Association for the Study of the Liver (EASL) recommendations announce a change of paradigm, for the management of patients infected with hepatitis C virus (HCV). The AFEF recommendations focus on the elimination of HCV infection on a national level by preventing reinfection, in less than ten years. This goal involves the facilitation of patients' management in a simplified pathway by increasing screening procedures and access to pangenotypic treatments mainly in the "reservoir" population of people who inject drugs and migrants. Even in the complex pathway of patients with previous comorbidities, AFEF takes the option of a therapeutic simplification. The EASL guidelines position themselves on the state of the art with a precise description of all therapeutic options available, without separating simplified and complex pathways even if they take into account the epidemiological evolution of difficult-to-treat populations.

Key words: French; European; Hepatitis C; Guidelines; Pangenotypic; Direct acting antiviral drugs; Eradication; People who inject drugs; Migrants

Core tip: New French and European guidelines for the management of hepatitis C virus infection take into account the rapid change in the epidemiology of the infection and the arrival of short treatments, based on pangenotypic drugs with very few side effects. However, the French guidelines have a strong bias towards viral eradication with the elaboration of a simplified pathway for patients who are far from traditional healthcare structures.

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Hepatitis C treatment history extends over approximately a quarter of a century from standard interferon for non-A non-B hepatitis, through combination with ribavirin at the end of the 1990s, to the availability of pegylated interferon in the early 2000s. It took 25 years to go from 5% to 55% of sustained virological response (SVR). The arrival of new direct-acting antiviral agents (DAAs) has revolutionized hepatitis C management in the last five years, even if the first protease inhibitors (PIs) initially associated with pegylated interferon and ribavirin greatly increased the global side effects. In fact, very quickly after just over a year, next generation DAAs in interferon-free regimen were available.

As a first step, the cost of drugs which were recommended for severe patients in most countries limited their use: In 2014-2015, sofosbuvir-based regimens combined with simeprevir, daclatasvir or ledipasvir were reimbursed by the French health insurance only for severe fibrosis, extra-hepatic manifestations, human immunodeficiency virus (HIV)-co-infected, transplanted and hemodialysis patients, and this, despite a tremendous decrease of side effects and a shortening of treatments. The extension of indications to F2 fibrosis according to the METAVIR classification, corresponded in 2016 with the marketing of ombitasvir paritaprevir/ritonavir and dasabuvir and finally, in 2017, universal treatment access in France was official. In 2018, thanks to pangenotypic associations' availability, a really ambitious median term goal of virus eradication becomes increasingly realistic.

During a 5-year period, multiple American, European and national guidelines were proposed trying to follow the tremendous therapeutic revolutions. The last 2018 recommendations that correspond to the marketing of pangenotypic associations are a real paradigm shift. We will focus on French (AFEF, Association Française pour l' Etude du Foie - French Association for Study of the Liver) and European Association for Study of the Liver (EASL) recent guidelines, highlighting the marked strategic differences.

The EASL recommendations are the state of art on hepatitis C in 2018[1]. They aim at "describing the optimal management of patients with chronic HCV infection in 2018". The French recommendations are brief, simplified, and avant-garde[2]. Their goal is "the elimination of hepatitis C virus (HCV) infection in France" before 2025 if possible (Table 1).

Of course, both guidelines highlight the epidemiological changes. In France, for example, it is estimated that the majority of HCV patients are represented by people who inject drugs (PWID, 95000 estimated patients), 46% of them being viremic and to be treated. The second most difficult population to assess is the migrant population (90035 estimated patients), with 57% of them estimated to be viremic. Today, 90% of patients transfused before 1992 are diagnosed and treated[3,4].

Among PWID, 30% attend addiction centers and the others, who are difficult to quantify, consult general practitioners who deliver opioid substitution treatments (OSTs): In the French survey HEPCORT 2011, the prevalence of HCV seropositivity was 26% in general practice on patients under OSTs with so-called "non-problematic" consumption[5]. Another important source of contamination is prison in which 70% of prisoners are PWID for whom prevalence of anti-HCV antibodies is 4.8%, 46.4% being HCV RNA (+): Thus, 2.5% of detainees are viremic for HCV[6].

According to the French recommendations, elimination of HCV infection could be possible by 2025, 2030 according to the United States Global Burden of Disease. The different guidelines advocate the eradication of the virus, made possible thanks to simple diagnostic methods and highly effective treatments, provided that screening policies are intensified and access to treatment promoted. The first proposal of AFEF recommendations is to screen every adult at least once in his life for combined HBV and HIV and HCV viruses, and a 100% reimbursement of the screening tests. Moreover, the principle of "all inclusive" in the management of particular target populations requires the use of new screening methods. In addition to the rapid diagnostic tests (RDTs) which were known to have excellent sensitivity and specificity (99%)[7,8], but only detect antibodies, EASL mentions the need for the development of Core Ag, dried blood spots, allowing HCV RNA rapid availability in patients who are difficult to collect. The principle of "reflex testing" is still in the experimental stage but is a way to obtain real time HCV RNA even if many problems remain to be solved including the cost.

The need for pre-therapeutic genotyping is addressed by AFEF and EASL. In the area of the availability of pangenotypic therapeutic associations, both guidelines consider that genotyping is not mandatory: In a "simplified pathway" for AFEF, or "in areas where genotyping is not available and/or not affordable, or simplify treatment access" for EASL. However, screening for initial fibrosis remains the key for both academic societies in simplified pathways for specific populations and in complex or specialized pathways. It determines the duration of the treatment and is essential for the follow-up especially the long-term detection of complications such as hepatocellular carcinoma or portal hypertension. FibroScan® (transient elastography) that measures liver stiffness in a non-invasive way is an educational and motivational tool for AFEF, qualities that were confirmed in several experiments available in addiction centers[9-11].

AFEF proposes FibroScan® or complex fibrosis biological tests thresholds, to rule out the diagnosis of severe fibrosis and therefore to identify patients who will not require prolonged follow-up after virological cure except for the presence of hepatic co-morbidities (Liver stiffness with FibroScan® < 10 kPa or FibroTest® ⩽ 0.58

Table 1 French and European Association for the Study of the Liver recommendations principal similitudes and differences

	French recommendations	EASL recommendations
Target audience	National	European, international
Philosophy	Goal of HCV eradication Maximum simplification of HCV management	State of art
Screening	Global "Test and treat"	Global "Test and treat"
Fibrosis	FibroScan®, FibroTest®, FibroMeter®	Enlarged to simple and accessible biological methods, APRI, Fib4
RAS screening	Only in case of previous failure to DAA treatment	May be used, in addition and if available, before treatment to optimize some non pangenotypic strategies
Prescribers	Hepatologists or general practitioners	Hepatologists
Regimens	Preferably pangenotypic associations sofosbuvir - velpatasvir 12 wk or glecaprevir - pibrentasvir 8 wk if no severe fibrosis	Pangenotypic and no pangenotypic associations according to genotype, viral load, degree of fibrosis, previous treatment, and eventual RAS No sofosbuvir - velpatasvir in case of G3 cirrhotic patients
In case of failure	RAS screening Only for first generation DAAs failures Sofosbuvir - velpatasvir - voxilaprevir 12 wk, sofosbuvir - velpatasvir - voxilaprevir with or without ribavirin 12-24 wk in G3 cirrhotic patients	RAS screening In addition, for patients with poorer prediction of response sofosbuvir - glecaprevir - pibrentasvir and sofosbuvir - velpatasvir - voxilaprevir 12-24 wk with or without ribavirin according to multidisciplinary decision
Decompensated cirrhosis	Regimen without protease inhibitors	Regimen without protease inhibitors
Renal insufficiency	Glecaprevir - pibrentasvir or, grazoprevir - elbasvir (G1) 12 wk	Glecaprevir - pibrentasvir or grazoprevir - elbasvir (G1), 8-12 wk

APRI: Aspartate aminotransférase to Platelet Ratio Index; DAA: Direct acting antiviral; EASL: European Association for the Study of the Liver; HCV: Hepatitis C virus; RAS: Resistance-associated substitutions.

or FibroMeter® $\leqslant$ 0.786). EASL retains APRI and FIB4 as an alternative in the absence of other local resources, even if the sensitivity and specificity are worse[12]. If FibroScan®, FibroMeter® and FibroTest® are easily available in France and many European countries, APRI and FIB4 can be instantly applied in all geographical area. For both academic societies, the screening strategy of particular populations in a "test and treat" goal, is therefore crucial and demonstrates an individual but also collective benefit.

The collective benefit, treating to prevent contamination in PWID has been demonstrated in various English, Australian and Icelandic experiments[13,14]. Interestingly, in several Eastern European countries, it has been shown that a global strategy - increasing screening, risk prevention with access to sterile syringes, in situ delivery of antiviral treatment associated with OSTs - reduced by almost 80% new HCV cases while the prescription of DAAs alone had an impact of only 10%[15]. Finally, one study unexpectedly suggested that accepting a diagnostic test for HCV in substitution centers, whether positive or negative, could have an impact on drug use[16].

Apart from these findings, the French recommendations commit themselves to a more proactive approach to facilitate diagnosis, treatment and eradication: "The treatment of hepatitis C must be prescribed by all doctors", "Treatment monitoring can be performed by non-medical caregivers", "Direct antiviral agents should be available in all pharmacies". Prescription by all doctors might be still a little premature and requires a culture change and systematic training. In a recent Australian experiment[17], dating from 2016, the opening of the prescription to general practitioners allowed access to treatment of rather disadvantaged populations, far from urban areas; however, much remains to be done as 58% of these prescriptions represented less than 12% of hepatitis C cases. Cost reduction and second-generation treatments generating fewer drug interactions, have allowed direct prescribing of DAAs without prior multidisciplinary consultation except in the following difficult cases: Prior DAA treatment failure, chronic renal disease, severe cirrhosis, liver cancer, co-infection with HBV or HIV, transplantation. Task delegation for therapeutic follow-up is possible as it was suggested that patients' attendance at consultations in addiction treatment centers was better with nurses than with general practitioners and specialists[18] and comparable results were experienced with the inmates[19].

Of course, according to AFEF recommendations, certain conditions are unavoidable for universal prescribing in a simplified pathway by non-specialists: Absence of HBV and/or HIV co-infection, severe renal insufficiency (eGFR < 30 mL/min per 1.73 m²), poorly controlled hepatic comorbidities (risky alcohol consumption, diabetes and obesity), severe hepatic disease, prior DAAs therapy. After ruling out the diagnosis of severe fibrosis by non-invasive methods, and in the absence of genotyping determination in the simplified pathway, the two pangenotypic therapeutic options recommended are: Sofosbuvir + velpatasvir for 12 wk and glecaprevir + pibrentasvir for 8 wk. A simple evaluation of the drug interactions is easy to do by consulting the website: https://www.hep-druginteractions.org/ or by using the smartphone app HEP iChart. Virological cure must be assessed by measuring viral load 12 wk after stopping treatment. All patients who do not meet these specifications are taken care of in a specialized pathway.

EASL guidelines do not distinguish between two types of patient pathways, even though specificities related to the management of PWID are clearly reported: Screening methods described above, *in situ* HCV RNA evaluation or easier, core antigen undetectability in serum or plasma 24 wk (SVR24) after the end of treatment, are an alternative endpoint of therapy in patients with detectable HCV core antigen prior to therapy.

In specialized patient pathways, AFEF recommendations also have a simplification bias focusing on the recommendations of pangenotypic associations. A minimal opening for non-pangenotypic options is left with the pre-requisite, of course, of systematic genotype knowledge: Sofosbuvir ledipasvir for G1 without severe fibrosis and grazoprevir elbasvir for genotype 1b, genotype 1a with an initial viral load ⩽ 800000 IU/mL and treatment naive genotype 4.

Some differences between both academic societies can be highlighted: EASL states the possibility of an 8-wk treatment with grazoprevir elbasvir for patients with genotype 1b[20] without severe fibrosis, and still finds relevant the ombitasvir paritaprevir dasabuvir combination for genotypes 1b or 4, during 8 or 12 wk whereas AFEF considers this combination obsolete. In many geographic areas however, this latter combination stays as a very good option, as studies from real life demonstrate its efficacy and safety in chronic hepatitis C, even in people with compensated liver cirrhosis[21].

A divergent point is also, according to EASL, the absence of recommendation of sofobusvir velpatasvir for G3 cirrhotic patients, the expected response being suboptimal (89% to 93% SVR)[22], while the AFEF maintains the indication of the association in this circumstance in a simplification goal.

The determination of pre-therapeutic resistance-associated substitutions (RAS) in situations that could have been demonstrated useful for some initial therapeutic options is no longer relevant for AFEF. On the other hand, EASL specifies that "in areas where only regimens that require optimization based on pre-treatment resistance testing are available, and physicians have easy access to a reliable test that evaluates HCV resistance to NS5A inhibitors", these analyses can guide decisions, as specified in the EASL recommendations for treatment of hepatitis C 2016[23].

In decompensated cirrhosis, conventionally managed by pangenotypic combinations without PIs and with ribavirin at standard doses, EASL states that increasing doses of ribavirin may be tested in terms of tolerance, and that a 24-wk regimen without ribavirin is possible in patients who have a contraindication or are intolerant to ribavirin.

AFEF only gives recommendations for patients who failed first-generation DAAs: Sofosbuvir + velpatasvir + voxilaprevir for 12 wk[24]. In genotype 3 patients without cirrhosis, the SVR was higher than in patients with cirrhosis, respectively 99% *vs* 93% and, sofosbuvir + velpatasvir + voxilaprevir with or without ribavirin for 12 to 24 wk was recommended for genotype 3 patients with cirrhosis (expert opinion). However, EASL offers solutions for patients with poorer prediction of response (several lines of treatment, advanced disease, complex RAS anti NS5a) in a multi-disciplinary decision: Sofosbuvir + glecaprevir + pibrentasvir for 12 wk[25,26] and for very difficult to retreat DAAs failures (NS5A RASs after at least two failures of a PI and an anti-NS5a): Sofosbuvir, velpatasvir, voxilaprevir + ribavirin or sofosbuvir + glecaprevir + pibrentasvir + ribavirin, and in case of intolerance to ribavirin an extension of treatment, from 16 to 24 wk (expert opinion).

For both academic societies, the post-transplantation treatment must be initiated early on stabilization (3 mo) of the patient and must include immunosuppression with therapeutic drug monitoring (TDM) of immunosuppressive (IS) treatments during DAAs treatment and after cessation. AFEF proposed sofosbuvir + velpatasvir for 12 wk or glecaprevir + pibrentasvir for 12 wk. EASL recommends mainly sofosbuvir + velpatasvir or sofosbuvir + ledipasvir without IS dose adjustments and glecaprevir + pibrentasvir only if eGFR < 30 mL/min per 1.73 m² and with IS dose adjustments. In fact, IS dose adjustment is essential regardless of the therapeutic associations used, even if the risk of imbalance of immunosuppression is greater with glecaprevir and pibrentasvir.

In case of renal insufficiency, for AFEF and EASL, if the eGFR is < 30 mL/min per 1.73 m^2, the available treatments are glecaprevir + pibrentasvir or, for genotype 1 infections grazoprevir + elbasvir. The AFEF advocates uniform treatment duration of 12 wk and EASL applies the classic rules of 8 to 12 wk even if the available clinical trials were carried out over 12 wk.

Finally, in this time of scarcity of grafts, organ transplantation from a HCV + RNA + patient to another HCV + RNA + patient is allowed. EASL offers the same option for HCV-RNA-patients provided that the patient's informed consent is obtained and that post-transplant antiviral therapy is available and very quickly proposed. In France, this possibility is not yet recognized by the official agencies but this should be the case in the near future.

In conclusion, the latest AFEF and EASL recommendations announce a change of paradigm, for the management of hepatitis C. The EASL recommendations are very detailed and describe almost all the therapeutic options. The AFEF recommendations focus on the simplification of HCV management with an eradication objective to prevent reinfection thus better taking into account the epidemiological evolution and the change of culture with respect to the disease, according to us. Patients including mainly PWID and migrants should be treated massively in a simplified way facilitated by the availability of very effective and devoid of side effects pangenotypic drugs. The philosophy of the "all inclusive" or the "talk, test and treat" will involve other actors than hepatologists in a global vision of health care of these

particular populations with a culture of task delegation.

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REVIEW

Aberrant expression of alternative isoforms of transcription factors in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide and the second leading cause of death among all cancer types. Deregulation of the networks of tissue-specific transcription factors (TFs) observed in HCC leads to profound changes in the hepatic transcriptional program that facilitates tumor progression. In addition, recent reports suggest that substantial aberrations in the production of TF isoforms occur in HCC. *In vitro* experiments have identified distinct isoform-specific regulatory functions and related biological effects of liver-specific TFs that are implicated in carcinogenesis, which may be relevant for tumor progression and clinical outcome. This study reviews available data on the expression of isoforms of liver-specific and ubiquitous TFs in the liver and HCC and their effects, including HNF4α, C/EBPs, p73 and TCF7L2, and indicates that assessment of the ratio of isoforms and targeting specific TF variants may be beneficial for the prognosis and treatment of HCC.

Key words: Alternative isoforms; Transcription factors; Hepatocellular carcinoma; Alternative splicing;Hepatic differentiation; Personalized treatment

Core tip: This paper aims to analyze existing data on the spectrum of isoforms of liver-specific transcription factors produced in the liver and hepatocellular carcinoma (HCC) and implicated in carcinogenesis, their distinct regulatory functions and subsequent isoform-

dependent biological effects which may be relevant for tumor progression and clinical outcomes in HCC patients.

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INTRODUCTION

According to the current estimations of The Human Genome Sequencing Consortium, the human genome is predicted to comprise less than 20000 protein-coding genes[1]. Due to concerted efforts of The Human Proteome Project, 85% of the predicted proteins have already been identified[2]. Multiple sources of evidence suggest that this amount may be underestimated due to the existence of protein isoforms arising from the usage of alternative promoters or translation start sites (TSSs), alternative splicing regulated by ubiquitous and tissue-specific splicing factors, which affects the transcripts of 92%-94% of genes, and alternative cleavage and polyadenylation[3-5]. Interestingly, the occurrence of both alternative promoters and multiple conserved TSSs positively correlates with alternative splicing events[4,6,7]. However, although most multi-exon genes produce several alternative isoforms, 85% of the transcriptome is generally represented by a single major gene transcript[8].

Deregulation of gene expression[9] and the generation of aberrant alternative isoforms are commonly observed in multiple cancer types[10,11]. Mechanisms underlying the misregulation of the production of alternative isoforms in cancer have been thoroughly described elsewhere[12-15] and are beyond the scope of this review.

Distinct transcriptional programs of particular cell types are principally controlled by tissue-specific transcription regulators[16]. Due to alternative promoter usage, transcription factors (TFs) produce a tissue-specific pattern of alternative isoform expression that may be altered in carcinogenesis[12], and the imbalance of isoform production in tumors contributes to a flexible context-dependent diversification of cell phenotypes[17,18]. Somatic mutations and abnormal activation of signaling pathways in cancer may cause a differential regulation of the abundance and activity of splicing factors and lead to an increase in the production of alternative transcripts, including those for TFs[14]. Additionally, the transcripts of genes encoding TFs tend to contain multiple conserved TSSs, which may contribute to the production of protein isoforms. In the case of TFs, these complex regulatory mechanisms specifying alternative isoform production frequently affect functional domains[19], and their disruption in cancer may result in the misregulation of networks of their target genes (Figure 1).

Hepatocellular carcinoma (HCC) is the most frequent type of liver cancer and the second leading cause of death among all cancer types. Although recurrent driver genes and somatic mutations have been identified in HCC, most of them cannot yet be considered as druggable targets for therapy[20]. Massive deregulation of the liver-specific TF network[16] and aberrant alternative splicing[14,21,22] have been reported in HCC. The latter arises from abnormal expression, altered transcript splicing and a high mutation rate of genes encoding splicing factors that exert an effect in hepatocarcinogenesis and result in profound changes in the isoform balance compared with the normal liver. Importantly, up to 9% of differentially spliced transcripts in HCC originate from TF-coding genes[23,24]. These profound changes in the ratios of TF isoforms should likely result in a substantial modification of gene expression programs and therefore produce tumor phenotype alterations with a distinct clinical impact. Thus, data on the induction of expression of tumor-specific TF variants may be useful for the prognosis and development of approaches to isoform-specific therapy for HCC.

Nevertheless, current data concerning the regulation of the TF network in hepatocarcinogenesis by alteration of their isoform balance are rather diverse. In this review, we consider structural properties, biological effects and the clinical impact of the most investigated TF isoforms specific to liver and HCC. Essential features of these TFs and their isoforms are summarized in Table 1.

HNF4α: DIFFERENTIATION IS THE KEY

Nuclear receptor HNF4α (NR2A1) is a key regulator of hepatocyte differentiation. Bound to DNA as a homodimer, it modulates the expression of nearly 42% of the genes expressed in hepatocytes, including a wide spectrum of hepato-specific genes, either directly or through activation of other liver-enriched TFs[25,26]. Apart from the regulation of differentiation and morphogenesis, HNF4α acts as a tumor suppressor in the liver. It has been shown to inhibit proliferation through the induction of *CDKN1A* and repression of BMP7 and MYC, to regulate the expression of the p53/p63-dependent apoptotic effector PERP and to interfere with epithelial-mesenchymal transition (EMT) *via* repression of SNAI1, SNAI2 and HMGA2 and the upregulation of CDH1[27,28].

HNF4A is transcribed from one of its alternative promoters, P1 or P2, which are regulated in a tissue- and developmental stage-specific manner. Additional exon inclusion and alternative splicing result in the generation of up to 12 HNF4α variants sharing common DNA-binding (DBD) and ligand-binding domains (LBD) and differing in the trans-activation domain (TAD) and repressor F domain[29]. The isoforms transcribed from P2 (HNF4α7-α12) are devoid of the TAD activation function (AF)-1 region involved in the interaction with co-activators, while demonstrating a significant decrease in trans-activation (TA) properties[30]. The full-length F domain of HNF4α1 and HNF4α7 variants impair their TA potential because it masks the AF-2-independent co-factor binding site. In

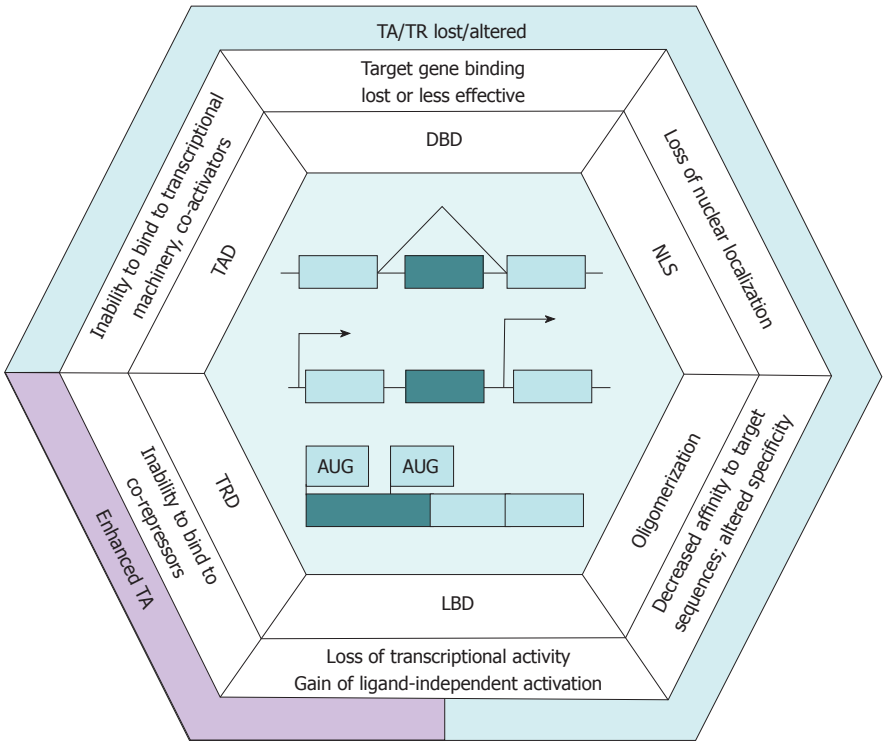

Figure 1 Alternative transcription factor isoforms possess regulatory properties distinct from those of canonical variants. Schematic representation of the common isoform formation mechanisms - alternative splicing, alternative promoter usage and alternative initiation of translation - is shown in the central part of the hexagon. Light boxes correspond to constitutive exons, and dark ones designate the exons harboring domains and regions indicated in the adjacent frame. TF isoform properties divergent from those of the canonical variants and resulting from functional domain loss are indicated at the corresponding hexagon edges on the outer side of the frame. The brace marks exon exclusion - a particular case of alternative splicing. The arrows represent transcription start sites. AUG: Start codon; TR: Transcription repression; TRD: Transcription repression domain; TF: Transcription factor; TAD: Trans-activation domain; LBD: Ligand-binding domains; DBD: DNA-binding; NLS: Nuclear localization signal.

contrast, the 10-amino-acid (AA) insert in the proline-rich F domain region of HNF4α2/α8 isoforms prevents masking, which increases the effectiveness of target gene activation[31]. Thus, the structural diversity of HNF4α variants determines a lower transcriptional activity of P2-derived isoforms (HNF4α7-α12) that lack AF-1, compared to P1-derived variants (HNF4α1-α6), and a higher transcriptional activity of isoforms that are devoid of the F domain, compared to HNF4α1/α7.

HNF4α3/α9 isoforms derive from skipping a splice site in exon 8 and contain an extended reading frame with an alternative termination site, thus encoding a protein with a completely different C-terminal F domain with undetermined function[32]. The existence of HNF4α4-α6 variants containing additional exons 1B and 1C has not been clearly proven[32]. HNF4α10-α12 isoforms bearing extended TAD due to exon 1E translation are expressed in various hepatoma cell lines, but their functions remain unclear[33].

P1 variants are predominant in the normal liver and regulate the expression of hepatocyte differentiation markers[32,34]. HNF4αP2 isoforms are prevalent at the early stages of embryogenesis; they preferentially activate promoters of early hepatic genes[35]. In the postnatal liver, the P2 promoter is repressed due to the binding of HNF4αP1 isoforms.

Deregulation of the expression of HNF4α isoforms is a frequent event in hepatocarcinogenesis. At the early stages of hepatocarcinogenesis, moderately differentiated HCC cells are characterized by activation of the expression of embryonic HNF4aP2 isoforms, which is associated with vascular invasion and poor overall survival in HCC patients[36]. In advanced dedifferentiated HCCs, which lose epithelial morphology, the expression of both P1 and P2 variants is repressed[34,37,38].

Exogenous expression of HNF4α1 in dedifferentiated HCC cells results in the restoration of epithelial morphology *via* the upregulation of epithelial markers (E-cadherin, connexin32, ZO-1), decrease in proliferation and reactivation of genes specific to differentiated hepatocytes, and decrease in *in vivo* tumor growth and metastatic potential[37,39,40]. Overall, these data indicate that HNF4αP1 reactivation might be beneficial as a therapeutic approach for HCC treatment. Further experiments are required to elucidate the role and clinical impact of particular HNF4α isoforms and to implement these findings into the development of therapeutic and prognostic approaches to HCC treatment.

ERα BALANCE IS SHIFTED TOWARDS DOMINANT-NEGATIVE VARIANTS DURING TUMOR PROGRESSION

Nuclear receptor ERα encoded by the ESR1 gene induces proliferation and exhibits anti-apoptotic and

Table 1 Transcription factors and their isoforms deregulated in hepatocarcinogenesis

TF	No. of isoforms expressed in the liver	Types of isoforms	Regulated processes (canonical isoform)	Upstream signaling pathways	Target genes expressed in the liver	Isoforms that presumably promote hepatocarcinogenesis	Isoforms that exhibit tumor-suppressive effects	Ref.
C/EBPβ	3	TA, DN	Metabolism POS: apoptosis, inflammatory response NEG: proliferation	TNF	UP(LAP1): cytochrome *p450* genes UP(LIP): GADD45B DOWN (LAP1): CCNA, CCNE, CDK2, PCNA DOWN (LIP): CLU, NUMB	C/EBPβ-LIP	C/EBPβ-LAP1, C/EBPβ-LAP2	[59,65,74,76,77, 78,139]
ERα	4	DN	POS: proliferation	Estrogen signaling	UP: CCND1, HNRNPH2, MYC, RET, WWC1	ERα-36, ERα-46, ER-αΔ5	ERα-66	[41,43,44,47,139,140]
HIF1α	3	DN	NEG: apoptosis, inflammatory response	PI3K/Akt, mTOR	UP: VEGFA	HIF1α1.1	HIF1α516, HIF1α736	[125,131,133,134,139]
HNF4α	9-12	TA	POS: angiogenesis	AMPK, Hippo, TGFβ	UP: CDH1, *CDKN1A*, HNF1a, PERP DOWN: BMP7, HMGA2, MYC, SNAI1, SNAI2	HNF4α7-α9	HNF4α1-α3	[28,29,37,139,141,142]
KLF6	3	TA, DN	POS: differentiation, morphogenesis, apoptosis	TGFβ	UP(wtKLF6): CCND1, CDH1, CDK4 UP(SV2): *CDKN1A* DOWN(wtKLF6): MDM2 DOWN(SV1): *CDKN1A*	SV1	wtKLF6, SV2	[105,108-114,143]
p73	3	TA, DN	NEG: proliferation, invasion, metastases	p53, Hippo, mTOR	UP: BCL2 family genes, caspases, CD95, TNF-R, TRAIL-R	ΔN-p73, ΔEx2p73, ΔEx2/3p73	TA-p73	[87-91,139,144]
PGC1α	3	TA, DN	POS: proliferation, differentiation, apoptosis	AMPK, Insulin signaling	UP: GLUT4, PDK4, PEPCK, PPARA, PPARG	PGC-1α1-a, L-PGC-1α, NT-Pgc 1α-a* (*promote replication and assembly of in HBV and HCV)		[49,52,54-56,139]
TCF4 (TCF7L2)	17	TA	POS: apoptosis	Wnt, Hippo	UP: AXIN2, CCND1, IRS1, JUN, MMP7, WISP1	TCF-4J, TCF-4C	TCF-4B, TCF-4K	[98-102,139,145]
WT1	4	DN	NEG: cell cycle progression		UP: BCL2 family genes, cFLIP DOWN: FADD, HNF4A, NF-κB	WT1(+/) 17AA(+)KTS	WT1(-)KTS	[119-122,146]
ZIP (ZGPAT)	2	DN	POS: gluconeogenesis		DOWN: CDC25A, EGFR, FGF5, FGF14, PDGFB, PTEN, RGS3, TBPL1, VCAM1	sZIP	ZIP(fl)	[135-137]

C/EBP: CCAAT enhancer binding protein; TF: Transcription factor; TA: Isoforms with differential transactivation properties; DN: Dominant-negative; POS: Positive regulation; NEG: Negative regulation; UP: Up regulation; DOWN: Down regulation.

anti-inflammatory activities[41] *via* estrogen-dependent binding to estrogen response elements of target genes. Non-canonical activation of ERα target genes *via* secondary messengers, activation of membrane-bound ERα isoforms or protein-protein interactions have also been described[42].

Canonical 66-kDa ERα (ERα-66) matches the conventional structure of nuclear receptors. Additionally, numerous ERα splice variants that differ in promoter usage and the presence of exons encoding TAD, DBD, LBD and the nuclear localization signal (NLS) region have been reported in normal and tumor tissues[42]. Apart from ERα-66, the expression of 3 dominant-negative (DN) isoforms has been reported in the liver, namely, ERα-46 lacking AF-1 TAD, ERαΔ5 harboring an incomplete LBD, and ERα-36, which is transcribed from an alternative promoter and is deficient in both AF-1 and LBD[41].

Investigation of the properties of truncated isoforms in various cell lines has demonstrated that the dimerization of a shorter isoform with the full-length one represses its transcriptional activity. Membrane-bound fractions of ERα-46 and ERα-36 are believed to interfere with the function of ERα-66. Moreover, ERα-36 lacks the NLS but is able to activate the MAPK/ERK cascade upon treatment

with estrogens or tamoxifen[41-45]. A constitutively active ER-αΔ5 variant exhibits approximately 10%-15% of the ligand-bound ERα-66 activity[46].

ERα-66 is highly expressed in the normal liver and, to a lesser extent, in cirrhotic tissue, while its transcription in HCC is substantially or completely suppressed. Higher ERα-66 expression is associated with a better overall and recurrence-free survival of HCC patients and is negatively correlated with the tumor size and extra-hepatic metastasis. The mRNA of DN ERα-46 is detected in non-tumor, cirrhotic and tumor liver tissues. Upregulation of ERαΔ5 in HCC causes repression of ERα-66 transcriptional activity through heterodimerization. An increase in ERα-36 isoform production has been reported in HCC and hepatoma cell lines; it correlates with the downregulation of the full-length isoform[43,44,47] and may arise from ESR1 promoter hypermethylation, which is frequently observed in HCC[48]. Thus, the shift in balance of ERα variants towards truncated isoforms with DN properties can essentially modulate the impact of the estrogen receptor signaling pathway in hepatocarcinogenesis and is likely associated with an unfavorable HCC prognosis.

PGC-1α, A CO-FACTOR OF NUCLEAR RECEPTORS THAT REGULATE METABOLISM AND INFLAMMATION

PGC-1α (PPARGC1A) is an inducible adaptor of the PPARγ co-activator 1 family. It has been shown to regulate adaptive reactions of energy metabolism in brain, cardiac and skeletal muscles, adipose tissue and liver[49]. PGC-1α is a key inducer of gluconeogenesis in the fasted liver[50]. A significant decrease in PGC-1α expression has been reported in primary human HCCs[51].

The N-terminus of PGC-1α protein contains a TAD with co-activator and nuclear receptor (HNF4α, PPARα, PPARγ, LXRα) binding sites[52] followed by phosphorylation and acetylation sites that modulate PGC-1α activity[53]. The NLS region, an arginine-serine-rich domain that enables binding to TFs and RNA-recognition motifs, is located in the C-terminal part of PGC-1α. C-terminal domains are involved in mRNA processing and splicing[53].

PGC-1α transcription is controlled by several tissue-specific promoters and is often coupled with alternative splicing. Processed alternative transcripts give rise to a set of TA-competent isoforms and DN variants lacking C-terminal domains or with truncations in their co-activator and co-repressor regions[53]. In human tissues with intense energy metabolism, the full-size PGC-1α1-a is a prevalent isoform[52]. In murine liver, mRNAs encoding *Pgc-1α1-a* and *NT-Pgc-1α-a* are transcribed from the proximal promoter. The *NT-Pgc-1α-a* variant preserves the co-activator binding domain and a portion of the co-repressor binding domain, but it lacks the C-terminal part[54]. Although less effective, *NT-Pgc-1α-a* is sufficient to stimulate gluconeogenesis in PGC-1α1-a$^{-/-}$ hepatocytes[55]. The hepato-specific promoter located downstream of the proximal one is active in human fasted liver[52] or in response to hepatitis C virus (HCV) induced endoplasmic reticulum (ER) stress. Its activation stimulates the expression of a liver-specific TA-isoform, L-PGC-1α, which is deficient in the first 127 aa but is able to co-activate most PGC-1α-interacting nuclear receptors except liver X receptor α (LXRα)[56]. LXRα has been shown to repress the FOXM1 transcriptional regulator and its target genes *CCNB1* and CCND1 in HCC cells[57]. Thus, the reduced LXRα activity may stimulate proliferation in hepatoma cell lines. The reduced LXRα activity also causes the deregulation of cholesterol metabolism, resulting in liver damage and inflammation, which facilitates HCC progression[58]. Hence, the elevated L-PGC-1α level is likely to be implicated in hepatocarcinogenesis. However, PGC-1α1-a induction occurs along with L-PGC-1α upregulation in the fasted liver and during HCV-induced ER stress. As the result, a higher PGC-1α1-a/L-PGC-1α ratio abolishes the effects of the alternative isoform. Currently, the expression of PGC-1α isoforms has been investigated in HCC cell lines but not in HCC clinical samples. Further research is required to determine a possible impact of the imbalance of PGC-1α isoforms in hepatocarcinogenesis.

INTERPLAY BETWEEN CCAAT ENHANCER BINDING PROTEINS AFFECTS PROLIFERATION AND HEPATIC DRUG METABOLISM

CCAAT enhancer binding proteins (C/EBPs) belong to a liver-enriched bZIP TF family; they regulate the expression of genes involved in proliferation, differentiation, inflammatory response and metabolism. C/EBPs bind to DNA as homo- and heterodimers, and the heterodimerization of C/EBPs can alter target site recognition[59].

Two C/EBP family members are predominant in normal liver. CEBPA is highly expressed in adult liver under normal conditions, whereas the expression of CEBPB is low in hepatocytes and can be induced by pro-inflammatory cytokines, hormones, cAMP and other agents[59,60]. The induction of hepatocyte proliferation is accompanied by C/EBPα downregulation and increases C/EBPβ levels[61]. Inactivation of the CEBPB gene significantly reduces the regenerative response in mice after a partial hepatectomy, whereas CEBPA-deficient murine hepatocytes demonstrate a high proliferative activity[59]. While CEBPA is mainly considered as a tumor suppressor that is downregulated in HCC[62], Lu *et al*[63,64] reported its upregulation in liver cancer, which was associated with escape from starvation-induced cell death and a poor prognosis. CEBPB expression is not altered in HCC, albeit the C/EBPβ protein level is decreased[65].

The N-termini of C/EBPα and C/EBPβ proteins contain a number of activation domains and negative regulatory regions that significantly differ between these TFs, while their C-termini contain a highly homologous basic

sequence-specific DNA recognition region and leucine zipper dimerization domain. Both CEBPA and CEBPB are intronless genes. Alternative protein isoforms arise due to the alternative usage of TSSs or regulated proteolysis[59].

The 42-kDa and 30-kDa C/EBPα isoforms differ in their amino termini with the p30 isoform possessing a lower activation potential than the predominant full-length variant due to the absence of two activation domains[59,62]. Apart from the DN activity, p30 has been proposed to bind and regulate a distinct set of target genes[66]. While both the p42 and p30 variants have been detected in liver, to date, the changes in the isoform ratio in HCC have not been quantitatively investigated, and reports of CEBPA upregulation leading to poor outcomes in HCC patients might arise from an isoform imbalance. Indeed, the induction of CEBPA expression in various models of liver disease leads to disease reversal and results in lower levels of liver damage markers and reduced tumor burden in rodents with cirrhotic HCC[67].

C/EBPβ isoforms are subdivided into 2 classes. Liver-enriched activating protein isoforms are able to induce the expression of target genes. C/EBPβ-LAP1 (LAP*) exhibits a stronger TA potential than C/EBPβ-LAP2 (LAP), which is deficient in the short N-terminal portion of the activation domain. C/EBPβ-LIP, a liver-enriched inhibitory protein, lacks the TAD but preserves a part of the negative regulation domain, and bZIP and acts as a DN regulator of transcription[59]. LAP1 is the major C/EBPβ variant expressed in hepatocytes[65]. According to Fang *et al*[65], LAP1 is significantly downregulated in HCC, whereas the LAP2 level is unaltered, and LIP is underrepresented. These observations are partly in line with reports on LAP1 isoform downregulation in squamous cell carcinoma[68] and breast cancer[69]. Albeit LAP1 variant expression is reduced in HCC, its expression is even weaker in liver cancer stem cells[70]. Conversely, LAP2 and LIP are induced in these tumor types, and a high LIP/LAP ratio is indicative of an advanced stage and poor prognosis in breast cancer[71].

In murine liver regeneration models, C/EBPβ regulates the proliferation of hepatocytes *via* activation of the transcription of E2F-regulated genes that are essential for DNA replication (*Mcm3, Cdc6*) and reparation (*Msh2, Msh5*) and *Cebpa* repression[72,73]. C/EBPβ-LAP expression leads to a delay of the G1/S transition, which results in the synchronization of hepatocytes after a partial hepatectomy due to the downregulation of CCNA, CCNE, PCNA and CDK2, whereas the expression of C/EBPβ-LIP induces proliferation. Intriguingly, overexpression of LAP leads to an increase in the C/EBPα p30/p42 ratio, while expression of the LIP variant upregulates both C/EBPα isoforms[74]. Exogenous expression of C/EBPβ-LAP (but not the –LIP variant) arrests cell cycle progression in HepG2 and Hep3B hepatoma cells[75,76]. C/EBPβ-LIP contributes to the survival of tumor cells. Drug-induced apoptosis is significantly reduced in Hep3B cells overexpressing the C/EBPβ-LIP but not the C/EBPβ-LAP variant[76].

Emerging data also link C/EBPβ expression to the metastatic potential of tumor cells. LAP1/2 overexpression represses Huh7 migration *in vitro* and metastasis *in vivo via* the induction of orsomucoid 2 (ORM2) gene expression, whereas LIP does not affect ORM2 levels[65]. *In vivo* experiments also demonstrate that LAP1-transfected hepatoma cells possess a reduced ability to form subcutaneous tumors in nude mice and that the expression of Ki-67 and cancer stem cell markers is reduced in tumors originating from these cells[70].

The complex impact of CEBP isoform variants on the fine regulation of liver-specific gene expression can be illustrated by the regulation of the *CYP3A4* gene encoding the most abundant type of cytochrome P450 in the liver. *CYP3A4* expression is modulated by liver-specific TFs, including C/EBPα and C/EBPβ, which have multiple binding sites in their regulatory region[77,78]. While TA-competent isoforms induce the expression of *CYP3A4*, the DN variant LIP inhibits it, and an increase in the LIP/LAP ratio results in the repression of *CYP3A4* in HepG2 cells[78]. As both TFs, including their DN variants, are able to heterodimerize, not only the levels of C/EBP proteins themselves but also the interactions between their isoforms, may affect the regulation of the metabolism of xenobiotics.

Taken together, the data on the abundance of C/EBPα and C/EBPβ variants in HCC remain limited and contradictory. CEBPA has previously been identified as a tumor suppressor that tends to be downregulated in HCC. Although there are limited data on the ratio of C/EBPα isoforms and their distinct effects in HCC, the capability of TA-competent and DN C/EBPα variants to form dimers with C/EBPβ isoforms increases the complexity of the network of target gene regulation and should be further investigated. In contrast, growing evidence of a variety of diverse effects arising from the regulation of expression by individual C/EBPβ isoforms indicates that evaluation of the ratio of LAP/LIP isoforms and their functions may be of value for HCC prognosis and treatment.

SEVERAL ISOFORMS OF p53 FAMILY PROTEINS ARE OVEREXPRESSED IN HCC AND FAVOR PROLIFERATION AND CELL DEATH EVASION

p53, p63 and p73 TFs of the p53 family regulate cell cycle progression and induce programmed cell death. Proteins of the p53 family have a similar domain structure, with TAD in their N-terminus followed by a proline-rich region, DBD and oligomerization domain. p63 and p73 possess a longer C-terminus encompassing the sterile α motif[79]. Since DBD shares substantial homology among the p53 family members, these TFs are able to regulate the expression of common target genes, including the regulation of each other's expression, although each transcription factor has specific target genes[80].

The p53 family proteins bind DNA as tetramers. The

wild-type p53 forms tetramers only with p53 variants, while mutant p53 variants can oligomerize with p63 and p73, thus reducing the activation of their target genes[81]. p63 and p73 can form mixed tetramers with varying transcriptional activity[82].

Alternative transcription and translation start sites and alternative splicing of the first exons encoding TAD result in the generation of a wide spectrum of N-terminally truncated (ΔN-) p53 family proteins that generally exert a DN effect over the full-length isoform activity. Additionally, the resultant proteins vary in their C-terminal domains due to alternative splicing[80]. p53 is frequently mutated or inactivated in cancer[80]. While p53 dysfunction is clearly associated with HCC progression, the only isoform proposed to play a significant role in hepatocarcinogenesis is the Δ40p53α variant translated from the second in-frame AUG of TP53 mRNA and lacking 39 AA of TAD. Δ40p53α has been demonstrated to be induced after doxorubicin treatment. Its exogenous expression suppresses proliferation, colony formation and induced senescence in hepatoma cells[83]. p63 is not expressed in the normal liver; however, the expression of ΔN isoforms is detected in p53-null hepatocytes. A trans-activating p63 variant (TAp63) is expressed in hepatoma cell lines irrespective of p53 status[84]. The TAp63 knockdown in HepG2 and Hep3B hepatoma cells leads to increased proliferation and colony formation. TAp63 expression negatively correlates with tumor size, intrahepatic metastasis, and distant metastasis and, according to the results of a retrospective analysis, a low TAp63 level is associated with poor survival in HCC patients[85,86].

The activation-competent TAp73 is absent in normal hepatocytes but is widely expressed in hepatoma cell cultures and human HCC samples[87-89]. Intriguingly, alternative splicing of full-length TAD-encoding transcripts originating from the first promoter (TA-promoter) is the main source of TAD-deficient pro-oncogenic p73 isoforms in HCC. The TA-promoter-driven and aberrantly spliced DN isoforms Δex2p73 and Δex2/3p73 that lack TAD, and ΔN'-p73 composed solely of TAD, are the most abundant p73 variants in HCC cells[88]. Both TAp73 and ΔTA variants including Δex2/3p73 are upregulated in hepatitis B virus-associated HCC[90]. ΔN-p73, an internal promoter-derived variant devoid of TAD, is detected both in hepatocytes and HCC and is overexpressed in 37% of tumors[87,89].

Normally, the induction of TAp73 expression inhibits cell proliferation; however, hepatoma cells tolerate its effects, presumably due to the co-expression of ΔTA variants[87]. The liver-specific expression of ΔNp73 in transgenic mice causes the formation of hepatic adenomas and subsequently to development of HCC in most animals[91]. Overexpression of the DN ΔN-p73 variant is associated with poor survival in HCC patients[89].

p63 and p73 initiate programmed cell death *via* a common mechanism. DNA damage induces TAp63 or TAp73-dependent TA of genes encoding the death receptors CD95, TNF-R and TRAIL-R, caspases and pro-apoptotic Bcl-2 proteins[91]. The expression of DN p63 and p73 variants abolishes the induction of pro-apoptotic genes and may cause resistance to chemotherapy[89,92]. Thus, since pro-oncogenic p53-family TF isoforms are generally expressed at low levels or are absent in non-transformed hepatocytes, targeting these variants may provide an option to counteract their DN effects on pro-apoptotic p53, p63 and p73 isoforms to improve the efficiency of drug therapy in HCC patients.

TCF-4 (TCF7L2) MODULATES A WIDE SPECTRUM OF TUMOR CELL PROPERTIES *VIA* INTERACTIONS WITH THE Wnt SIGNALING PATHWAY AND COMPETITION WITH HNF4α

TCF-4 (TCF7L2) is a basic helix-loop-helix (bHLH) TF of the LEF/TCF family that modulates the expression of Wnt signaling pathway target genes involved in proliferation, differentiation, apoptosis, cell polarity and motility upon β-catenin binding[93]. The conserved domain structure of TCF-4 is characteristic of the LEF/TCF family; it comprises the N-terminal β-catenin-binding domain (BCBD), followed by the context-dependent regulatory domain, high-mobility group DBD, and E-tail, which includes the second sequence-specific DBD, C-clamp and binding region for the transcriptional repressor CtBP[94].

The context-dependent regulatory domain harbors several regulatory motifs: LVPQ and SxxSS, implicated in the repression of β-catenin-mediated TA, and the Groucho/TLE co-repressor binding site. The C-clamp contains a 30 AA motif that is required for binding to weak Wnt response elements[93,95]. Approximately 20 TCF-4 isoforms generated by alternative splicing and alternative transcription start site usage have been reported[96]. Variants transcribed from the internal transcription start sites 2 and 3 lack BCBD and act as transcriptional repressors of β-catenin target genes[97]. Additionally, alternatively spliced isoforms differ by the presence of exon 4, which confers transcription repressor function, LPQV- and SxxSS motifs, and by the splicing pattern of exons 13-17 encoding a part of the C-clamp[98,99].

TCF-4 is highly expressed in hepatocytes. The pattern of expression of TCF-4 variants depends on the degree of cell differentiation[98]. The isoform TCF-4B that is predominant in normal liver lacks exon 4, the SxxSS, C-clamp and CtBP-binding region, which makes it a potent β-catenin-mediated trans-activator[98]. TCF-4B expression is maintained in HCCs and in differentiated hepatoma cell lines[98,100].

LPQV and SxxSS motif-containing variants expressed in normal liver are not substantially altered in liver tumors[100], except for a TCF-4K isoform. TCF-4K retains a portion of the C-clamp and the full CtBP-binding site[98]; it is significantly downregulated in HCC[100]. Conversely,

TCF-4C and -4J are upregulated in HCC, specifically in poorly differentiated tumors. Along with TCF-4B, TCF-4C demonstrates the highest transcriptional activity among the TCF-4 isoforms due to the lack of any repressor motifs and domains. TCF-4J, that is deficiency in most repressor units, preserves the LVPQ motif and CtBP-binding region, which results in a reduced TA ability[98,99,101].

The exogenous expression of TCF-4J in hepatoma cell cultures induces the expression of genes that are overexpressed in poorly differentiated HCCs and are distinct from SxxSS-containing TCF-4K activated targets. Additionally, TCF-4J has been demonstrated to interact with the liver-specific master regulator HNF4α, affecting its target gene expression[96]. Thus, hepatic differentiation may be affected by the balance of certain TCF-4 isoforms[102]. Alternatively, it was proposed that HNF4α competes with TCF-4 for its binding sites *in vitro* in human colorectal cancer cells in an isoform-dependent manner, where the HNF4α2, but not the HNF4α8, P2-driven variant, might displace TCF-4 in the AP-1 transcriptional complex[103]. Although there are no data on their interplay in the liver, this possible interaction may also be important for the indirect regulation of gene expression mediated by the Wnt signaling pathway and relevant for hepatocarcinogenesis.

The exogenous expression of SxxSS-deficient TCF-4B, -4C and -4J isoforms in hepatoma cell lines results in the upregulation of Wnt-responsive genes, thus promoting cell proliferation. TCF-4C and -4J overexpression also induces colony formation and promotes cell migration in hepatoma cell lines. In contrast, a SxxSS-containing TCF-4K variant reduces the proliferation rate and colony formation but stimulates cell migration[98,99,101,102].

TCF-4J-expressing hepatoma cells demonstrate higher tumorigenicity than TCF-4K-overexpressing ones. Tumors derived from TCF-4J-overexpressing cells also express high levels of HIF-2a and EGFR, which confer hypoxia resistance characteristic of an aggressive tumor phenotype[100]. Overall, these data indicate that the imbalance of TCF-4 isoforms observed in HCC contributes to multiple processes including dedifferentiation, increased proliferation, survival and metastatic potential of tumor cells and thus confers an aggressive tumor phenotype.

FULL-LENGTH KLF6 ACTS AS A TUMOR SUPPRESSOR IN LIVER AND IS DOWNREGULATED EARLY IN HEPATOCARCINOGENESIS

The tumor suppressor KLF6 belongs to the C2H2 zinc finger domain Sp1/KLF family of TFs, which are essential regulators of proliferation, differentiation and migration. Biological functions of KLFs are disrupted in a wide variety of tumors[104]. In HCC, KLF6 is frequently inactivated due to the loss of heterozygosity or point mutations[105].

The KLF6 molecule contains N-terminal TAD region, an NLS and three zinc fingers located in the C-terminus that allow DNA binding. Several KLF6 isoforms are produced *in vivo* due to the activation of cryptic splice sites[104]. In the liver, the full-length wtKLF6 isoform is prevalent, while truncated SV1 and SV2 variants are also expressed[106]. In contrast to wtKLF6, which is accumulated in the nucleus, SV1 and SV2 proteins lacking the NLS are mainly localized in the cytoplasm[107]. The SV1 variant is also defective in DNA-binding due to the absence of all zinc fingers[104]. The downregulation of wtKLF6 in dysplastic nodules is an early event in hepatocarcinogenesis, while its further decrease is observed in highly malignant HCCs and is associated with a poor prognosis[108,109]. The downregulation of wtKLF6 in HCC is often accompanied by a decrease in SV2 production and upregulation of the SV1 isoform, while higher SV1/wtKLF6 ratios correlate with an advanced tumor stage[106,108,110].

Several mechanisms regulating the balance of SV1 and wtKLF6 in HCC have been proposed. HGF- or Ras-dependent activation of the PI3K/AKT signaling pathway induces alterations of KLF6 splicing, which leads to enhanced generation of the SV1 variant[111,112]. In addition, miRNA-1301, which is abundant in HCC samples, and miRNA-210 have been found to specifically target wtKLF6 but not the SV1 variant[113].

The SV1 isoform counteracts wtKLF6 and SV2 and demonstrates obvious oncogenic properties. It has been proposed to carry out DN functions *via* the cytoplasmic sequestration of wtKLF6, which leads to its subsequent proteasomal degradation[110]. Both wtKLF6 depletion and SV1 overexpression significantly accelerate tumorigenesis in diethylnitrosamine-treated mice[110]. In human hepatoma cell lines, wtKLF6 depletion and SV1 exogenous expression enhance proliferation *via* the downregulation of *CDKN1A* and *CCNB1* target genes[110-112], whereas SV2 expression has an opposite effect[106].

KLF6 variants are implicated in the regulation of apoptosis. The exogenously expressed wtKLF6 induces transcriptional repression of the *MDM2* gene, thus securing the stabilization of the p53 level[109]. SV2 overexpression results in p53-dependent upregulation of the apoptosis inducers Bax and PUMA[106].

SV1 promotes the migration of hepatoma cell lines; it is proposed to favor metastasis *via* inhibition of the function of wtKLF6 implicated in the regulation of Rho family GTPase activity[114]. Overexpression of SV1 also upregulates the expression of mesenchymal marker genes, whereas wtKLF6 is essential for the maintenance of E-cadherin expression but does not affect other EMT-associated markers[113,114]. Thus, since the DN SV1 isoform of the KLF6 tumor suppressor performs obvious pro-oncogenic functions and is upregulated in HCC, it can be considered as a prognostic factor and candidate target for siRNA-mediated therapeutic inactivation in

liver tumors. Alternatively, targeting miRNAs implicated in wtKLF6 downregulation may also result in the restoration of the KLF6 isoform balance to reduce the pro-oncogenic effects of the SV1 TF variant.

ACTIVATION OF THE PRODUCTION OF MAJOR WT1 ISOFORMS IN HCC

WT1, a TF of the C2H2-type zinc-finger protein family, is involved in the regulation of cell differentiation, proliferation and apoptosis. Depending on the tissue-specific context, WT1 exhibits either tumor-suppressive or oncogenic properties[115], but in most solid tumors, including HCC, the upregulation of WT1 is associated with a poor prognosis[116,117]. The N-terminal proline-rich region of WT1 contains a homodimerization domain and trans-repression and trans-activation domains that are responsible for co-factor binding. The C-terminus comprises DBD with 4 C2H2-type Zn-fingers[115].

Although up to 36 alternative isoforms of WT1 produced by a combination of alternative start codon usage, alternative splicing and RNA editing have been predicted, 4 major isoforms that vary in exon 5 (17-AA insertion - "17AA") and/or exon 9 (3 AA insertion - "KTS") splicing have mostly been investigated[115,118]. The 17AA insertion located between the proline-rich region and the first zinc finger and the KTS triplet between the third and fourth Zn-fingers have been demonstrated to change the DBD conformation and diminish WT1 DNA-binding[115]. Therefore, the WT1 isoforms harboring these insertions have been suggested to be less transcriptionally active[119]. While WT1 expression in the normal liver is very low, in the cirrhotic liver and HCC tissue, all 4 major WT1 variants are upregulated[116,120,121].

The functional differences in the KTS-variable WT1 isoforms have been investigated in HCC model systems. In HepG2 and Hep3B hepatomas, exogenously expressed WT1 KTS(-) isoforms, but not KTS(+), act as tumor suppressors, triggering a p53-independent apoptotic program[119]. Conversely, in Huh7 and HLE cells, the major 17AA(+) KTS(+) isoform inhibits apoptosis *via* transcriptional activation of the cFLIP apoptotic inhibitor and repression of FADD, a caspase cascade activator[122]. These data imply that cell fate decisions may be dependent on the ratio of KTS(+)/KTS(-) isoforms in HCC cells.

The WT1 knockdown in PLC/PRF/5 HCC cells stimulates differentiation through upregulation of the key hepato-specific regulator HNF4α and leads to the loss of resistance to apoptosis[121]. In contrast, ectopic equimolar expression of 4 major WT1 variants in a primary rat hepatocytes culture induces a decrease in HNF4α expression and dedifferentiation[120]. Overall, the existing ambiguity regarding the impact of WT1 on different tumors could be explained not only by the tissue-specific context but also by a different spectrum of expressed WT1 variants. Importantly, the expression of minor WT1 isoforms, in addition to the major ones, and their impact on HCC development, progression and prognosis has not been thoroughly investigated. Thus, further investigation of WT1 isoform properties and the development of approaches for WT1 silencing or shifting the balance towards DN variants that counteract its pro-oncogenic and apoptosis resistance functions may benefit HCC prognosis and treatment. Apart from gene silencing, enforced expression of DN WT1 variants may be considered a therapeutic option for HCC to oppose the dedifferentiation and acquisition of resistance to apoptosis conferred by extrinsically expressed WT1.

MINOR ISOFORMS OF HIF1α FOUND IN HEPATOMA CELLS ARE ABLE TO COUNTERACT THE ADAPTIVE RESPONSE TO HYPOXIA

bHLH-PAS protein HIF1α promotes tumor progression through the regulation of the adaptive response to hypoxia[123]. HIF1α has been proven to be the most potent inducer of the expression of vascular endothelial growth factors and other hypoxia-responsible element-containing genes in various cell type-dependent contexts[124-126]. HIF1α is not detected in hepatocytes under normoxia; its expression is triggered by low oxygen levels in the murine liver[127]. HIF1α expression is induced in dysplastic liver nodules during malignization and is further increased in HCC[128], where its upregulation correlates with vascular invasion and a poor prognosis[129]. In Hep3B hepatoma cells, HIF1α modulates the expression and alternative splicing of its target genes to facilitate tumor cell metabolism adaptation to hypoxia[130].

The amino-terminal region of HIF1α harbors a bHLH DBD followed by the PAS domain, which enables its heterodimerization with HIF1β, a component of the TA-competent HIF1 complex, and by the oxygen-dependent degradation domain (ODDD). ODDD contains proline residues that are modified under normoxia and potentiate binding to VHL ubiquitin ligase to provide ubiquitin-dependent degradation of the TF. The C-terminal part of HIF1α contains 2 TADs and the NLS region[125,131].

HIF1α transcription is regulated by the universal I.1 promoter that drives expression of the major HIF1α1.1 isoform or by two tissue-specific promoters, I.2 and I.3, which give rise to minor HIF1α1.2 and HIF1α1.3 variants[132]. Alternative splicing of the HIF1α1.1 transcript leads to frameshifts and generates isoforms with impaired activity. DN cytoplasm-localized HIF1α516 and HIF1α557 variants are devoid of the NLS and both TADs as a result of the exclusion of exons 11/12; thus, they escape hypoxia-induced nuclear translocation and lack TA properties. The HIF1α736 variant lacks the C-terminal TAD due to the exclusion of exon 14 and demonstrates weaker TA properties compared with HIF1α1.1[123,133,134].

HIF1α1.1 is a predominant isoform generated under hypoxic conditions in hepatoma cell lines. The expression of minor fractions of HIF1α516 and HIF1α736 that inhibit the activity of HIF1α1.1 through competitive binding to HIF1β and thereby reduce the expression of HIF1 target genes has also been identified in hepatoma cells[123,134]. Although the biological role of these variants in hepatocarcinogenesis is underexplored and no survival analysis addressing their impact has been carried out to date, further investigation of DN HIF1α variants is required to define the possible utility of distinct isoforms in prognosis and therapy.

TRANSCRIPTIONAL REPRESSOR ZIP (ZGPAT)

ZIP (ZGPAT) is a DNA-binding transcriptional repressor that contains a C3H1-type zinc finger, TUDOR, G-patch, coiled-coil domains. ZIP-mediated transcriptional repression of target genes is achieved through the recruitment of the nucleosome remodeling and deacetylase (NuRD) complex[135]. Dimerization of ZIP is essential for its DNA binding ability[136]. ZIP target genes encode growth factors and their receptors, cell cycle regulators, components of the MAPK signaling pathway, actin cytoskeleton, and tight and gap junctions, particularly EGFR, PTEN, CDC25A, FGF5, FGF14, PDGFB, RGS3, and VCAM1[135].

Two ZIP isoforms have been identified. The full-length one acts as a transcriptional repressor, whereas a truncated sZIP variant deficient in DBD but capable of competitive binding with NuRD and, presumably, of dimerizing with ZIP decreases the inhibitory effect of ZIP[136,137]. Unlike most cell types, hepatocytes express both truncated and full-length ZIP variants[135]. Induced ZIP overexpression in HepG2 cells result in EGFR transcriptional downregulation, growth inhibition and a reduced clonogenic potential. In contrast, sZIP overexpression or ZIP/sZIP co-expression induce clearly opposite effects, indicating that the shortened isoform can counteract the tumor-suppressing activity of ZIP[137].

CONCLUSION

Aberrant alternative splicing has recently been proposed to be an additional hallmark of cancer[138]. We believe it reasonable to consider this hallmark in a broad sense, *i.e.*, as the generation of transcripts and protein variants that are not characteristic of a particular cell type. Such an interpretation stems from the observation that the mechanism of isoform generation is not limited to alternative splicing and can be based on additional mechanisms that are listed above or, frequently, on their combination. The production of aberrant isoforms is certainly not only a hallmark of cancer but also the cause of the development of other distinguishing characteristics of tumor cells. This statement is particularly relevant in regard to TF isoforms since TFs act as hubs that convert various incoming signals to a wide variety of target genes to modulate their expression, and the processes presumably regulated by these atypical isoforms might also be relevant for hepatocarcinogenesis (Figure 2).

The existence of TF isoform variants enables their functional diversification for the tight tissue- and condition-specific regulation of target genes. In contrast, aberrant production of TF variants with different TA properties can result in a significant alteration of the transcriptional program of the cell and, eventually, in its malignization. For instance, the overproduction of DN isoforms of TFs that are able to dimerize with functionally active variants of the corresponding TFs or compete for co-factors and/or binding to target gene promoters causes a reduction or inhibition of the functional activity of such TFs. The abnormal isoform production may result in mRNA degradation or structural changes that cause cytoplasmic localization, constitutive activation of TFs or enhanced TA ability and other alterations that eventually affect the expression of target genes.

The rapid development of transcriptome sequencing and proteomic technologies facilitates the detection, quantitative assessment and acquisition of statistically significant data on alterations of TF isoform ratios. Recently, global profiling of alternative splicing in hepatitis-associated and virus-free HCCs using a TCGA-LIHC dataset revealed deep perturbations in RNA splicing concomitant with altered expression levels and isoform patterns of splicing factors. Among the spectrum of alternatively spliced transcripts, those that are common for HCC or those that change depending on hepatitis infection status have been identified[24]. Notably, 7.6% to 9.0% of differentially spliced transcripts in HCCs originate from TF-encoding genes, including those involved in the regulation of the specification, differentiation or malignant transformation of hepatocytes (HNF1B, FOXM1, PPARA, NR1I3/CAR, NR3C1/GR)[24]. However, very few of the identified TFs have been previously investigated with regard to the biological effects or clinical implications of their isoforms. The available data on TF isoforms that are produced in the liver and HCC are summarized in Table 1.

The application of methods that allow the differential detection of TF isoforms appears to be crucial for the identification of clinically relevant alterations. Data based on the evaluation of the total level of gene expression or protein synthesis may be ambiguous, as such approaches mask the contribution of individual TF isoforms possessing distinct transcriptional activities to the overall pool of the corresponding TF. For example, several oncogenic TCF4 isoforms implicated in hepatocarcinogenesis are substantially upregulated in HCC, whereas the expression of other variants is usually decreased or unaltered.

Additionally, the ratio and functional impact of TF isoforms may significantly depend on the tissue-specific context. In the liver, HNF4a isoforms that are translated from mRNA transcribed from the P1 promoter are

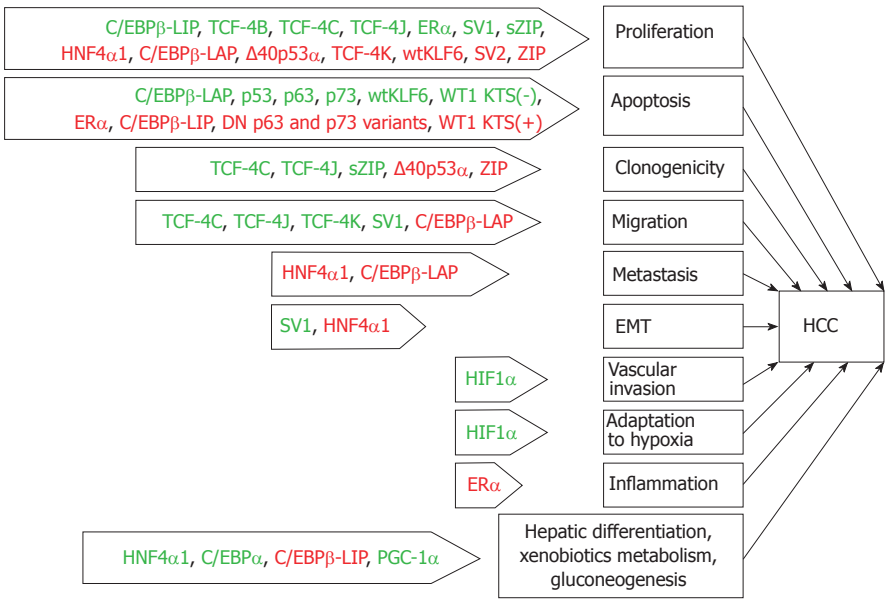

Figure 2 The key processes implicated in hepatocarcinogenesis that are regulated by transcription factors or their specific isoforms. TFs with stimulatory effects on these properties are depicted in green color, and TFs acting as repressors are colored in red. Sharp arrows indicate the stimulation of HCC development, and inhibition is indicated by blunt arrows. TF: Transcription factor; HCC: Hepatocellular carcinoma; EMT: Epithelial-mesenchymal transition.

predominant and clearly demonstrate tumor-suppressive properties[37]. In the colon epithelium, HNF4A isoforms transcribed from both promoters are normally expressed. Unlike HCC cells, non-isoform-specific HNF4A knockdown in colorectal carcinoma cell lines inhibits proliferation[147], while HNF4αP1 downregulation *in vivo* is associated with a higher metastatic potential in CRC, raising the possibility that this isoform group nevertheless implements a tumor suppressive function[148]. Thus, the isoform-discerning approach may resolve the existing contradictions in understanding the roles of certain TFs that are believed to exert opposing effects in different types of tumors.

Overall, knowledge of the effects of particular isoforms, their tissue-specific expression profiles and shifts in their ratios in disease may be of practical value for HCC prognosis and outlining potential therapeutic targets. Unfortunately, the available data on TF isoforms are mostly based on *in vitro* studies, the results of which are not to be directly extrapolated since gene expression, splicing and the effects of those variants may differ *in vivo*[149,150]. Few of the isoforms discussed above have been investigated in terms of their prognostic significance in HCC, although their aberrant production is associated with clinical features and outcomes in other types of cancer. To date, only the overproduction of HNF4αP2, ΔN-p73 and the downregulation of the TAp63 and ERα-66 isoforms have been reported to be significantly associated with the poor survival in HCC patients. A comprehensive understanding of the functions and regulation of particular isoforms may be further applied to the development of new therapeutic approaches. For instance, the upregulation of the anti-apoptotic Bcl-x(L) isoform of Bcl-2-like protein 1 is frequently observed in HCC. Its knockdown in hepatoma cell lines through RNA-interference has been demonstrated to induce apoptosis after staurosporine treatment in originally resistant cells[151]. A similar approach may be applied to inhibit isoforms that exert definite oncogenic effects, such as SxxSS-deficient TCF4 variants, which are overexpressed in HCC and possess high transcriptional activity due to the absence of co-repressor binding or posttranslational modifications that normally decrease TCF4 activity.

Identification and targeting of the regulators that control the production of particular TF variants would also be beneficial. For instance, tumor-suppressive HNF4α and KLF6 isoforms are frequently downregulated in HCC and can induce at least partial reversion of the malignant phenotype. In contrast, c-MET- and EGFR-mediated activation of signaling pathways that are essential for hepatocarcinogenesis has been demonstrated to drastically alter the expression of multiple splicing factors and induce the production of DN KLF6 and p73 isoforms[14]. Thus, targeted inhibition of the indicated signaling pathways may be considered as an additional strategy to prevent aberrant isoform synthesis. Since the expression of genes encoding splicing machinery components is altered in HCC, their inhibition may be considered as an additional way to reduce aberrant splicing. Several small molecule splicing modulators targeting basal splicing machinery or splicing factors and their regulators have been identified. Such modulators effectively inhibit tumor growth *in vivo,* but they lack specificity; in addition, they have been demonstrated to be toxic in xenograft experiments and clinical trials[152]. Alternatively, whereas some components of the splicing regulatory network are recurrently mutated in HCC[23], targeting

these mutant proteins with specific small molecules or antibodies may be relevant. Thus, further expansion of knowledge on the functions of TF variants, mechanisms of their generation and switching in HCC should shed light on additional mechanisms of hepatocarcinogenesis. Hopefully, it will also facilitate the discovery of new clinically relevant prognostic markers and therapeutic targets and contribute to the development of novel approaches to cancer treatment.

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REVIEW

Complements are involved in alcoholic fatty liver disease, hepatitis and fibrosis

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Abstract

The complement system is a key component of the body's immune system. When abnormally activated, this system can induce inflammation and damage to normal tissues and participate in the development and progression of a variety of diseases. In the past, many scholars believed that alcoholic liver disease (ALD) is induced by the stress of ethanol on liver cells, including oxidative stress and dysfunction of mitochondria and protease bodies, causing hepatocyte injury and apoptosis. Recent studies have shown that complement activation is also involved in the genesis and development of ALD. This review focuses on the roles of complement activation in ALD and of therapeutic intervention in complement-activation pathways. We intend to provide new ideas on the diagnosis and treatment of ALD.

Key words: Alcoholic liver disease; Complement system proteins; Complement regulator; Liver cells; Hepatocyte injury

Core tip: In this review, we cited evidence that the complement system is involved in the pathogenesis of each stage of alcoholic liver disease (ALD) that include fatty liver, alcoholic hepatitis, and fibrosis/cirrhosis, and we also summarized the complement regulation in ALD. We intend to provide new ideas on the treatment of ALD.

Lin CJ, Hu ZG, Yuan GD, Lei B, He SQ. Complements are involved in alcoholic fatty liver disease, hepatitis and fibrosis. *World J Hepatol* 2018; 10(10): 662-669 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i10/662.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i10.662

INTRODUCTION

Liver disease caused by long-term excessive ethanol drinking is a major cause of chronic liver disease. As the global incidence of alcoholic liver disease (ALD) increases year by year, it has become a serious threat to human health. Almost all heavy drinkers have fatty liver, 10%-20% of which develop into alcoholic hepatitis, cirrhosis, and even hepatocellular carcinoma[1]. Exploration of the mechanisms of alcohol-induced liver injury and repair is extremely important in developing methods for preventing and treating ALD.

The complement system plays an important beneficial role in the immune system: Complement activation promotes target-cell lysis, with the associated elimination of exogenous pathogens. Yet, the complement system is a "double-edged sword", as the excessive activation of complement can induce inflammation and lead to autoimmune diseases, such as autoimmune kidney disease, glomerular nephritis, acute lung injury, and others[2-5]. Most plasma complement components are synthesized in liver cells. Thus, the liver becomes the main target of damage by complement activation[6-8]. This connection is likely due to the direct effects of alcohol that activate complement, but not because the liver is a major producer of complement proteins. Several studies have illustrated that complement activation is involved in the development of ALD[8-14] (Table 1).

METABOLIC PATHWAYS OF ETHANOL

Most ingested ethanol is absorbed into the blood circulation, and soon reaches each organ of the body. About 90% of the ingested ethanol is metabolized in the liver[15], and most is metabolized by alcohol dehydrogenase and aldehyde dehydrogenase to form acetic acid, which can be used as substrate in the tricarboxylic acid cycle to produce energy. With excessive drinking, the body can activate another metabolic pathway, *i.e.*, the microsomal ethanol oxidation system (MEOS), which catalyzes ethanol mainly by cytochrome P450 2El in Kupffer cells. The MEOS can over produced reactive oxygen species and reactive nitrogen species, which may exceed the body's antioxidant capacity. Free radicals produced *via* the MEOS pathway exert a series of toxic effects: Membrane-lipid peroxidation, intracellular protease degeneration, oxidative modification of DNA, and others, which eventually lead to necrosis or apoptosis of hepatocytes[14,16-18]. A small percentage of ethanol is metabolized by fatty acid ethyl ester synthase to produce fatty acid ethyl ester through the non-oxidative pathway. Fatty acid ethyl ester has cytotoxicity, which can further injure the liver and pancreas[19]. Thus the liver becomes the main organ damaged by ethanol. Chronic ethanol exposure results in decreased protease activity in liver cells, imbalance of the liver's detoxification function, and overproduction of acetaldehyde, thus inducing hepatic oxidative stress and complement activation; all these activities can injure hepatocytes[20].

COMPLEMENT ACTIVATION PATHWAY

The complement system consists of more than 30 kinds of proteins with enzyme-like activities which are inherent components, regulatory proteins, and complement receptors. Complement regulatory proteins include plasma soluble factors, membrane binding proteins, homologous restriction factor, and membrane inhibitors of reactive lysis. Because the complement system is involved in inflammation and immune regulation, it plays an important role in regulation of pathophysiological functions[21].

Complement is activated by three pathways: The classical, mannan-binding lectin (MBL), and alternative pathways. The three pathways start with different mechanisms, but they end with a common terminal pathway, as shown in Figure 1. The classical pathway is the main mechanism of immune responses. In it, C1q identifies immune complexes, followed by the activation of C1r and C1s. Activated C1s cleaves C2 and C4 to form C3 convertase (C4bC2a), which cleaves C3 to form C5 convertase (C4bC2aC3b). In contrast to activation of the classical pathway, activation of the lectin pathway does not depend on immune complexes. In this pathway, the cascade of enzymatic reactions proceeds in this sequence: MBL identifies the pathogens to form MBL-associated serine proteases (MASP1, MASP2); MASP1 directly cleaves C3 to form C3 convertase (C3bBb), MASP2 cleaves C4 and C2 in a manner similar to that of C1s, forming C3 convertase (C4bC2a), which continues to cleave C5 to form C5 convertase (C4bC2aC3b). Thus, this pathway can cross-promote the classical and alternative pathways. The alternative pathway is activated with hydrolysis of C3 into C3(H_2O), factor B and factor D, the activation of which is also independent of immune complexes, and participates in the defense mechanisms of the early stage of inflammation[13,22-24]. The above three pathways merge into the terminal pathway, in which C5 convertase cleaves C5 to form C5a and C5b, and C5b combines with C6, C7, C8 and C9 to form the membrane attack complex (MAC). Formation of the MAC leads to cell lysis and induces cells to release inflammatory cytokines.

COMPLEMENT ACTIVATION IN ALD

ALD progresses in three distinct stages: Fatty liver, alcoholic hepatitis, and fibrosis/cirrhosis. In this review, we cite evidence that the complement system is involved

Table 1 Important publications on complement in alcoholic liver disease

Study	Complement component	Alcoholic liver disease	Species
Järveläinen *et al*[10]	C1, Crry, CD59	Alcohol-induced injury	Rat
Bykov *et al*[11]	C3	Liver steatosis	Mouse
Pritchard *et al*[8]	C3, C5, DAF	Fatty liver	Mouse
He *et al*[7]	C3	Liver steatosis	Mouse
Cohen *et al*[13]	C1q	Alcohol-induced injury	Mouse
Wlazlo *et al*[27]	C3	Liver steatosis	Human
Shen *et al*[6]	C3	Alcoholic hepatitis	Mouse
Maslowska *et al*[30]	Factor D	Alcoholic hepatitis	Mouse

Crry: Complement receptor 1-related protein y; DAF: Decay accelerating factor.

in the pathogenesis of each of these stages.

Complement activation in alcoholic fatty liver disease

The liver is the main site of fat metabolism. Disorders of fat metabolism, caused by various factors, can lead to excessive fat accumulation in the liver cells, *i.e.*, fatty liver. Long-term heavy drinking is the main independent risk factor of fatty liver disease[25], but its pathogenesis is not clearly defined. Liu *et al*[26] found that gut microbiota played a synergistic role in the liver response, and the complement system was suppressed in fatty liver which was partially due to increased blood lactic acid from enriched Lactobacillus. Abnormal complement activation reportedly enhances the sensitivity of steatotic livers to ischemia and reperfusion injury, which leads to the development of fatty liver[27,28]. Järveläinen *et al*[10] found that deposition of complement C1, C3, and C8 was increased, and the expression of membrane-binding proteins, complement receptor 1-related protein y (Crry), and CD59 was decreased in the liver cells of a mouse ALD model. These findings proved that alcohol-induced complement activation can result in ALD, at least in an experimental model. In a study in mice chronically exposed to ethanol, Cohen *et al*[13] found that lipid deposition in liver cells as well as values of liver-related serum enzymes (alanine aminotransferase and aspartate amino transferase) increased significantly; various degrees of liver cell apoptosis were also found. Moreover, with knock out of the C1q gene, hepatic steatosis in the mice was significantly decreased[13]. This study illustrated that complement activation could be associated with ethanol-induced hepatic steatosis.

Bykov *et al*[11] fed C3+/+ and C3-/- mice a high fat and high alcohol diet, respectively, and found that hepatic steatosis and significant increases in triglyceride values occurred in the C3+/+mice, whereas C3-/-mice were protected from ethanol-induced liver injury; research by Stewart *et al*[9] yielded similar results.

At the complement activation pathways, C3a converted to C3adesAg, C3adesArg which known as acylation stimulating protein had been shown to have lipogenic activity *via* its receptor C5L2, and promoted triglyceride storage in adipocytes[29,30] It was also found that C3adesArg was involved in the triglyceride metabolism[31].

Thus, activation of complement C1 and C3 appears to play a significant role in promoting fatty accumulation in the liver. Further definition of the relationship between activated complement C1, C3 and lipid metabolism in the liver may aid in the development of methods for intervention and treatment of alcoholic fatty liver disease. Besides C1 and C3, complement C5 also is involved in lipid metabolism. Bavia *et al*[32,33] found that the activation of complement C5 by high-dose ethanol exposure can affect the distribution of lipid in liver cells and serum. Less lipid and cholesterol is deposited in hepatocytes of C5- mice than in hepatocytes of C5+mice, and values of IL-17, which are involved in the synthesis and metabolism of lipid and cholesterol, are higher in C5- mice than in C5+mice[34,35]. The above-cited reports indicate that activation of C5 may play a role in the development of alcoholic fatty liver.

Complement activation in alcoholic hepatitis

ALD has many potential pathogenic factors, such as endotoxin, which may lead to complement activation and deposition in the liver cells. Shen *et al*[6] found that complement activation was involved in humans with ALD. Cohen *et al*[13] found that long-term alcohol exposure can lead to apoptosis of liver cells, and the degree of apoptosis is positively correlated with liver injury. However, whether short-term alcohol exposure can cause hepatocyte apoptosis was not known. Further research found that short-term alcohol exposure did not cause hepatocyte apoptosis, but it did promote the deposition of complement C3b and the expression of inflammatory cytokines (tumor necrosis factor and IL6). After the Cq gene was knocked out, the expression of inflammatory cytokines was significantly reduced compared to that in wild-type animals[12,13]. Experiments by Païdassi *et al*[36] and Lu *et al*[37] supported these observations.

Complement C5, a core component of the complement activation pathway, is involved in the occurrence and development of alcoholic hepatitis, in addition to fatty liver[6,8,38]. Bavia *et al*[38] documented this in a hepatitis model induced by alcohol; they found that values of pro-inflammatory cytokines (IL-6, IFN-γ, IL-1β, and others) in B6C5+ mice were significantly higher than those in B6C5- mice, and anti-inflammatory factors (IL10 and IL17) were secreted significantly more in B6C5- mice. These findings illustrated that activated C5 induced the expression of proinflammatory cytokines after alcohol exposure. Up-regulated expression of pro-inflammatory cytokines (IL-6, IFN-γ, IL-1β, and others) aids the body's defense against pathogenic microorganisms, but it also participates in the pathogenesis of alcoholic fatty liver and alcoholic hepatitis[8,39-41].

Complement activation in alcoholic hepatic fibrosis

Intrahepatic inflammatory reaction and a decrease

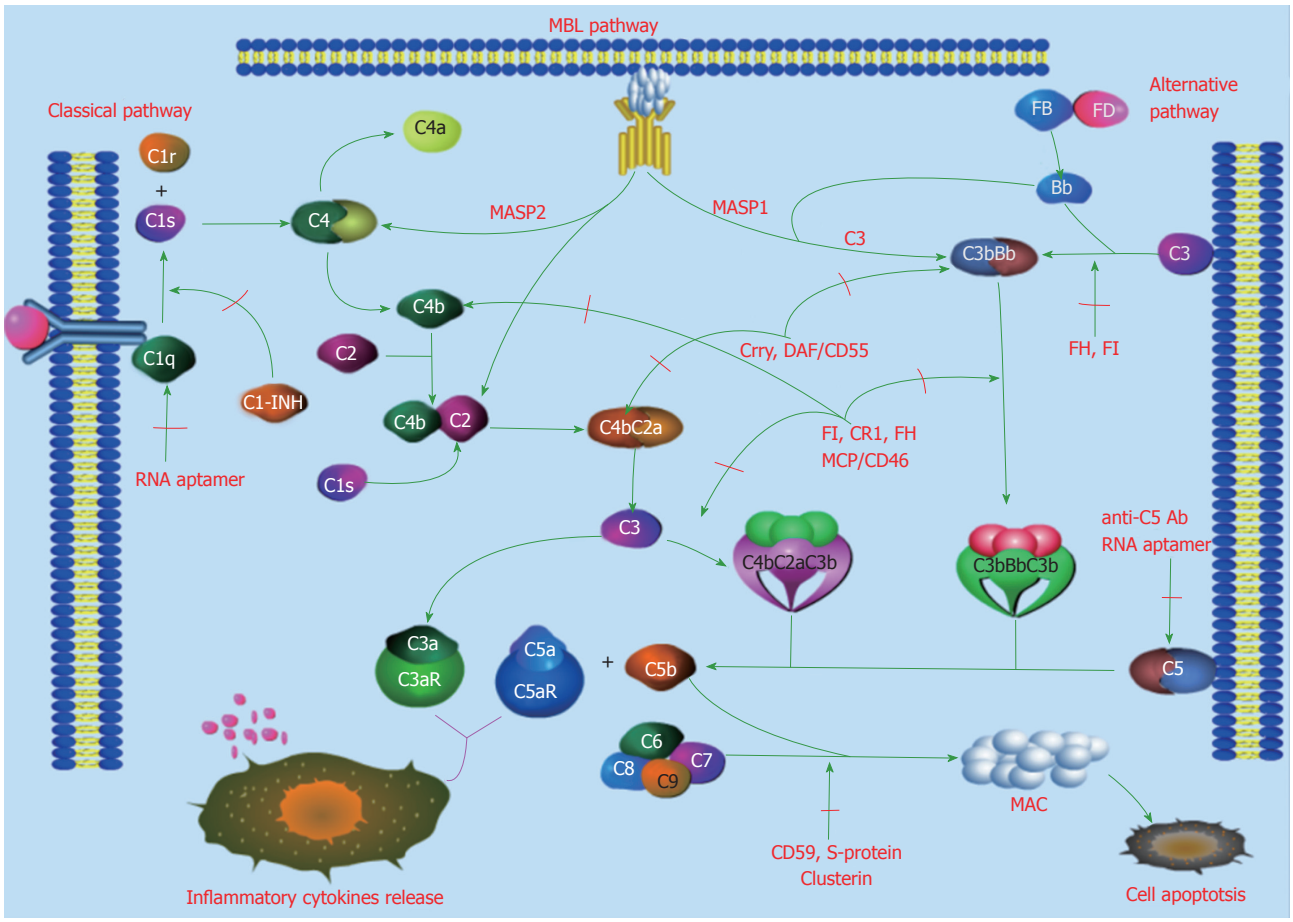

Figure 1 Complement activation pathway and regulation. Three pathways (classical, pathway, mannan-binding lectin, and alternative) are involved in complement activation. Green arrows without a red line indicate the process of complement activation. Green arrows with red line indicate the the process of inhibiting complement activation. MBL: Mannan-binding lectin; MASP: MBL-associated proteases; FB: Factor B; FH: Factor H; FI: Factor I.

in structural integrity of hepatic sinusoidal endothelial cells after long-term alcohol exposure are important inducements to liver injury. Sinusoidal endothelial cells express C5R1, which is the foundation of C5 activation-induced alcoholic hepatic fibrosis[42]. In recent years, the pathogenesis of alcoholic hepatic fibrosis has attracted worldwide attention , but the cause of the fibrosis is still not fully defined[43-45]. According to published reports[46,47], complement C3, C4 and activation of the MBL pathway are involved in the development of fibrosis. Bavia *et al*[38] using the mouse model of ALD, found that values of TGF-β, which promotes hepatic fibrosis, were significantly higher in B6C5+ mice than in B6C5- mice[38,48]. Hillebradt *et al*[49] found that the C5 gene was involved in the regulation of hepatic fibrosis on human chromosome, and further study found that C5- mice had decreased hepatic fibrosis. Thus, the evidence indicates that activation of complement C5 may promote hepatic fibrosis. Exploration of the relationship between complement activation and alcoholic hepatic fibrosis, and of possible intervention in ALD by reversing the progression of hepatic fibrosis in its early stage, seems worthwhile goals.

Complement-induced Kupffer cells activation in ALD

Kupffer cells, located in liver sinusoids, are an important part of the mononuclear phagocyte system. Alcohol exposure in the early stage can promote apoptosis of Kupffer cells, but longer exposure usually is needed[13,50,51]. Ethanol-induced activation of complement component C1q at the early stage of ALD promotes the release of inflammatory cytokines from Kupffer cells, which further promote alcoholic liver injury[51-56]. Furthermore, Kupffer cells can express C3R and C5R, then induce prostaglandin release and synthesis of pro-inflammatory cytokines[57-60]. However, in certain pathological conditions, activated C5 combines with C5R, inducing the upregulation of fibrinogen on Kupffer cells, an interaction that is believed to lead to hepatic fibrosis[22,61]. In addition, alcohol-induced upregulation of CD14 leads to Kupffer cells combining with lipopolysaccharide, which induces liver damage through the activation of TLR4 in Kupffer cells and inflammatory signaling pathways; these events can further aid in the development of hepatic fibrosis or cirrhosis[62]. Thus, Kupffer cells seem to be extensively involved in the development of ALD[63-66].

COMPLEMENT REGULATION IN ALD

Reducing inflammatory reactions by inhibiting amplification of the complement cascade and blocking the

Table 2 Complement regulators

Type of regulators	Regulators	Functions
Complement regulatory protein	DAF/CD55, Crry, FH, FI	Inhibit C3, C5 convertase
	CD59, protein S, clusterin	Inhibit MAC
Complement inhibitor	C1-INH	Inhibit C1r, C1s
Targeted inhibitor	h5G1, 1-ScFv	Inhibit C5 activation
RNA aptamer	Specifically bind C1q, C5	

DAF: Decay accelerating factor; Crry: Complement receptor 1-related protein y; FH: Factor H; FI: Factor I; MAC: Membrane attack complex.

combination of complement with the corresponding complement receptors are being pursued worldwide. Excessive activation of complement can be inhibited by self-regulation of the body (Table 2). For example, the complement regulatory protein decay accelerating factor (DAF) can inhibit C3, C5 convertase, thereby inhibiting amplification of the complement cascade. The complement regulatory protein Crry can cooperate with DAF and factor H to accelerate dissociation of C3 and C5 convertase and to cleave C3b and C4b, so that the cells avoid being attacked by autologous complement[67-69]. Deficiency of CD55/DAF and complement regulatory factors aggravate liver injury[8,11], whereas factor H can control the activity and stability of C3 convertase *via* binding with C3b[70,71]. Also, defects in the factor H gene can cause persistent activation of complement pathways and trigger various diseases[72-75]. By contrast, factor H-related proteins (FHRs), including FHR1-5, can either promote or inhibit complement activation. The degree of complement activation depends on the homeostasis between factor H and FHR[71]. However, the relationship between factor H and ALD has not been clarified and needs further research. McCullough *et al*[76] found FD-dependent amplification of complement is an adaptive response that promotes hepatic healing and recovery in response to chronic ethanol. In other complement regulatory activities, CD59, protein S and clusterin inhibit the formation of the MAC through limiting the binding of complement C9[77-81]. Membrane cofactor protein (MCP) and factor I can inhibit cells from binding with C3b and C4b[82,83].

Specific epitope structures of complement, such as anti-complement antibody, complement antisense strand, and complement mutants[84-91] have been invented, with the intent of inhibiting complement activation. In addition, complement inhibitors and RNA aptamer are being used to inhibit progression of complement-related diseases[92,93], and C1-INH and CR1 have been used in the treatment of ALD and other diseases[10].

CONCLUSION

Mounting evidence indicates that complement activation is involved in the development of ALD at all its stages - fatty liver, alcoholic hepatitis, and fibrosis/cirrhosis. Moreover, all three pathways of complement activation (classical, MBL, and alternative) promote the development of ALD. Therapeutic strategies, using various measures to inhibit complement activation, might prevent the development of ALD. Thorough understanding of the relationships between complement activation and ALD may aid in developing new approaches for the treatment of ALD.

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MINIREVIEWS

Era of direct acting anti-viral agents for the treatment of hepatitis C

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Abstract

Hepatitis C infection is universal and the most common indication of liver transplantation in the United States. The period of less effective interferon therapy with intolerable side effects has gone. Now we have stepped into the era of direct acting anti-viral agents (DAAs) against hepatitis C virus. Treatment of hepatitis C is now extremely effective, tolerable and requires a short duration of intake of oral agents. Less monitoring is required with the current therapy and drug-drug interactions are less than the previous regimen. The current treatment options of chronic hepatitis C with various DAAs are discussed in this article.

Key words: Direct acting anti-viral agents; Hepatitis C virus infection; Post-liver transplant; Hepatitis C virus/human immunodeficiency virus co-infection

Core tip: Treatment of hepatitis C has now become much easy and simple with the advent of direct acting anti-viral agents (DAAs) against hepatitis C virus (HCV). Although the DAAs are highly effective in eradicating HCV infection, they have different mechanisms of action, side effects, resistance factors and drug-drug interactions. The treatment also varies in special situations like HCV/ human immunodeficiency virus co-infection and post-liver transplant patients. Physicians treating patients with HCV infection should have a clear knowledge about the DAAs as well as the current guidelines.

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INTRODUCTION

Chronic hepatitis C infection is common in the United

States and throughout the world. About 3 million people in the United States and 177.5 million people in the world suffer from chronic hepatitis C infection[1]. Hepatitis C virus (HCV) contains structural proteins - core or C protein, E1 and E2 envelope proteins, and nonstructural proteins - P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B[2]. HCV contains error prone RNA dependent RNA polymerase and mutation is present during each viral replication[3]. As a result, HCV exists as a quasispecies characterized by the presence of various genetically distinct variants which protect the HCV from host defenses as well as anti-HCV agents[4]. DAA are specific antiviral agents targeting the critical steps of HCV replication. With the development of direct acting anti-viral agent (DAA), the treatment of chronic hepatitis C has been completely revolutionized. In 1990's when we had only Interferon therapy, the sustained virological response (SVR) was 17% with many side effects[5]. Then Pegylated Interferon and Ribavirin came after few years but still the SVR was 40% to 50%[6]. In 2011, the first generation NS3/4A protease inhibitors (Telaprevir and Boceprevir) were launched to be used with pegylated interferon and ribavirin for the treatment of hepatitis C genotype 1 infection. The SVR improved to about 70% at the expense of many side effects, drug-drug interactions and complex regimen of administration of medications and cost[7]. Now we have 2nd generation NS3/4A protease inhibitors, NS5A inhibitors and NS5B inhibitors. They have markedly improved the efficacy of treatment of HCV infection. The different DAAs with their dose, efficacy, side effects, drug-drug interactions as well as specific treatment against different genotypes of HCV will be discussed in the following sections (Figure 1).

NS3/4A protease inhibitors

HCV NS3/4A is a multifunctional protein composed of a membrane-targeted serine protease domain and a helicase domain. NS3/4A protease is responsible for maturation of a viral polyprotein that cleaves and generates NS3, NS4A, NS4B, NS5A and NS5B. Thus NS3/4A protease is essential for viral replication[8]. NS3/4A protease inhibitors (PIs) are classified into first generation PIs and second generation PIs[9]. The first generation PIs include boceprevir and telaprevir. They are no longer used in clinical practice. The second generation PIs includes simeprevir, paritaprevir, grazoprevir, glecaprevir and voxilaprevir. They are highly potent DAAs but they have low barrier to resistance and limited genotypic coverage.

Simeprevir is a once daily macrocyclic 2nd - waive NS3/4A protease inhibitor approved to be used with pegylated interferon and ribavirin for the treatment of chronic hepatitis C genotype 1 patients in 2013. In QUEST-1 and QUEST-2 trials (simeprevir + PEG + RBV *vs* PEG + RBV in treatment-naïve genotype 1 patients), 80% to 81% of treatment-naïve patients achieved SVR12, *i.e.*, negative viral load 12 wk after completion of treatment[10,11]. Patients who had Q80K polymorphism had only 58% SVR12. In PROMISE trial, Simeprevir was given with pegylated interferon and ribavirin to treatment (Interferon)-experienced hepatitis C genotype 1 patients. The SVR12 was 79.2%. The SVR 12 also varied with the host IL28B genotype. Patient's IL28B genotype is involved in the host immune response to HCV infection. There are 3 IL28B subtypes: CC, CT and TT. The IL28B CC genotypes had the highest response and the IL28B TT genotypes had the lowest response[12]. In COSMOS trial, combination of simeprevir and sofosbuvir (NS5B inhibitor) with or without ribavirin were given to HCV genotype 1 infected patients - both treatment-naïve patients and non-responders to pegylated interferon and ribavirin for 12 wk and 24 wk. Ninety-two percent to 94% of patients achieved SVR12[13]. In OPTIMIST-1 trial, combination of simeprevir and sofosbuvir was given to treatment-naïve non-cirrhotic HCV genotype 1 infected patients. Ninety-seven percent of patients who received this combination achieved SVR12 in the 12 wk arm whereas only 83% achieved SVR12 in the 8-wk arm[14]. In OPTIMIST-2 trial, cirrhotic patients due to HCV genotype 1 infection received combination of simeprevir and sofosbuvir for 12 wk. SVR12 was achieved in 83% of patients: 88% in treatment-naïve patients and 79% in treatment-experienced patients[15].

The main side effects of Simeprevir include headache, fatigue, nausea, photosensitivity and skin rash[16]. Latent HBV infection could be reactivated if patient is on simeprevir. Simprevir can also cause hepatic failure if used in decompensated cirrhosis of liver. Simeprevir has sulphur moiety which may cause sulphur allergy. Patients on simeprevir should not take moderate to high intensity enzyme-inducers or enzyme-inhibitors as they may decrease or increase the serum levels of simeprevir. Prior to the use of Simeprevir, it is recommended to do Q80K polymorphism screening (HCV GenoSure NS3/4A Assay) which has been found in 35% of patients infected with HCV genotype 1, and in more than 40% of patients infected with HCV genotype 1a[17]. In the Interferon free era, combination of simeprevir 150 mg/d and sofosbuvir 400 mg/d for 12 wk is recommended as an alternative regimen in the treatment of treatment-naïve genotype 1a or 1b patients without cirrhosis as per the American Association for the Study of Liver Diseases (AASLD).

Drug-drug interactions: Common medications which are not recommended to be co-administered with Simeprevir include systemic antibiotics like clarithromycin, erythromycin, systemic antifungals like fluconazole, ketoconazole, itraconazole, antimycobacterials like rifampin, rifabutin, anti-hepatitis C medication ledipasvir, anti-human immunodeficiency virus (HIV) medications like ritonavir, darunavir, efavirenz, nevirapine, etravirine, delavirdine, atazanavir, nelfinavir, saquinavir, indinavir, fosamprenavir, tipranavir, anti-arrhythmic drug like amiodarone, anticonvulsants like phenytoin, phenobarbital, carbamazepine, dexamethasone, cyclosporine, milk thistle and St. John's Wort[18].

Paritaprevir is a macrocyclic NS3/4A protease inhibitor used in combination with low dose ritonavir which is a strong CYP3A inhibitor, and thus increases

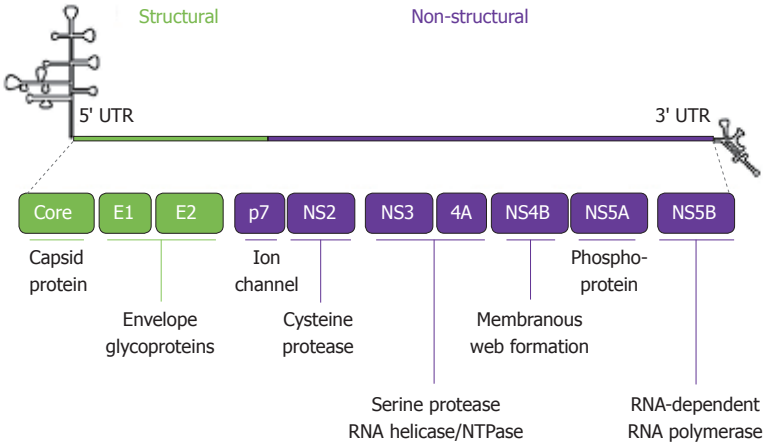

Figure 1 Hepatitis C virus RNA (genome).

peak and trough level of Paritaprevir[19]. Paritaprevir/ritonavir-ombitasvir (NS5A inhibitor) - a 3 drug fixed dose combination tablet co-packaged with dasabuvir (NS5B inhibitor) tablet in Viekira Pak, and dasabuvir, ombitasvir, paritaprevir and ritonavir - a 4 drug fixed dose combination tablet in Viekira XR with or without ribavirin are approved for the treatment of chronic hepatitis C genotype 1[20]. Paritaprevir/ritonavir-ombitasvir (in TECHNIVIE) with ribavirin is approved for the treatment hepatitis C genotype 4 without cirrhosis of liver. Viekira Pak and Viekira XR carry a higher pill burden than other regimen. They are also contraindicated in advanced cirrhosis of liver (Child-Pugh B and C). They are metabolized mainly in the liver. As a result, no dose adjustment is needed even in severe renal failure including those on dialysis[21]. If the patient has HBV/HCV co-infection and not receiving HBV antiviral therapy, HBV reactivation can occur leading to severe hepatitis, hepatic failure and death. In RUBY-1 trial, 90% of patients with HCV genotype 1 infection and end-stage renal disease (CKD 4 and CKD 5) had SVR12, *i.e.*, negative serum HCV RNA after 12 wk of therapy with dasabuvir, ombitasvir, paritaprevir and ritonavir[22]. In case of HCV genotype 1a infection - Viekira should be continued for 12 wk in non-cirrhotics and 24 wk in cirrhotics. In case of HCV genotype 1b infection - Viekira should be given for 12 wk irrespective of cirrhosis or non-cirrhosis status[23]. In liver transplant recipients with normal liver function and mild hepatic fibrosis, Viekira pak or Viekira XR with weight-based ribavirin should be given for 24 wk. The common side effects of Viekira include nausea, insomnia, itching and asthenia.

Drug-drug interaction: Common medications which are contra-indicated with Viekira include lipid lowering agent gemfibrozil, HMG co-reductase inhibitors - simvastatin, atorvastatin, lovastatin, anti-arrhythmic drug dronedarone, α-1 adrenoreceptor blocker alfuzosin, anti-anginal medication ranolazine, phosphodiesterase inhibitor sildenafil, anticonvulsants like phenytoin, phenobarbital, carbamazepine, sedative/hypnotics triazolam, midazolam, anti-gout medication colchicine, antimycobacterial medication rifampin, antipsychotic medication lurasidone and pimozide, ergot agents methylergonovine, ergotamine and dihydroergotamine, ethinyl estradiol containing medications, prokinetic agent cisapride, herbal agent St. John's Wort, immunosuppressive agents tacrolimus, sirolimus and everolimus, and anti-HIV medication efavirenz[24].

Grazoprevir is a macrocyclic NS3/4A serine protease inhibitor against HCV genotype 1 and 4. It is used in combination with Elbasvir (NS5A inhibitor) for the treatment of HCV genotypes 1 and 4. In clinical trials, combination of elbasvir and grazoprevir (Zepatier) has been shown to achieve SVR in 94% to 97% of cases of genotype 1 infection and 97% to 100% of cases of genotype 4 infection. The overall SVR for non-cirrhotics was 97% and for cirrhotics 95.7%[25]. The regimen was effective in stage 4 to 5 chronic kidney disease[26]. In case of HCV genotype 1a infection, NS5A resistance testing should be done prior to initiation of elbasvir and grazoprevir therapy. In 10% to 15% of cases, NS5A polymorphism is positive and in that case weight-based ribavirin (less than 66 kg = 800 mg/d, 66 to 80 kg = 1000 mg/d, 81 to 105 kg = 1200 mg/d, greater than 105 kg = 1400 mg/d administered in two divided doses with food) should be added and the duration should be increased from 12 to 16 wk. Otherwise HCV genotype 1a or 1b infection requires only 12 wk of Zepatier therapy irrespective of exposure to pegylated interferon and ribavirin (PEG/RIBA), and presence of cirrhosis of liver. In case of HCV genotype 4 infection, the duration of Zepatier therapy is 12 wk for treatment-naïve patients, but it should be extended to 16 wk if PEG/RIBA experienced with prior on-treatment virologic failure[27]. In C-ISLE study, Elbasvir/Grazoprevir plus Sofosbuvir was given to patients with compensated cirrhosis due to HCV genotype 3 infection for 12 wk. The SVR12 was 96% in treatment-naïve patients and 100% in PEG-interferon/Ribavirin experienced patients[28]. HBV reactivation can occur on grazoprevir/elbasvir therapy if the patient is co-infected with HBV and not receiving anti-HBV therapy. HBV serology (HBsAg and anti-HBc) and HCV resistance

associated polymorphisms as mentioned before should be tested prior to initiation of Zepatier therapy. Common side effects include headache, nausea and fatigue[29].

Common medications which are not recommended to be used with Zepatier include antibiotic Nafcillin, antifungal oral ketoconazole, anti-HIV medication etravirine, cobicistat containing medications like tenofovir, emtricitabine, cobicistat, HMG Co-A reductase inhibitors atorvastatin (dose should not exceed > 20 mg/d), rosuvastatin (dose should not exceed > 10 mg/d), simvastatin, lovastatin, fluvastatin (should be closely monitored for myopathy), nacolepsy medication modafinil, immunosuppressant tacrolimus, and endothelin antagonist bosentan[29].

Drug-drug interaction: Glecaprevir is a NS3/4A protease inhibitor coformulated with NS5A inhibitor pibrentasvir (Mavyret). Glecaprevir-pibrentasvir combination has been shown to have pangenotypic anti-HCV activity. In EXPEDITION-1 study, glecaprevir (300 mg) - pibrentasvir (120 mg) coformulation when given daily for 12 wk to treatment-naïve or treatment experienced (pegylated interferon plus ribavirin or sofosbuvir plus ribavirin) patients with HCV genotypes, infection and compensated cirrhosis, 99% of patients achieved SVR at 12 wk[30]. Another study showed glecaprevir-pibrentasvir combination when given for 8 wk to patients with HCV genotype-1 and 3 infection had SVR 12 of 99.1% and 95% respectively[31]. Patients with chronic kidney disease (CKD) have more chronic hepatitis C, particularly in patients on hemodialysis, 8.4% hemodialysis patients had chronic hepatitis C in the year of 2000. When patients with severe renal failure (stage 4 or 5 CKD or dialysis dependence) and HCV genotype 1, 2, 3, 4, 5 or 6 infection with or without compensated cirrhosis, treatment-naïve or treatment-experienced (pegylated interferon, ribavirin, sofosbuvir or a combination of these medications), were treated with glecaprevir-pibrentasvir combination for 12 wk, they had sustained SVR12 of 98%[32]. No dose adjustment was required in patients with severe renal failure or in hemodialysis patients[33].

Three tablets of fixed dose Glecaprevir 100 mg/ Pibrentasvir 40 mg (*i.e.*, 300 mg/120 mg total dose) PO once daily is given with food.

In treatment-naïve patients[33], the recommended duration is 8 wk for genotypes 1 to 6 infection without cirrhosis. But the treatment duration is 12 wk for genotypes 1 to 6 infection with compensated cirrhosis (Child-Pugh A). In treatment-experienced patients. In non-cirrhotics: Genotype 1 and NS5A inhibitor prior treatment: 16 wk. Genotype 1 and NS3/4A protease inhibitor prior treatment: 12 wk. Genotypes 1, 2, 4, 5, or 6 (prior treatment with boceprevir, or telaprevir or simeprevir with pegylated interferon and ribavirin, simeprevir and sofosbuvir): 8 wk. Genotype 3 (prior treatment with boceprevir, or telaprevir or simeprevir with pegylated interferon and ribavirin, simeprevir and sofosbuvir): 16 wk.

In treatment-experienced patients[33] with compensated cirrhotics (Child-Pugh A): Genotype 1 and NS5A inhibitor prior treatment: 16 wk. Genotype 1 and NS3/4A protease inhibitor prior treatment: 12 wk. Genotypes 1, 2, 4, 5, or 6 (prior treatment with boceprevir, or telaprevir or simeprevir with pegylated interferon and ribavirin, simeprevir and sofosbuvir): 12 wk. Genotype 3 (prior treatment with, boceprevir, or telaprevir or simeprevir with pegylated interferon and ribavirin, simeprevir and sofosbuvir): 16 wk. Common side effects of Mavyret include headache, fatigue, nausea and diarrhea.

Commonly used medications which can cause drug interaction with Mavyret include HMG Co-A reductase inhibitors atorvastatin, lovastatin, simvastatin, rosuvastatin, pravastatin, pitavastatin, fluvastatin, ethinyl estradiol containing medications, anticoagulant dabigatran, antiarrhythmic digoxin, anticonvulsant carbamazepine, antimycobacterial rifampin, anti-HIV medications efavirenz, ritonavir, lopinavir, darunavir, atazanavir, herbal medication St. John's Wort, and immunosuppressant cyclosporine[34].

Voxilaprevir is a NS3/4A protease inhibitor. It is used in the fixed dose combination of sofosbuvir and velpatasvir (NS5A inhibitor), commercially available as Vosevi. In POLARIS-1, sofosbuvir (400 mg)/velpatasvir (100 mg)/voxilaprevir (100 mg) single pill was given daily for 12 wk to NS5A inhibitor-experienced patients with HCV genotype 1-6 infection. In POLARIS-4, sofosbuvir/velpatasvir/voxilaprevir was given daily for 12 wk to DAA experienced (but not NS5A experienced) patients with HCV genotype 1-3 infection, and also to patients with HCV genotype 4 infection. Forty-six percent of all these patients had compensated cirrhosis of liver. In POLARIS-1, the SVR was 96%, and in POLARIS-4, the SVR was 98%[35]. Currently, Vosevi is approved for retreatment of: (1) HCV genotype 1-6 infection in adults who were previously treated with an NS5A inhibitor-containing regimen; and (2) HCV genotype 1a or 3 infection in adults who were previously treated with sofosbuvir-containing regimen without an NS5A inhibitor[36]. Vosevi can be given to patients without cirrhosis or with compensated cirrhosis, and no dose adjustment is required even in severe renal impairment with glomerular filtration rate (GFR) < 30 mL/min or end stage renal disease. If the patient is HBV coinfected and not receiving anti-HBV therapy, HBV reactivation can occur during or after completion of treatment with Vosevi. So, serological evidence of HBV infection (HBVsAg and anti-HBVc antibody) should be looked for before initiation of therapy with Vosevi. Common side effects of Vosevi include headache, nausea, diarrhea insomnia, asthenia and fatigue.

Drug-drug interaction: Certain medications are not recommended with Vosevi. These include anatacids like aluminum or magnesium hydroxide, H2 receptor antagonists like Famotidine, proton pump inhibitor like omeprazole, anticoagulant like dabigatran, antiarrhythmic agents like digoxin, amiodarone, HMG Co-A reductase inhibitors atorvastatin, lovastatin, fluvastatin,

simvastatin, rosuvastatin, pravastatin, pitavastatin, anticonvulsants like phenytoin, phenobarbital, carbamazepine, oxcarbazepin, anti-HIV medications efavirenz, tenofovir, lopinavir, atazanavir, tipranavir/ritonavir, anti-mycobactrial agents like rifampin, rifabutin, rifapentin, immunosuppressant cyclosporine, and herbal supplement St. John's Wort[37].

NS5A inhibitors

Inhibit hyperphosphorylation of NS5A phosphoprotein which is necessary for HCV RNA replication, and they also cause transfer of NS5A from the endoplasmic reticulum to lipid droplets in HCV replicon-containing cells leading to significant reduction of HCV RNA in cell culture[38]. Ledipasvir, ombitasvir, daclatasvir, elbasvir, velpatasvir and pibrentasvir are NS5A inhibitors. They are highly potent DAA with multigenetic coverage and intermediate barrier to resistance.

Ledipasvir is a NS5A inhibitor used as part of combination therapy with Sofosbuvir (NS5B inhibitor) for the treatment of chronic hepatitis C. The fixed dose combination called Harvoni (90 mg of Ledipasvir and 400 mg Sofosbuvir) developed by Gilead Sciences was approved by the FDA in 2014.

Effectiveness, duration and need for addition of ribavirin with this medication depend on viral load, genotype, compensated or decompensated cirrhosis, treatment naïve or treatment-experienced status, and pre or post-transplant status.

In ION-1 phase 3 clinical trial, 99% of treatment naïve patients with chronic hepatitis C genotype 1 who received Ledispavir-Sofosbuvir combination for 12 wk achieved SVR[39]. In ION-2 phase 3 clinical trial, 99% of patients with previously treated genotype-1 HCV infection who received Ledispavir-Sofosbuvir combination for 24 wk had SVR[40]. Patients with HCV genotype-1 infection and compensated cirrhosis were studied with Ledispavir-Sofosbuvir combination for 12 and 24 wk in treatment naïve and treatment experienced patients with or without ribavirin[41]. The overall SVR was 96%: 98% in treatment-naïve group *vs* 95% in treatment-experienced group, 95% in 12 wk therapy group *vs* 98% in 24 wk therapy group, 95% without ribavirin *vs* 97% with ribavirin. The only group who did not do well was the treatment-experienced group who received Ledispavir-Sofosbuvir combination for only 12 wk and without ribavirin. Their SVR was 90%[41]. As a result, this combination is recommended to be continued for 24 wk in treatment-experienced HCV genotype-1 infection with compensated cirrhosis. Patients with HCV genotype-4 with or without compensated cirrhosis were treated with Ledispavir-Sofosbuvir combination with or without ribavirin. Overall SVR12 was 95.4% in non-cirrhotics and 93.2% in cirrhotics[42]. Patients with HCV genotype 5 with or without compensated cirrhosis were treated with Ledspavir-Sofosbuvir for 12 wk in a multi-center open-label study. The SVR12 was 95% in both treatment-naïve and treatment-experienced groups; 89% of cirrhotics and 97% of non-cirrhotics achieved SVR12[43]. A small study showed when fixed dose combination of Ledispavir-Sofosbuvir was given to treatment-naïve and treatment-experienced patients with HCV genotype 6 infection for 12 wk, 96% of them achieved SVR12[44]. At the present time, Ledispavir-Sofosbuvir combination is indicated for the treatment of HCV genotype 1, 4, 5 and 6 infections with or without compensated cirrhosis. It is also used in HCV genotype 1 infection with decompensated cirrhosis in combination with ribavirin. Post liver transplant recipients who have HCV genotype 1 or 4 infection with or without compensated cirrhosis can be treated with Ledispavir-Sofosbuvir plus ribavirin for 12 wk. In one study, 96% of patients who had F-0 to F-3 fibrosis or compensated cirrhosis achieved SVR[45].

Common side effects of Harvoni include headache, fatigue, nausea and diarrhea. Harvoni like other DAAs can reactivate HBV if the patient is HBV/HCV co-infected and not receiving anti-HBV therapy. So prior to initiation of Harvoni, serological evidence of HBV infection should be tested and treated if positive.

Drug-drug interaction: Co-administration of amiodarone with Harvoni may cause serious symptomatic bradycardia. Dose adjustment or regimen change may be required for certain medications. These include antiarrhythmic drug digoxin, HMG Co-A reductase inhibitor rosuvastatin, anticoagulant warfarin, acid reducing agents proton pump inhibitors, H2 receptor antagonists, antacids, anticonvulsants phenytoin, phenobarbital, carbamazepine, oxcarbazepine, antimycobacterials rifampin, rifabutin, rifapentine, anti-HCV medication simeprevir, anti-HIV medications tenofovir DF, emtricitabine, cobicistat, elvitegravir, tipranavir/ritonavir, regimen containing tenofovor DF and an HIV protease inhibitor/ritonavir or cobicistat: darunavir/ritonavir or cobicistat + emtricitabine/tenofovir DF, atazanavir/ritonavir or cobicistat + emtricitabine/tenofovir DF, lopinavir/ritonavir + emtricitabine/tenofovir DF, and herbal supplement St. John's Wort[46].

Ombitasvir is a NS5A inhibitor. After absorption, it binds to NS5A and blocks the activity of NS5A to prevent HCV replication. It is used in combination with paritaprevir, retinovir and dasabuvir with or without ribavirin. In Viekira pak, paritaprevir, ritonavir and ombitasvir packaged in a single table, and dasabuvir is a different tablet. The daily dose of Viekira pak is 2 tablets of paritaprevir, ritonavir and ombitasvir in the morning, and one tablet of dasabuvir twice a day. In Viekira XR, each tablet contains partaprevir, ritonavir, ombitasvir and dasabuvir. 3 tablets have to be taken daily for 12 to 24 wk.

Daclatasvir is a highly selective pangenotypic NS5A inhibitor[47]. It has dual mode of action as it binds to the N-terminal of NS5A and thus prevents viral replication and viral assembly. It is the first oral medication used in combination with sofosbuvir for 12 wk for the treatment of hepatitis C genotype 3 infection. HCV genotype 3 is the most aggressive (having faster disease progression) and

most treatment resistant genotype affecting 12% of all HCV genotypes in United States. In Ally-3 trial, a 12 wk therapy of daclatasvir plus sofosbuvir achieved SVR12 in 96% of non-cirrhotic patients infected with HCV genotype 3 irrespective of prior treatment experience[48]. In Ally 3+ trial, daclatasvir plus sofosbuvir plus ribavirin were given to treatment-naïve and treatment-experienced patients with advanced fibrosis or compensated cirrhosis for 12 or 16 wk. The SVR12 was 90% in the 12 wk and 92% in the 16 wk group[49]. In Ally-2 trial, 12 wk course of daclatasvir plus sofosbuvir achieved SVR12 of 97% in patients infected with HCV genotype 1 to 4 coinfected with HIV[50]. The efficacy of daclatasvir plus sofosbuvir in cirrhotic patients infected with HCV genotype 1 to 6 is being studied in Ally-1 trial[51].

Common side effects include headache, fatigue, nausea and diarrhea. Certain drugs are contraindicated to be used with daclatasvir. These include anticonvulsants phenytoin, carbamazepine, antimycobacterial agent rifampin and herbal supplement St. John's Wort.

Drug-drug interactions: Medications which can cause significant drug interactions with daclatasvir include statins, digoxin, dabigatran, ketoconazole, itraconazole, clarithromycin, nafcillin, dexamethasone, buprenorphine, modafinil, bosentan, anti-HIV medications - protease inhibitors: atazanavir with ritonavir, indinavir, nelfinavir, saquinavir; Non-nucleoside reverse transcriptase inhibitors: Efavirenz Etravirine Nevirapine; and cobicistat-containing antiretroviral: atazanavir/cobicistat, elvitegravir/cobicistat/emtricitabine/tenofovir disoproxil fumarate[52].

Elbasvir is a potent NS5A inhibitor. It is used in combination with grazoprevir which is a NS3/4A protease inhibitor. The combination is effective against HCV genotypes 1 and 4. Elbasvir can be less effective when there are many resistance- associated variants or substitutions (RAVs or RASs) of NS5A[53]. Elbasvir-Grazoprevir can be taken in empty stomach or with food. In C-EDGE trial, treatment-naïve HCV infected patients had a good response (SVR12) rate to 12 wk therapy of Elbasvir-Grazoprevir: 92% with genotype 1a, 99% with genotype 1b, 100% with genotype 4 and 80% with genotype 6; 97% in cirrhotics and 94% in non-cirrhotics[54]. In C-EDGE TE trial, patients with prior exposure to pegylated interferon and ribavirin, Elbasvir/Grazoprevir with or without ribavirin was highly effective in inducing an SVR12 in patients with HCV genotype 1, 4 and 6 including patients with cirrhosis[55].

Velpatasvir is a pangenotypic second generation NS5A inhibitor and is much more potent with a higher barrier to resistance than the first generation NS5A inhibitors (ledispavir and Daclatasvir). It acts as a defective substrate for NS5A and prevents HCV replication. It is used as a fixed dose co-formulation with sofosbuvir (Epclusa). In a double blind placebo-controlled study, sofosbuvir-velpatasvir combination was given daily for 12 wk to untreated and previously treated patients with HCV infection genotypes 1-6 including those with compensated cirrhosis. The SVR was 99%[56]. Another study showed that when sofosbuvir-velpatasvir combination plus ribavirin were given daily for 12 wk to decompensated cirrhotics (Child - Pugh - Turcotte class B) due to HCV infection genotype 1-6, the SVR was 94%[57].

Common side effects of Epclusa include headache, fatigue, insomnia, nausea and diarrhea. If the patient is HBV/HCV coinfected and not receiving ant-HBV therapy, administration of Epclusa may cause reactivation of HBV and fulminant hepatitis during or after treatment with Epclusa.

Drug-drug interactions: Similar to Harvoni, co-administration of Amiodarone with Epclusa may lead serious symptomatic bradycardia. Dose alteration or regimen change may be recommended for certain medications. These include acid suppressant agents proton pump inhibitors, H2 receptor blockers, antacids (aluminum and magnesium hydroxide), HMG Co-A reductase inhibitors atorvastatin, rosuvastatin, anti-arrhythmic drug digoxin, anticonvulsants phenytoin, phenobarbital, carbamazepine, oxcarbazepine, anti-mycobacterials rifampin, rifabutin, rifapentine, anti-HIV medications efavirenz, etravirine, nevirapine, tipranavir/ritonavir, Regimens containing tenofovir DF, anti-cancer medication topotecan, and herbal supplement St. John's wort[58].

Pibrentasvir is a 2^{ed} generation NS5A inhibitor coformulated with glecaprevir (Mavyret). As mentioned before glecaprevir/pibrentasvir combination is pangenotypic and can be used in severe renal failure, including hemodialysis patients.

NS5B inhibitors

They act on the catalytic site of NS5B polymerase. They cause HCV RNA chain termination after being incorporated into the RNA chain. Nucleotide NS5B inhibitors are already activated. They act on the active site of NS5B polymerase. Non-Nucleoside NS5B inhibitors need to be activated by cellular kinase 3 times to become the triphosphate which is the active form. They act on different allosteric sites (thumb, finger and palm domains) to downregulate NS5B polymerase[59]. Nucleotide NS5B inhibitors are moderately potent DAA with pangenotypic coverage and have high barrier to resistance. Non-nucleoside NS5B inhibitors are moderately potent DAA with limited genotypic coverage and low barrier to resistance.

Sofosbuvir is a nucleotide NS5B polymerase inhibitor[60]. It is coformulated with Ledipasvir for the treatment of hepatitis C genotype 1, 4, 5 and 6 infection. A meta-analysis showed no additional benefit when ribavirin was added to sofosbuvir/ledipasvir for the treatment genotype 1 infection[61]. As mentioned before, sofosbuvir/daclatasvir combination is effective for the treatment of hepatitis C genotypes 1 to 4[62]. Patients with HCV genotypes 2 and 3 infections were treated with sofosbuvir and ribavirin for 12 wk and 24 wk respectively: SVR 12 was 93% for genotype 2 infection, and 85% for genotype

Table 1 Direct acting anti-viral agents with posology

No.	Trade name	Generic name with doses
1	Sovaldi	Sofosbuvir 400 mg
2	Olysio	Simeprevir 150 mg
3	Daklinza	Daclatasvir 30 mg/60 mg/90 mg
4	Harvoni	Ledipasvir 90 mg/Sofosbuvir 400 mg
5	Viekira Pak	1 d pack contains Paritaprevir 75 mg/Ombitasvir 12.5 mg/Ritonavir 50 mg tablet × 2 and Dasabuvir 250 mg tablet × 2
6	Viekira XR	Extended release tablet contains Dasabuvir 200 mg/ombitasvir 8.33 mg/Paritaprevir 50 mg/Ritonavir 33.33 mg
7	Technivie	Ombitasvir 12.5 mg/Paritaprevir 75 mg/Ritonavir 50 mg
8	Epclusa	Sofosbuvir 400 mg/ Velpatasvir 100 mg
9	Zepatier	Elbasvir 50 mg/ Grazoprevir 100 mg
10	Mavyret	Glecaprevir 100 mg/ Pibrentasvir 40 mg
11	Vosevi	Sofosbuvir 400 mg/Velpatasvir 100 mg/Voxilaprevir 100 mg

3 infection. In non-cirrhoitc HCV genotype 3 infection, the SVR was 91% whereas in cirrhotic genotype 3 infection, the SVR was 68%[63]. Sofosbuvir has been coformulated with other NS5A inhibitor or NS3/4A protease inhibitor to make the combination more effective against HCV and also pangenotypic.

Common side effects of Sofosbuvir include headache, nausea, insomnia and fatigue. Reactivation of HBV with fulminant hepatitis can occur if the patient is HBV/HCV co-infected and not receiving anti-HBV therapy.

Drug-drug interactions: Dose alteration or change in regimen is recommended for certain medications. These include anticonvulsants phenytoin, phenobarbital, carbamazepine, oxcarbazepine, anti-HIV medications tipranavir/ritonavir, antimycobacterial agents rifampin, rifabutin, rifapentine, and herbal supplement St. John's Wort[64].

Dasabuvir is a non-nucleoside NS5B polymerase inhibitor. It is used in combination with Paritaprevir/ritonavir-ombitasvir ((in Viekira Pak and Viekira XR) with or without ribavirin for the treatment of hepatitis C genotype 1 infection with or without compensated cirrhosis (Child-Pugh A). It binds to the palm domain of NS5B, and thus prevents elongation of HCV RNA. The binding site is poorly conserved across other HCV genotypes. As a result, Dasabuvir is only effective against HCV genotype 1 infection. Every year new DAAs are being added in our clinical practice. Currently, the various DAAs available in formulation are as follows (Table 1).

Individualized treatment options

In 2018, we treat hepatitis C with combination of at least two DAAs or one DAA with ribavirin. Several factors are to be considered before planning treatment for hepatitis C. These include HCV genotype, HCV viral load, treatment-naïve or treatment-experienced (PEG/RIBA, NS3/4A protease inhibitor, NS5A inhibitor, NS5B inhibitor) patient, cirrhotic or non-cirrhotic patient, absence or presence of baseline NS5A resistance-associated substitutions (RASs), patient's current medications considering any drug-drug interactions (DDI), patient's renal function, co-infection with HIV, post-liver transplant infection, and of course, patient's insurance and financial status. Guidelines for the treatment of hepatitis C was updated by the European Association for the Study of Liver (EASL) in 2016 and the American Association for the Study of Liver Diseases (AASLD) and the Infectious Diseases Society of America (IDSA) in 2017[65,66]. Individualized treatments as per the AASLD guidelines[66] are listed in Tables 2 and 3.

Genotype 1 infection-treatment-experienced

Glecaprevir/Pibrentasvir (Mavyret): Duration of treatment depends on previous regimen and presence or absence of compensated cirrhosis. Elbasvir/Grazoprevir (Zepatier): duration depends on viral load irrespective of no cirrhosis or compensated cirrhosis as per EASL guideline. Ledipasvir/Sofosbuvir (Harvoni): Applicable for both non-cirrhotics and compensated cirrhotics. Sofosbuvir/Velpatasvir (Epclusa): treatment is same for both non-cirrhotics and compensated cirrhotics. Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi): applicable for both non-cirrhotics and compensated cirrhotics. Paritaprevir/ritonavir/ombitasvir and dasabuvir (in Viekira Pak and Viekira XR) with weight-based ribavirin (Tables 3-6).

HCV/HIV Co-infection

Twenty percent to 30% of the 34 million HIV infected individuals in the world are co-infected with HCV[67]. HCV/HIV co-infection carries increased morbidity and mortality including advanced hepatic fibrosis and cirrhosis[68]. HCV/HIV co-infected patients have higher HCV viral load, more chance of developing chronic HCV infection and rapid development of advanced liver disease than HCV mono-infected patients. The main challenge of treating these group of patients is drug-drug interactions, *i.e.*, interactions between anti-HCV DAAs and anti-retroviral medications[69]. But the SVR and side effects are similar to those of HCV mono-infected patients[70]. Ideally, HCV/HIV co-infection should be managed by or in collaboration with a HIV practitioner. No change in antiretroviral therapy should be done without consultation of the HIV practitioner. Certain guidelines given by AASLD and IDSA are as follows[66]: (1) Ledipasvir/Sofosbuvir (Harvoni): This combination has certain interactions with anti-retrovirals mentioned before. As it increases serum level of tenofovir disoproxil fumerate which may cause renal toxicity, renal function should be monitored and tenofovir disoproxil fumerare should be used with

Table 2 Genotype 1a and 1b infection - treatment-naïve (with compensated cirrhosis) and non-cirrhotic

No.	First line therapy	Alternative regimen
Genotype 1a infection - treatment-naïve and non-cirrhotic		
1	Glecaprevir/Pibrentasvir (Mavyret) - 8 wk	Paritaprevir/ritonavir/ombitasvir and dasabuvir (in Viekira Pak and Viekira XR) with weight-based ribavirin - 12 wk
2	Elbasvir/Grazoprevir (Zepatier) for patients without baseline NS5A RAVs for Elbasvir - 12 wk	Simeprevir (Olysio) plus Sofosbuvir (Sovaldi) - 12 wk
3	Ledipasvir/Sofosbuvir (Harvoni) - 12 wk < Ledipasvir/Sofosbuvir (Harvoni) - 8 wk in non-black, HIV-uninfected individuals with serum HCV RNA < 6 million units/mL	Daclatasvir(Daklinza) plus Sofosbuvir (Sovaldi) - 12 wk
4	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	Elbasvir/Grazoprevir (Zepatier) with weight-based ribavirin for patients with baseline NS5A RAVs for Elbasvir - 16 wk
Genotype 1a infection - treatment-naïve with compensated cirrhosis		
1	Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	Elbasvir/Grazoprevir (Zepatier) with weight-based ribavirin for patients with baseline NS5A RASs for Elbasvir - 16 wk
2	Elbasvir/Grazoprevir (Zepatier) for patients without baseline NS5A RAVs for Elbasvir - 12 wk	
3	Ledipasvir/Sofosbuvir (Harvoni) - 12 wk	
4	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	
Genotype 1b infection - treatment-naïve and non-cirrhotic		
1	Glecaprevir/Pibrentasvir (Mavyret) - 8 wk	Paritaprevir/ritonavir/ombitasvir and dasabuvir (in Viekira Pak and Viekira XR) - 12 wk
2	Elbasvir/Grazoprevir (Zepatier) - 12 wk	Simeprevir (Olysio) plus Sofosbuvir (Sovaldi) - 12 wk
3	Ledipasvir/Sofosbuvir (Harvoni) - 12 wk < Ledipasvir/Sofosbuvir (Harvoni - 8 wk in non-black, HIV-uninfected individuals with serum HCV RNA < 6 million units/mL	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) - 12 wk
4	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	
Genotype 1b infection - treatment-naïve with compensated cirrhosis		
1	Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	Paritaprevir/ritonavir/ombitasvir and dasabuvir (in Viekira Pak and Viekira XR) - 12 wk
2	Elbasvir/Grazoprevir (Zepatier - 12 wk	
3	Ledipasvir/Sofosbuvir (Harvoni) - 12 wk	
4	Sofosbuvir/Velpatasvir (Epclusa - 12 wk	

RAVs: Resistance- associated variants; HIV: Human immunodeficiency virus; HCV: Hepatitis C virus.

Table 3 Genotype 1 infection-treatment-experienced

Glecaprevir/Pibrentasvir (Mavyret): Duration of treatment depends on previous regimen and presence or absence of compensated cirrhosis.		
Previous regimen	No cirrhosis	Compensated cirrhosis
Pegylated IFN, ribavirin and/or Sofosbuvir but no prior exposure to NS3/4A PI or NS5A inhibitor	8 wk	12 wk
NS3/4A PI but no prior exposure to NS5A inhibitor	12 wk	12 wk
NS5A inhibitor but no prior exposure to NS3/4A PI	16 wk	16 wk
Elbasvir/Grazoprevir (Zepatier): Duration depends on viral load irrespective of no cirrhosis or compensated cirrhosis as per EASL guideline.		
Previous regimen	HCV RNA ⩽ 800000 IU/mL	HCV RNA > 800000 IU/mL
Pegylated IFN and ribavirin	12 wk without ribavirin	16 wk with ribavirin
Ledipasvir/Sofosbuvir (Harvoni): Applicable for both non-cirrhotics andcompensated cirrhotics.		
Previous regimen	With ribavirin	Without ribavirin
PEG IFN and ribavirin	12 wk	24 wk
Sofosbuvir/Velpatasvir (Epclusa): Treatment is same for both non-cirrhotics and compensated cirrhotics.		
Previous regimen	No cirrhosis	Compensated cirrhosis
PEG IFN and ribavirin	12 wk without ribavirin	12 wk without ribavirin
Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi): Applicable for both non-cirrhotics and compensated cirrhotics.		
Previous regimen	With ribavirin	Without ribavirin
PEG IFN and ribavirin	12 wk	24 wk
Paritaprevir/ritonavir/ombitasvir and dasabuvir (in Viekira Pak and Viekira XR) with weight-based ribavirin.		
Previous regimen	No cirrhosis	Compensated cirrhosis
PEG IFN and ribavirin	12 wk	24 wk

IFN: Interferon; PI; Protease inhibitors; HCV: Hepatitis C virus.

GFR > 60 mL/min. Tenofovir alafenamide could be an alternative option; (2) Paritaprevir/ritonavir/ombitasvir and dasabuvir (in Viekira Pak and Viekira XR): The anti-HIV medications which do not have substantial interaction with Vikira Pak and Viekira XR include atazanavir, dolutegravir, emtricitabine, enfuvirtide,

Table 4 Genotype 2-4 infection - treatment-naïve (with compensated cirrhosis) and non-cirrhotic

No.	First line therapy	Alternative regimen
Genotype 2 infection - treatment-naïve and non-cirrhotic		
1	Glecaprevir/Pibrentasvir (Mavyret) - 8 wk	Daclatasvir(Daklinza) plus Sofosbuvir (Sovaldi) - 12 wk
2	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	
Genotype 2 infection - treatment-naïve with compensated cirrhosis		
1	Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	Daclatasvir(Daklinza) plus Sofosbuvir (Sovaldi) - 16 to 24 wk
2	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	
Genotype 3 infection - treatment-naïve and non- cirrhotic		
1	Glecaprevir/Pibrentasvir (Mavyret) - 8 wk	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) - 12 wk
2	Sofosbuvir/Velpatasvir (Epclusa - 12 wk	
Genotype 3 infection - treatment-naïve with compensated cirrhosis		
1	Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	Vosevi - Sofosbuvir 400 mg/ Velpatasvir 100 mg/ Voxilaprevir 100 mg when Y93 is present - 12 wk
2	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) with or without weight-based ribavirin - 24 wk
Genotype 4 infection - treatment-naïve and non-cirrhotic		
1	Glecaprevir/Pibrentasvir (Mavyret) - 8 wk	Ombitasvir 25 mg/Paritaprevir 150 mg/ Ritonavir 100 mg (Technivie) with weight-based ribavirin - 12 wk
2	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	
3	Elbasvir/Grazoprevir (Zepatier) - 12 wk	
4	Ledipasvir/Sofosbuvir (Harvoni) - 12 wk	
Genotype 4 infection - treatment-naïve with compensated cirrhosis		
1	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	Ombitasvir 25 mg/Paritaprevir 150 mg/ Ritonavir 100 mg (Technivie) with weight-based ribavirin - 12 wk
2	Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	
3	Elbasvir/Grazoprevir (Zepatier) - 12 wk	
4	Ledipasvir/Sofosbuvir (Harvoni) - 12 wk	

Table 5 Genotype 5 or 6 infection - treatment-naïve with and without compensated cirrhosis

No.	DAA	No cirrhosis	Compensated cirrhosis
1	Glecaprevir/Pibrentasvir (Mavyret)	8 wk	12 wk
2	Sofosbuvir/Velpatasvir (Epclusa)	12 wk	12 wk
3	Ledipasvir/Sofosbuvir (Harvoni)	12 wk	12 wk

DAA: Direct acting anti-viral agent.

lamivudine, raltegravir, and tenofovir; (3) Sofosbuvir/Velpatasvir (Epclusa): Can have drug-drug interactions with anti-retrovirals mentioned before. As velpatasvir can increase the serum level of tenofovir disoproxil fumerate, renal function should be checked and tenofovir disoproxil fumerate should not be used with a GFR of less than 60 mL/min. Tenofovir alafenamide could be used instead; (4) Elbasvir/Grazoprevir (Zepatier): Certain antiretrovirals do not have significant drug-drug interactions with elbasvir/grazoprevir combinations. These include tenofovir, lamivudine, emtricitabine, abacavir, dolutegravir, raltegravir, rilpivirine, and enfuvirtide; (5) Glecaprevir/Pibrentasvir (Mavyret): The anti-retrovirals which do not have significant drug-drug interactions with Glecaprevir/Pibrentasvir include lamivudine, tenofovir, emtricitabine, abacavir, dolutegravir, raltegravir, rilpivirine, and enfuvirtide; (6) Sofosbuvir/Velpatasvir/Voxilaprevir (Vosevi): Has some drug-drug interactions with certain anti-retrovirals mentioned before. But some of the anti-retrovirals do not have significant interactions with this combination (Vosevi). These include lamivudine, emtricitabine, dolutegravir, enfuvirtide, raltegravir and rilpivirine; (7) Simeprevir (used in combination with another DAA): Does not have significant drug-drug interactions with certain anti-retrovirals which include lamivudine, tenofovir, emtricitabine, abacavir, dolutegravir, raltegravir, rilpivirine, enfuvirtide and maraviroc; and (8) Daclatasvir (used in combination with another DAA): Has significant drug-drug interactions with certain anti-retrovirals mentioned before. Certain dose adjustment include: cobicistat/atazanavir (decrease dose to 30 mg/d), cobicistat/elvitegravir (decrease dose to 30 mg/d), ritonavir/atazanavir (decrease dose to 30 mg/d), and efavirenz or etravirine (increase dose to 90 mg/d).

HCV infection in post-liver transplant patients

Recurrence of hepatitis C infection following liver transplantation is universal in all patients with pre-transplantation viremia. The course varies from asymptomatic infection to fibrosing cholestatic hepatitis[71]. About one third of post-transplant HCV-infected patients develop allograft cirrhosis 5 to 7 years after transplantation[72]. As per AASLD and IDSA guidelines published on September 21, 2017 the various treatment options are list in Table 7[66].

There are drug-drug interactions between DAA and

Table 6 Genotype 2-6 infection - treatment-experienced

	First line therapy	Alternative regimen
Genotype 2 infection - treatment-experienced		
Pegylated IFN/ribavirin-experienced without cirrhosis	Glecaprevir/Pibrentasvir (Mavyret) - 8 wk or Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) - 12 wk
Pegylated IFN/ribavirin-experienced with compensated cirrhosis	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk or Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) - 16 to 24 wk
Sofosbuvir plus ribavirin-experienced with or without compensated cirrhosis	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk or Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	
Genotype 3 infection - treatment-experienced		
Pegylated IFN/ ribavirin-experienced without cirrhosis	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) - 12 wk or Glecaprevir/Pibrentasvir (Mavyret) - 16 wk or Sofosbuvir/Velpatasvir/Voxilaprevir - 12 wk
Pegylated IFN/ ribavirin-experienced with compensated cirrhosis	Elbasvir/Grazoprevir (Zepatier) - 12 wk or Sofosbuvir/Velpatasvir/Voxilaprevir (Vosevi) -12 wk	Sofosbuvir/Velpatasvir (Epclusa) plus weight-based ribavirin - 12 wk or Glecaprevir/Pibrentasvir (Mavyret) - 16 wk
DAA-experienced including NS5A inhibitors with or without compensated cirrhosis	Sofosbuvir/Velpatasvir/Voxilaprevir (Vosevi) - 12 wk or in case of NS5A inhibitor failure and cirrhosis - Vosevi plus weight-based Ribavirin - 12 wk	
Genotype 4 infection - treatment-experienced		
Pegylated IFN/ ribavirin-experienced without cirrhosis	Sofosbuvir/Velpatasvir (Epclusa) -12 wk or Glecaprevir/Pibrentasvir (Mavyret) - 8 wk or Elbasvir/Grazoprevir (Zepatier) in virologic relapse - 12 wk or Ledipasvir/Sofosbuvir (Harvoni) - 12 wk	Ombitasvir 25 mg/Paritaprevir 150 mg/Ritonavir 100 mg plus weight based Ribavirin - 12 wk or Elbasvir/Grazoprevir (Zepatier) with weight-based Ribavirin (in case of prior on-treatment virologic failure) - 16 wk
Pegylated IFN/ ribavirin-experienced with compensated cirrhosis	Sofosbuvir/Velpatasvir (Epclusa) - 12 wk or Elbasvir/Grazoprevir (Zepatier) in virologic relapse - 12 wk or Glecaprevir/Pibrentasvir (Mavyret) - 12 wk	Ombitasvir 25 mg/Paritaprevir 150 mg/ Ritonavir 100 mg plus weight based Ribavirin - 12 wk or Elbasvir/Grazoprevir (Zepatier) with weight-based Ribavirin (in case of prior on-treatment virologic failure - 16 wk or Ledipasvir/Sofosbuvir (Harvoni) plus weight-based Ribavirin) - 12 wk
DAA-experienced including NS5A inhibitors with or without compensated cirrhosis	Sofosbuvir/Velpatasvir/Voxilaprevir (Vosevi) - 12 wk	
Genotype 5 or 6 infection - treatment-experienced (recommended regimen)		
Pegylated IFN/ ribavirin-experienced with or without compensated cirrhosis	Glecaprevir/Pibrentasvir (Mavyret) - 8 wk for patients without cirrhosis and 12 wk for patients with compensated cirrhosis or Ledipasvir/Sofosbuvir (Harvoni) plus weight-based Ribavirin - 12 wk or Sofosbuvir/ Velpatasvir (Epclusa) - 12 wk	
DAA-experienced including NS5A inhibitors with or without compensated cirrhosis	Sofosbuvir/Velpatasvir/ Voxilaprevir (Vosevi) - 12 wk	

IFN: Interferon; DAA: Direct acting anti-viral agent.

calcineurin inhibitors: cyclosporine A and Tacrolimus. When Sofosbuvir is used with cyclosporine A, serum level of sofosbuvir is increased by 4.5 fold but GS -331007 metabolite remains unchanged. So no dose adjustment is required[73]. Sofosbuvir has no drug interaction with Tacrolimus[74]. When Paritaprevir/ritonavir/ombitasvir and dasabuvir- (PrOD) in Viekira Pak and Viekira XR are used with cyclosporine A, there is 5.8 fold increased serum level of cyclosporine A suggesting use of 1/5th of dose of cyclosporine A and requirement of monitoring of serum level of cyclosporine A. When PrOD are used with tacrolimus, there is 57 fold increase in tacrolimus level in the blood, suggesting use of 0.5 mg of tacrolimus once a week, and monitoring of tacrolimus level in the blood[75]. When elbasvir/grazoprevir combination is used with cyclosporine A, there is 15 fold increases in grazoprevir level in the blood. As a result, this combination is not recommended to be used. When elbasvir/grazoprevir are used with tacrolimus, there is 43% increase in tacrolimus level, and although no prior dose adjustment is required, frequent monitoring of tacrolimus level, tacrolimus-associated side effects and renal function should be done[76]. When glecaprevir/pibrentasvir is used with higher dose (400 mg) of cyclosporine A, there is 5 fold increase in glecaprevir level in the blood. This combination is not recommended in patients requiring more than 100 mg of cyclosporine A[77]. When glecaprevir/pibrentasvir combination is used with tacrolimus, there is 1.45 fold increase in tacrolimus level suggesting no prior dose adjustment but tacrolimus level should be monitored. When sofosbuvir/velpatasvir/voxilaprevir combination is used with cyclosporine A, there is 9.4 fold increase in voxilaprevir. This combination is not recommended[78].

Table 7 Recommended and alternative therapy

Genotype	Treatment-naïve and - experienced patients with HCV infection in the allograft without cirrhosis	Treatment-naïve and - experienced patients with HCV infection in the allograft with compensated cirrhosis	Treatment-naïve and - experienced patients with HCV infection in the allograft with decompensated cirrhosis
Recommended therapy			
1, 4, 5 or 6	Glecaprevir/Pibrentasvir (Mavyret) - 12 wk or Ledipasvir/Sofosbuvir (Harvoni) for 12 wk	Ledipasvir/Sofosbuvir (Harvoni) with weight-based ribavirin - 12 wk	Ledipasvir/Sofosbuvir (Harvoni) with initial low dose of ribavirin (600 mg), increase the dose as tolerated - 12 wk
2 or 3	Glecaprevir/Pibrentasvir (Mavyret) - 12 wk or Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) with initial low dose of ribavirin (600 mg), increase the dose as tolerated for 12 wk	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) with initial low dose of ribavirin (600 mg), increase the dose as tolerated - 12 wk	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) with initial low dose of ribavirin (600 mg), increase the dose as tolerated for 12 wk or Sofosbuvir/Velpatasvir (Epclusa) with weight-based ribavirin - 12 wk
Alternative therapy			
1, 4, 5 or 6	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) with initial low dose of ribavirin (600 mg), increase the dose as tolerated for 12 wk or HCV genotype 1 or 4 infection only: Simeprevir (Olysio) plus Sofosbuvir (Sovaldi) with or without weight-based ribavirin	Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) with initial low dose of ribavirin (600 mg), increase the dose as tolerated for 12 wk or HCV genotype 1 or 4 infection only: Simeprevir (Olysio) plus Sofosbuvir (Sovaldi) with or without weight-based ribavirin	
2 or 3		Glecaprevir/Pibrentasvir (Mavyret) for 12 wk or Sofosbuvir/Velpatasvir (Epclusa) with weight-based ribavirin for 12 wk	

HCV: Hepatitis C virus.

DAA and hepatocellular carcinoma

Concern emerged after the study done by Reig *et al*[79] in 2016 showing higher rate of hepatocellular carcinoma (HCC) recurrence after DAA treatment in patients with prior HCC. Subsequently few studies were done to evaluate this finding. Large VA cohort study did not find any increased risk of developing HCC after DAA therapy[80]. Fortunately, the concern has lost its importance. But all cirrhotic patients should be under surveillance for HCC after DAA therapy.

DAA failure

DAA failure or relapse has occurred in less than 10% of HCV infected patients in clinical trials. In a multicenter real life study, the overall failure rate was found to be 3.6%[81]. As mentioned before, HCV exists as a quasispecies with production of various genetically distinct variants due to replication errors. Due to this high mutation rate of HCV genome, changes in the critical coding regions may occur making the virus less susceptible to anti-HCV therapy. Viral variants containing polymorphisms or substitutions resistant to DAA are also called baseline resistant-associated substitutions (RASs) and they exist in a small percentage of patients with chronic HCV infection prior to anti-HCV therapy. Sometimes RASs develop after initiation of DAA therapy when they are called treatment-emergent or treatment-selected RASs. Subtherapeutic DAA level may predispose to the development of RASs. NS5A and NS3 RASs are frequently seen in cases of failure of DAA containing NS5A or NS3 inhibitor[82]. On the other hand, there is rare development of NS5B RASs even after exposure to a failed DAA therapy containing. NS5B inhibitor, possibly due to binding of NS5B inhibitor to the highly conserved catalytic site of the viral genome making generation of RASs extremely difficult[83].

AASLD and IDSA recommend testing for NS5A RASs if DAAs containing NS5A inhibitors fail[82]. Testing for baseline NS5A RASs is recommended in patients infected with HCV genotype 1a prior to initiation of elbasvir and grazoprevir therapy[84]. Baseline NS5A RASs testing should also be considered in patients with cirrhosis of liver due to HCV genotype 1a infection prior to sofosbuvir plus daclatasvir therapy[85]. NS5A RASs testing should be considered in certain situations: (1) treatment-naïve HCV genotype 3 infection with compensated cirrhosis of liver prior to treatment with either Sofosbuvir/Velpatasvir (Epclusa) for 12 wk or Daclatasvir (Daklinza) plus Sofosbuvir (Sovaldi) for 24 wk; (2) treatment-experienced HCV genotype 3 infection without cirrhosis prior to treatment with Daclatasvir(Daklinza) plus Sofosbuvir (Sovaldi) for 12 wk; and (3) treatment-experienced HCV genotype 3 infection with or without cirrhosis prior to treatment with Sofosbuvir/Velpatasvir (Epclusa) for 12 wk. If Y93H is found, weight-based ribavirin should be added[82]. DAA failure has been associated with certain predisposing factors like presence of RASs, sofosbuvir/ribavirin treatment, HCV genotype 3, and cirrhosis of liver[81,86].

CONCLUSION

DAAs have changed the treatment plan and outcome of chronic hepatitis C infection in this pegylated interferion free era. There are various DAAs available in the market and these include second generation NS3/4A protease inhibitors, NS5A and NS5B inhibitors. AASLD, IDSA and EASL recommend combination of DAAs with or without ribavirin. As the success rate is high, side effects are low, drug-drug interactions are less, and duration of

therapy is relatively short, more and more patients are getting treatment for the cure of hepatitis C infection. Some DAAs require preliminary testing to find out the effectiveness of that particular DAA, for example Q80K polymorphism in case of Simeprevir, and NS5A resistance testing in case of elabasvir. HBV co-infection testing should be done prior to initiation of any DAA therapy to prevent fulminant hepatitis and liver failure. Drug-drug interaction, and specific conditions like HCV/HIV co-infection and HCV infection in post-liver transplant patients should considered before initiating any treatment plan. Although DAA can eradicate HCV infection, it does not decrease the chance of developing HCC in cirrhotic patients. So they should be under regular HCC surveillance. The next challenge will be the development of HCV vaccine to reduce the incidence of HCV infection in the world.

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MINIREVIEWS

Nutritional support in chronic liver disease and cirrhotics

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Abstract

The liver is a major organ and an essential component in maintaining an appropriate nutritional status in healthy individuals through metabolism of protein, carbohydrates, and fat. In individuals with chronic liver disease (CLD), along with a number of other essential functions that the liver serves, its role in nutrition maintenance is severely impaired. Common causes of CLD include hepatitis C, alcoholic liver disease, and non-alcoholic liver disease. Amongst this population, the most common manifestation of impaired nutritional maintenance is protein-calorie malnutrition. Aside from inherent abnormalities in metabolism, such as malabsorption and maldigestion, CLD can be associated with anorexia as well as increased metabolic requirements, all of which contribute to a state of malnutrition. Given the systemic implications and impact on prognosis of malnutrition, proper nutritional assessment is essential and can be achieved through a thorough history and physical, as well as biochemical investigations and anthropometry as needed. Following an appropriate assessment of a patient's nutritional status, an approach to management can be decided upon and is based on the extent of malnutrition which directly reflects the severity of disease. Management options can be grossly separated into enteral and parenteral nutrition. The former is usually sufficient in the form of oral supplements in less severe cases of malnutrition, but as the CLD worsens, parenteral nutrition becomes necessary. With appropriate assessment and early intervention, many of the complications of CLD can be avoided, and ultimately better outcomes can be achieved.

Key words: Chronic liver disease; Cirrhosis; Energy requirements; Nutrition; Malnutrition; Anthropometry; Liver

Core tip: This paper highlights the most recent evidence in the clinical approach to dealing with nutrition in patients with chronic liver disease and cirrhotics.

We will review the pathophysiology of liver disease, etiology, and management of nutrition.

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INTRODUCTION

The liver is a major organ involved in maintaining appropriate nutritional status. Its role includes metabolism of protein, carbohydrates and fat, which are essential sources of nutrition for the body. Accordingly, patients who suffer from chronic liver disease (CLD) often experience debilitating and severe malnutrition that both reflects the severity of the disease, and thereby disease prognosis, but is also an independent predictor of mortality[1,2]. Malnutrition in CLD is a function of multiple factors including, but not limited to, impaired absorption and/or digestion, increased metabolic requirements, as well as anorexia and overall decreased oral intake. The permanent functional deficits in cirrhosis result in nutritional deficiencies with systemic impacts, and coupled with the several mechanisms by which these nutritional deficiencies are realized, approaches to management and support are increasingly complicated[3]. Given the prognostic implications of nutritional status in patients with CLD, further insight into the assessment and therapy of these patients is essential to appropriate management. This literature review aims to summarize a general approach to nutritional support in patients with CLD, including exploring etiology, clinical assessment and key investigations, as well as management.

ETIOLOGY AND PREVALENCE

CLD involves a process of continuous inflammation and regeneration that eventually results in permanent fibrosis and cirrhosis. The most common cause of this condition is hepatitis C virus infection[4]. Other common causes include alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD) and hepatitis B virus infection. Although it is difficult to assess, experts estimate that over 844 million people worldwide have CLD, and this associated with a mortality rate of approximately 2 million deaths per year. Of those affected by CLD, approximately 20% with compensated cirrhosis and 65%-95% with decompensated cirrhosis have protein-calorie malnutrition (PCM)[5]. PCM is a condition involving cachexia due to nutritional deficiencies in calories and protein. This decline in essential macronutrients can lead to extensive body wasting as well as other related sequelae. Micronutrient deficiencies are also present in patients with CLD, albeit with a more subtle presentation than PCM. In the context of cirrhosis, patients with associated malnutrition have higher rates of hepatic encephalopathy, infection, ascites and variceal bleeding[6]. Multiple studies have documented increased complications and overall length of hospital stay in malnourished patients[7]. It is no question that malnutrition complicates patient management significantly, leading to increased rates of morbidity and mortality in CLD patients.

MALNUTRITION

Dietary intake

The underlying cause of malnutrition in patients with CLD and cirrhosis is multifactorial, and is typically related to a loss of appetite, malabsorption and increased metabolic requirements. Low dietary intake is commonly found among cirrhotic patients. Davidson *et al*[8] describes the hepatic role in appetite regulation through clearance of chemical mediators like cholecystokinin, which contributes to feelings of satiety. The liver also contributes to splanchnic production of cytokines that reduce the hypothalamic-mediated drive for appetite. Furthermore, considering that ascites is a common complication of CLD, the mechanical compression may possibly lead to a premature feeling of fullness. In addition to a low overall dietary intake, there is an alteration in the pattern of fuel consumption in the body. There is a higher rate of fat oxidation in the fasting state of CLD patients. During an overnight fast, a research study showed that 58% of energy came from fat oxidation in cirrhotics, whereas healthy controls derived 55% of their energy from carbohydrates[9]. This may reflect the low hepatic glycogen stores found in patients with cirrhosis. Additionally, in a study by Nielsen *et al*[10], they demonstrated that body size in cirrhotic patients increased during refeeding therapy. This indicates that the volume of dietary intake was in fact a contributing factor to the decline in body mass and overall malnutrition in CLD patients.

Malabsorption

Malabsorption is also an important consideration in patients who present with malnutrition in the setting of CLD. Liver disease that leads to a decline in the bile-salt pool can contribute to fat malabsorption[11]. This is commonly found in patients who suffer from comorbid biliary and pancreatic disease, as there is a decrease in fat and fat-soluble vitamin absorption. The extent of malabsorption in CLD patients without related cholestatic disease is currently a topic of controversy. Some studies claim that regardless of etiology, CLD patients experience bacterial overgrowth

from small bowel hypomotility and portal hypertension that can contribute to malabsorption[12]. While others have found that neither fat nor protein are noticeably malabsorbed unless there is co-existing biliary or pancreatic disease.

Energy requirements

Whether or not increased metabolic requirements contribute to malnutrition in CLD is considered uncertain, with varying levels of evidence to support this claim. Müller *et al*[13] found that the average resting energy expenditure (REE) in a study of 473 cirrhotic patients was found to be normal. However, 34% of patients had an increased REE of over 120% of the expected value[13]. Various causes for hypermetabolism in CLD have been proposed, including infection, ascites and portal hypertension. The link between energy expenditure and malnutrition in cirrhotics remains unclear and further research on this topic is required. In addition to metabolic changes, the overall protein requirement is also increased in patients with CLD. Druml *et al*[14] credits this to a decrease in the production of protein, and an increase in the rate of protein degradation. Low glycogen reserves in the liver trigger increased rates of gluconeogenesis from amino acids, which are derived from protein breakdown. These elevated protein requirements in cirrhotic patients can contribute to a state of malnourishment.

NUTRITIONAL ASSESSMENT (PHYSICAL EXAM AND SERUM MARKERS)

Considering that PCM and micronutrient deficiencies have major prognostic implications in patients with CLD, it is essential to effectively and regularly assess nutritional status. Clinicians should consider and analyze multiple factors in order to make a comprehensive nutritional assessment. This typically includes a medical history, extensive physical examination, laboratory data and more.

History

A thorough medical history can offer significant insight as a preliminary assessment of nutritional status. Discussing the patient's eating behaviors and dietary intake through 24-h recall can help the clinician identify possible sources of malnutrition. Recent weight loss is also important to review, as it can point towards the severity of nutritional deficit. This can be complicated in cases of decompensated cirrhosis, as water retention and ascites can lead to inappropriate increases in weight. It is also imperative to identify comorbidities, as they can indirectly impact nutritional status. For example, underlying nausea, vomiting, or anorexia can decrease dietary intake and contribute to malnourishment irrespective of the underlying CLD. Additionally, the severity of liver disease should be assessed through various clinical tools like the Child-Pugh score or Model for End-Stage Liver Disease (MELD) score[15]. The clinical suspicion and onset of malnourishment is directly related to the extent of liver disease in patients.

Physical examination

An appropriate physical examination is a critical component of an effective nutritional status assessment. Macronutrient and micronutrient deficiencies can have a variety of unique physical manifestations. Common examples include pallor in iron deficiency, dermatitis in vitamin A deficiency, bruising in vitamin K or C deficiency and many more[16]. Of particular importance is being able to recognize and assess sarcopenia, which is the generative loss of skeletal muscle mass. Sarcopenia is the most common complication of cirrhosis and so will often be the initial or only presentation of someone with malnutrition secondary to CLD[17]. One of most notable and effective measures of nutritional assessment in clinical practice is known as the subjective global assessment (SGA). The SGA is a standardized array of questions and physical exam findings that are collectively scored to provide a comprehensive rating of nutritional status[18]. In the medical history component, patients are asked a variety of questions about weight changes, dietary intake, associated symptoms, and functional capability. The physical examination includes an assessment of muscle wasting, peripheral edema, ascites, and fat loss. They are then graded with a letter score: Grade A - well nourished, Grade B - Moderately malnourished, Grade C - Severely malnourished. The SGA has been found to serve as a good prognostic indicator in post-liver transplant and chronic dialysis patients[19]. Unfortunately, the SGA is a subjective assessment and it has been found to underestimate the severity of malnutrition in cirrhotic patients compared to the handgrip strength (HG) tool[20]. However, despite being a subjective assessment, there is an 80% inter-rater reproducibility of SGA results[19]. A variation of the SGA includes Royal Free Hospital Global Assessment (RFHGA) tool. This assessment tool adds anthropometric measurements and incorporates gender differences into its final rating of nutritional status[3].

Serum markers

In addition to an appropriate history and physical, there are various laboratory markers that help evaluate the nutritional status of a patient. Currently, various plasma proteins, vitamin levels, and creatinine are considered useful for nutritional assessment. Albumin, pre-albumin, and occasionally transferrin are major plasma proteins that are included in biochemical investigations. Prealbumin, also known as

transthyretin, is a hepatic protein that has been found to correlate well with the body protein status. With a half-life of approximately 2 d, its serum concentration closely reflects recent dietary intake. Production is measurably decreased roughly 14 d following insufficient dietary protein intake, which is less than 60% of the required amount[21]. Devoto *et al*[22] found that prealbumin levels correlated well with the Detailed Nutritional Assessment (DNA) tool, which was used as a reference standard for detecting PCM. They concluded that prealbumin is a good screening tool for protein malnutrition. Furthermore, low prealbumin levels as a nutritional marker have been shown to correlate with higher rates of complications and mortality[21]. In an independent study of dialysis patients, prealbumin was found to be the best nutritional predictor of survival in comparison to serum albumin, cholesterol and creatinine[23]. A serum level less than 15 mg/dL denotes concern for malnutrition[21]. Limitations to the use of prealbumin include inappropriate fluctuations after an alcoholic binge, prednisone use, or active infection and inflammation[24].

In addition to prealbumin, serum albumin was historically considered a good nutritional marker for protein status. However, recent studies have placed significant doubt on this claim and reported no significant link between albumin levels and nutritional status[25]. In malnourished patients, albumin levels were effectively maintained despite the clinical presence of severe PCM. Furthermore, albumin has a half-life of 20 d, making it a particularly slow tool for use in tracking clinical improvement or decline[25]. The serum concentration is also heavily impacted by active inflammation, where hepatic protein synthesis is reduced in order to prioritize the production of acute phase reactants[26]. Several studies have therefore recommended against the use of albumin for nutritional assessment, claiming that it does not serve as a marker for PCM. Similar to albumin, transferrin is also major hepatic protein that was historically measured to assess nutritional status. Transferrin functions as a transport protein for iron. Its use as a marker for protein status is limited because of the large number of factors that influence serum levels[27].

Finally, another major serum marker that has historically been used as a measure of protein status includes creatinine. Researchers hypothesized that urinary creatinine can be used as a serum indicator because it is almost entirely derived from processes in muscle tissue. A creatinine-height index (CHI) was developed as a method of assessing protein status[28]. However, this tool requires 24-h urine measurements and this can serve as a limitation to clinical application. Nevertheless, studies have shown that CHI as a nutritional marker serves as a better prognostic indicator than total body protein and serum albumin.

In terms of micronutrient malnutrition, serum measurements of various vitamins and minerals can serve as a rough measure of total body stores. The specific micronutrients that warrant clinical attention can depend on the etiology of cirrhosis. For example, alcoholic liver cirrhosis is often associated with a thiamine deficiency and cholestatic liver disease can include deficiencies in fat-soluble vitamins.

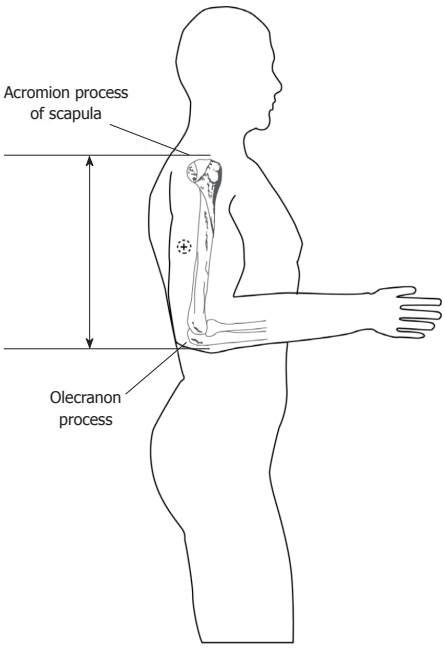

Figure 1 Position for mid-arm muscle circumference measurement[49].

Anthropometry

The mainstay of nutritional assessment almost always involves a history, physical and biochemical investigation. However, patients with a more complicated picture of malnutrition can undergo anthropometric ancillary tests to better characterize their nutritional status. One of the simplest methods of anthropometric assessment includes the body mass index (BMI). BMI evaluates patient height and weight to determine if they are underweight, normal, or overweight. However, the use of BMI in liver disease is very limited as patients often have volume overload complications, which can lead to an overestimation of nutritional status[29]. An anthropometric test that is less affected by fluid status is known as mid-arm muscle circumference (MAMC). MAMC can be used as a measurement of lean tissue levels and muscle bulk (Figure 1). Multiple studies have demonstrated that MAMC correlates well with various other markers in estimating muscle mass[18]. Specifically, it has been shown to correlate well with body cell mass (BCM) measurement, which is a proven marker for PCM in CLD patients. There is also prognostic value to MAMC measurements, as it is associated with mortality risk in CLD patients[1]. In addition to MAMC, triceps skinfold

Figure 2 Position and technique for triceps skinfold thickness measurement[49].

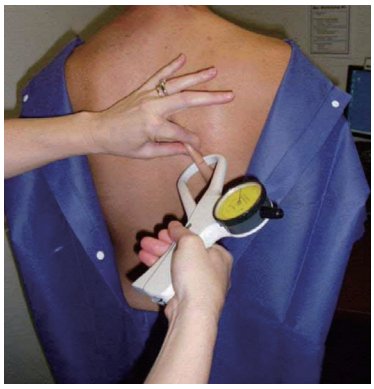

Figure 3 Position and technique for subscapular skinfold thickness measurement[49].

thickness (ST) is another anthropometric test that uses a caliper to measure fat reserve (Figure 2). ST was found to correlate with DEXA scan as a marker of body fat stores. Both ST and MAMC compare results on a percentile score, with values below the 5th percentile indicating severe malnutrition[20]. Anthropometric measurements are widely available, inexpensive and easy to complete. This makes them quite invaluable as bedside tools for nutritional status assessment. However, a major limitation to the use of anthropometric measurements as nutritional markers includes poor inter-rater reproducibility of results[30] (Figure 3) .

Functional measurements have also gained popularity as tools to assess nutritional status in CLD patients. In particular, HG has been shown to be a strong predictor of malnutrition. In a study by Alvares-da-Silva *et al*[20], HG was found to be more sensitive than SGA in predicting malnutrition and the incidence of major complications at 1-year in cirrhotic patients. This functional assessment is completed with a dynamometer and is useful for tracking clinical change in patients. Refer to Table 1 for a summary of these anthropometric measurements and their unique limitations.

Miscellaneous

Less commonly used tests for nutritional status assessment include a DEXA scan, bioelectrical impedance analysis, and *in vivo* neutron activation analysis (IVNAA). These tests are rarely used, primarily due to their limited availability and high cost[30]. DEXA scans can accurately assess the fat mass in CLD patients. Sarcopenia is the most common complication of cirrhosis and DEXA scans can also be used to assess skeletal muscle mass[31]. Additionally, CT and MRI scans can be used to measure the extent of sarcopenia as they both can determine muscle cross-sectional area. These two tests are not used as commonly as DXA scans would be in assessment of sarcopenia however because CT scans are expensive and expose the patient to significant radiation, while MRI scans are also expensive and less available[17]. Bioelectrical impedance analysis uses electrodes to accurately estimate fat content. IVNAA is used to measure total body protein and can serve as a good indicator of PCM[30]. Nevertheless, these are not typical tools used in a standard nutritional assessment.

MANAGEMENT - ENTERAL AND PARENTERAL

Therapeutic interventions to maintain adequate nutritional status in CLD patients can be divided into enteral or parenteral forms. The indications and contraindications for each mode of therapy vary widely depending on the patient and the extent of disease.

Generally speaking, guidelines suggest that the required energy intake for cirrhotic patients is 35-40 kcal/kg-BW per day and a protein intake of 1.2-1.5 g/kg-BW per day[32,33]. The dietary plan for an average 70 kg adult does not significantly differ from a practically "normal" diet, as long as the above caloric and protein intake guidelines are met (usually through protein supplementation). In fact, the diet of CLD patients is largely based on a standard diet with added supplements as needed[31]. It is however important to discuss variations in a patient's condition, as they may concomitantly have hepatic encephalopathy or hepatic-renal syndrome, and these must be addressed as well. With regards to hepatic encephalopathy, the general recommendation currently is to simply continue usual diet, while maximizing appropriate treatment with lactulose rifaximin, and so on[33]. In hepato-renal syndrome, there do not appear to be any recommendations as per the current literature to adjust nutrition, and the current approach remains to address the underlying mechanism causing HRS (*e.g.*, correcting hypovolemia with albumin infusions)[34].

Note that CLD can arise through different pathologies, including NAFLD. This is of particular importance because the pathophysiology through which NAFLD arises is metabolic syndrome and diet/

Table 1 Anthropometric techniques: Benefits and limitations

Technique	Benefits	Limitations
BMI	Weight (kg)/height (m^2) Indicator of choice for chronic undernutrition in adults Probability of misclassifying nutritional status on basis of BMI considered to be very small	Confounded in cirrhotics with ascites and peripheral edema
Mid-arm muscle circumference	Measured in centimeters using flexible measuring tape (halfway between olecranon and acromion process) Less influenced by patient fluid status (upper limbs less commonly edematous) Recognize malnutrition earlier relative to BMI Better at recognizing mild-moderate malnutrition	Possibly significant inter-observer variability Poorly recognizes patients with severe malnutrition
Skinfold thickness (triceps, biceps, subscapular, suprailiac)	Measured in millimeters using skinfold caliper Less influenced by patient fluid status Recognize malnutrition earlier relative to BMI Better at recognizing mild-moderate malnutrition	Possibly significant inter-observer variability Poorly recognizes patients with severe malnutrition
Handgrip strength	Measured in kilogram force, using hydraulic dynamometer adjusted to patient hand size Highly sensitive indicator of functional impairment, reflective of protein-calorie malnutrition Correlates with severity of clinical outcome in different disease states	Requires certain equipment to measure which may not be widely available

BMI: Body mass index.

lifestyle is largely implicated[35]. Given this, addressing diet in patients with CLD from NAFLD is of utmost importance. General recommendations are to reduce total fat, saturated fats, trans fats, and fracture, while simultaneously increasing intake of polyunsaturated fats, and monosaturated fats[35]. These changes will likely benefit any patient with CLD but are especially important in those with NAFLD.

As prognosis is closely linked to nutrition in patients with chronic liver pathology, the goal of any therapeutic measure is to achieve the recommended intake amount.

Enteral

Enteral means of nutritional administration implies that food is absorbed primarily through the digestive processes of the gastrointestinal tract. The intake of nutritional substances can be done orally, directly into the stomach, or from the rectum. In the setting of comorbidities or inability to consume food orally, a nasogastric (NG) or percutaneous endoscopic gastrostomy (PEG) tube can be inserted for direct gastric administration.

Oral

The content and distribution of diet must be regulated to ensure adequate nutritional intake in CLD and cirrhotic patients. The general diet recommendation is to have multiple (5-6) small meals that are rich in complex carbohydrates, while lipids may compose 20%-30% of the overall caloric intake[32]. Initially, it was thought that protein restriction was essential as it was shown to decrease the incidence of encephalopathy in end-stage liver disease. However, recent insight into the value of protein restriction has shown that there is minimal impact on the onset of encephalopathy and rather that overall protein intake should be increased since requirements are higher[36]. A diet low in protein should therefore be avoided.

One of the largest topics of study in patients with liver disease includes the possible benefits of oral supplement use. More specifically, there have been multiple research studies on the use of branched chain amino acids (BCAAs) for nutritional support. BCAAs include leucine, isoleucine, and valine, which cannot be synthesized in the body and must be obtained through diet[37]. Patients with CLD or cirrhosis are known to have low levels of BCAAs, which impacts a variety of bodily functions including ammonia detoxification. Furthermore, inadequate levels have been shown to worsen hepatic encephalopathy and ultimately contribute to a worsened clinical outcome[36]. Multiple research studies have demonstrated benefits of using BCAA supplementation for patients with severe liver disease. A randomized clinical trial showed that long-term supplementation with oral BCAAs helped prevent progressive hepatic failure[37]. They have also been noted to improve cases with pre-existent hepatic encephalopathy. In addition to BCAAs, usage of a controlled diet with nutritional supplements like casein-based protein mixtures were associated with lower bilirubin levels, improved prothrombin time and an overall reduction in infection[37]. A 2012 systematic review by Koretz *et al*[32] identified that the use nutritional supplements in oral feeding for patients with liver disease were associated lower rates of ascites, infection and hepatic encephalopathy. Finally, multivitamins are also recommended but there is

limited research on the benefits in CLD patients.

Increasing numbers of patients with CLD are being found to have coexisting celiac disease and this introduces an additional barrier with regards to tailoring feeds to achieve appropriate nutrition[38-40]. Given that general recommendations for diet include meals that are rich in complex carbohydrates, it is essential that patients with concomitant celiac disease ensure the carbohydrates that they intake are gluten-free. Many BCAA supplements do not contain gluten, and further supplementation with protein mixtures can be achieved by ensuring the protein mixture acquired is gluten-free[41]. Although celiac disease does in fact introduce an additional barrier, much of it can be overcome with a fastidious approach to which foods are consumed. These principles apply to other methods of nutritional intake explored below as well.

Tube feeding

If it is evident that nutritional requirements cannot be met through oral feeding, the next line of therapy is tube feeding. In regards to the use of a NG or PEG tube, clinicians are often concerned about the possibility of an NG tube causing gastrointestinal bleeding. However, literature has found that the risk of GI bleeding in NG tube placement is quite low and should not deter clinicians away from this form of tube feeding[42]. The European Society for Parenteral and Enteral Nutrition (ESPEN) guidelines originally indicated that tube feeding could be used in patients who were not able to maintain adequate nutritional status through oral intake, even in the presence esophageal varices. Though, this was modified shortly after the results of a small-randomized trial identified the possibility of tube feeding causing recurrence of bleeding[42]. The guidelines were then changed to state that tube feeding in patients with esophageal varices is dangerous, and that patients should be closely monitored should this therapy be initiated[42].

When NG tubes are needed for extended periods of time, smaller diameter NG tubes are recommended to avoid irritation of the nasal mucosa when in use for extended periods of time. However, in cases where long term enteral tube feeding is needed, there is typically discussion on the merits of using a PEG tube. Major problems with using PEG tubes in this patient population are that there are various instances in liver disease where PEG tubes are contraindicated; specifically in the setting of ascites, bleeding varices, coagulopathy or other sequela of decompensated cirrhosis[43]. Due to this, clinicians are rarely inclined to use a PEG tube for CLD or cirrhotic patients.

Research comparing oral diets to enteral-tube feeding has shown variable results in determining which mode of nutrition provides better outcomes. Studies by Kearns *et al*[44] and Cabre *et al*[45] have shown significant clinical improvements in serum albumin, Child's score, and a reduction in mortality with patients who were on enteral-tube feeding compared to oral diet controls[46]. Accordingly, a recent multicenter trial in 99 cirrhotic patients randomly placed half on enteral tube-feeding for 4 wk, followed by oral supplements for 8 wk while the other half was kept on a strict oral diet[47]. Using short-term enteral tube feeding allowed investigators to achieve the recommended caloric intake in 70% of patients, but there was no difference in 1-year survival, or other liver parameters between the 2 groups[47]. Considering the multitude of studies on the topic of oral nutrition *vs* tube feeding, Hasse *et al*[48] performed an extensive review of enteral nutrition in the setting of liver disease. This review indicated that evidence for starting a patient on oral or tube feeding is currently inconclusive, as the majority of data show highly variable results[48]. However, the discussion of when to use oral or tube feeding is typically not a matter of preference, but more so what a patient is able to tolerate. Currently, this review of enteral nutrition outlines that patients should maximize oral intake with a targeted diet and supplements initially. At this stage, individuals should be closely monitored to see if oral feeding is able to achieve the desired caloric and nutrient intake. If nutritional status continues to decline, it is recommended that patients begin tube feeding within 1 wk of inadequate oral intake[42,48]. Delaying the onset of tube feeding is associated with worse outcomes and delayed improvement in nutritional status. NG tubes are recommended, since PEG tubes are often contraindicated in CLD patients.

There are various formulas that are suitable for tube-feeding depending on the individual patient disease. They may typically be a standard formula that is protein rich, or nutrient-dense in those who are on fluid restrictions. These can also be hydrolyzed for those who have impaired digestion, include more BCAAs or offer immune complexes for patients that are immunocompromised. These formulas are highly variable and can be altered for individual patient needs.

Parenteral

Parenteral nutrition therapy is an intervention in which feeding is done entirely through an intravenous line. In patients with liver disease, parenteral nutrition is often recommended when caloric and nutritional intake is insufficient through either oral or enteral means. It can also be considered in short-term situations where patients must undergo prolonged fasts for procedural considerations. It is also a consideration in patients with compromised airways, encephalopathy or impaired swallowing reflexes that would make oral and sometimes tube feeding difficult.

Cirrhotic patients require a caloric intake that is

approximately 1.2-1.3 times the REE[43]. Nutrient intake is divided into carbohydrates, lipids, proteins and micronutrients. Through parenteral nutrition, carbohydrates are provided in the form of glucose to make up for approximately 50%-60% of non-protein energy requirements. Lipids are provided as emulsions of unsaturated fatty acids that make up approximately 40%-50% of non-protein energy requirements. Protein requirements are met through an infusion of amino acids that ranges from 1.2-1.5 g/kg per day depending on the patient's current disease severity[43]. These amino acid solutions typically contain a larger proportion of BCAAs and a lower fraction of aromatic amino acids[16]. The inclusion of micronutrients into parenteral formulas is a topic of controversy as many studies have failed to show actual therapeutic benefits. Nevertheless, various water and fat-soluble vitamins, and electrolytes are included in the overall infusion by clinicians. This is primarily because CLD is often associated with significant micronutrient deficiencies, specifically those that have an alcohol-related etiology[44]. Once again, the recommendations for beginning parenteral nutrition are when oral and enteral means have been tried and failed.

There are a variety of complications involved with parenteral nutrition. Since it often requires chronic vascular access, there is risk of catheter infection and clotting. Furthermore, there is risk of total parenteral nutrition (TPN) induced liver disease, particularly due to interactions between linoleic acid (major lipid source of calories) and the liver parenchyma[43]. Furthermore, patients may experience severe hunger pains because TPN use completely bypasses the GI system. Refeeding syndrome is also a consideration in patients with severe cirrhosis or CLD that have been experiencing severe nutrient deficiencies for a prolonged period of time[9].

CONCLUSION

In summary, given the essential role of the liver in maintaining appropriate nutritional status, the importance of prompt recognition of nutritional deficiency in patients with CLD cannot be understated given the immense implications it has on overall morbidity and mortality. The etiology of this malnutrition is multifactorial, including decreased intake, increased metabolic requirements, and malabsorption/maldigestion. Recognizing and assessing nutritional deficiencies can be challenging in patients with CLD. Although a history and physical examination can prove helpful, often they are insufficient and further investigations in the form of serum markers and anthropometry are required for proper assessment. Ultimately, management is in the form of enteral or parenteral nutrition, with the appropriate choice being reflective of disease severity. Although enteral nutrition in oral form with appropriately selected supplements can be sufficient in managing the majority of patients with CLD and malnutrition, often with worsening disease and malabsorption/maldigestion, a step-wise approach moving from oral, to tube feeding, and if necessary, parenteral nutrition is required. Early recognition and intervention is beneficial as the systemic implications of malnutrition greatly impact the prognosis and can limit management options as the disease progresses. With an appropriate approach to malnutrition in CLD, many of the associated complications can be avoided and overall improve the outcomes in this patient population.

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MINIREVIEWS

Anthropometric indicators of visceral adiposity as predictors of non-alcoholic fatty liver disease: A review

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Abstract

The objective was to critically analyze studies that evaluated the predictive capacity of indicators of visceral adiposity in non-alcoholic fatty liver disease (NAFLD). The bibliographic research was carried out using the electronic database PubMed, LILACS and SciELO, references of selected articles. Although we found few studies, they have already used several indicators of visceral adiposity as waist circumference, waist-to-hip ratio, waist-to-height ratio, Lipid accumulation product, Body Shape Index, Body Roundness Index and most them were good predictors of NAFLD. Thus, the anthropometric indicators may contribute for the diagnosis of NAFLD in a simple, low-cost and non-invasive way, allowing early therapeutic measures to prevent the evolution to non-alcoholic steatohepatitis.

Key words: Anthropometry; Adiposity; Non-alcoholic fatty liver disease; Abdominal fat; Pediatrics; Predictive value

Core tip: Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide and presents evolutional potential for more severe forms of the disease such as cirrhosis and hepatocellular carcinoma. The most effective treatment is based on changes in lifestyle, diet and exercise; however, this presents the challenge of having to be performed for

a long time. The diagnosis, especially of non-alcoholic steatohepatitis (NASH), requires invasive examination such as liver biopsy. The anthropometric clinical indicators of visceral obesity, of easy applicability and low cost, have been very promising in the prediction of NAFLD. Thus, future studies could be conducted to use them in the prediction of NASH, besides assisting in the therapeutic and preventive conduction NASH.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a relevant metabolic disorder with a high evolutional potential, including from isolated hepatic steatosis to non-alcoholic steatohepatitis (NASH), and if left untreated, can progress to fibrosis, cirrhosis and even liver failure[1].

Excess weight, mainly the accumulation of visceral fat, is considered the main risk factor for NAFLD, being closely related to the severity of the disease[2-4]. Currently, clinical anthropometric indicators such as waist-to-height ratio (WHtR) and lipid accumulation product (LAP) are described in the literature as the most sensitive and specific for discriminating visceral fat compared to the classic parameters as waist circumference (WC) and body mass index (BMI)[5].

Some formulas recommended in the literature for NAFLD prediction already use anthropometric indicators such as BMI and WC[6,7]. However, there are few studies that have evaluated predictive capacity as well as the cutoff points of these new indicators in individuals with NAFLD[8-11]. This review aimed to critically analyze studies that assessed the ability of anthropometric indicators of adiposity to predict NAFLD.

LITERATURE SEARCH

To perform the search and retrieval of scientific articles, we used the PubMed (National Library of Medicine), Scielo (Scientific Eletronic Library Online) and LILACS (Literatura Latino-Americana e do Caribe em Ciências da Saúde) databases. The "OR" and "AND" connectors were used to combine the descriptors: ("non-alcoholic fatty liver disease" or "non-alcoholic steatohepatitis" or "fatty liver" or "NAFLD") and ("adult") and ("waist circumference" or "body mass index" or "waist-to-height ratio" or "conicity index" or "lipid accumulation product") and ("discriminatory performance" or "optimal cutoff points"). The articles of interest listed in the references have also been identified and reviewed.

Inclusion criteria were: the availability of the full text in the database; publication in the last ten years (Feb 2008-Feb 2018); written in Portuguese, English or Spanish. Exclusion criteria were: review studies, repeated studies in more than one database, research outside the previously determined context.

After analyzing the titles and abstracts, according to the eligibility criteria, the studies were selected for reading in full. One thousand eight hundred and sixty-three studies were found in the databases. After completing the selection of the studies based on the reading of the titles and abstracts, 1858 studies were excluded: 1845 did not meet the eligibility criteria; 1 duplicated, 1 Chinese, 1 cohort study; 1 evaluated individuals with hepatitis B and C virus, and 2 studies were included after the screening of the references.

Thus, eleven articles were included in the review. The main information of the studies as authorship and year of publication, where the study was conducted, the study population characteristics (number of individuals and age), anthropometric indicators evaluated BMI, WC, WHtR, LAP, Waist-To-Hip Ratio (WHR), Body Shape Index (ABSI), Body Roundness Index (BRI) and diagnosis criteria of NAFLD (Table 1).

CLINICAL ANTHROPOMETRIC INDICATORS OF VISCERAL ADIPOSITY

Classical anthropometric indicators, such as BMI and WC, are disseminated in clinical practice to assess nutritional status, since they are relatively simple and inexpensive tools[12,13]. Visceral obesity is the most important risk factor for NAFLD[2-4] and recent studies have been investigating the relevance of WC and other new clinical indicators of visceral adiposity (WHtR and LAP) in NAFLD diagnosis (Table 2).

In the studies selected here it is observed that different methods were used to identify hepatic steatosis: liver biopsy[9], computed tomography[14], proton magnetic resonance spectroscopy[8,15] being that the great majority realized abdominal ultrasonography[10,11,16-20].

Most of these methods such as ultrasonography, computed tomography and magnetic resonance imaging are important in the diagnosis of NAFLD, however these techniques cannot distinguish benign steatosis from steatohepatitis, severity of inflammation and grade and degree of fibrosis or stage of disease[21]. The liver biopsy, although not used in routine clinical practice remains the only available procedure to grade and to stage NAFLD and to exclude other causes of liver disease. It has been considered a gold standard method[22].

WAIST CIRCUMFERENCE

The WC is an anthropometric indicator widely used

Table 1 Studies evaluating the anthropometric indicators as predictors of non-alcoholic fatty liver disease

Ref.	Country	Population (*n*)	Age (yr)	Anthropometric indicator	NAFLD diagnosis
Yoo *et al*[14]	South Korea	NAFLD (77) Non-NAFLD (379)	20-88	WC and WHtR	Tomography
Zheng *et al*[9]	China	NAFLD (250) Non-NAFLD (240)	36.6 ± 11.1 37.3 ± 10.2	WHR, BMI, WC and WHtR	Liver biopsy
Ju *et al*[10]	South Korea	NAFLD (2553) Non-NAFLD (6606)	42.5 ± 5.1 41.6 ± 4.9	BMI and WC	Ultrasonography
Cuthbertson *et al*[8]	England and Germany	NAFLD (168) Non-NAFLD (168)	50.3 ± 11.9 48.6 ± 10.9	LAP	Proton magnetic resonance spectroscopy
Monteiro *et al*[16]	Brazil	Obese with NAFLD (45) Obese without NAFLD (100)	11-17	WC	Ultrasonography
Zhang *et al*[17]	China	NAFLD (362) Non-NAFLD (6867)	7-18	WHtR, BMI and WC	Ultrasonography
Motamed *et al*[11]	Iran	NAFLD (2048) Non-NAFLD (2824)	48.6 ± 12.7 39.0 ± 15.4	WHtR, WHR, ABSI, BRI	Ultrasonography
Özhan *et al*[18]	Turkey	Obese without NAFLD (130) Obese with NAFLD (202)	11.6 ± 2.7 12.1 ± 2.6	WHtR	Ultrasonography
Lin *et al*[19]	Taiwan	NAFLD (167) Non-NAFLD (1043)	18.8 ± 1.9 15.1 ± 2.8	WHtR, WHR	Ultrasonography
Lee *et al*[15]	United States	Black (94) and White (58) overweight and obese adolescents	14.7 ± 1.8 14.5 ± 1.5	WC	Proton magnetic resonance spectroscopy
Dai *et al*[20]	China	NAFLD (12150) Non-NAFLD (28309)	18-94	LAP	Ultrasonography

NAFLD: Non-alcoholic fatty liver disease; WHR: Waist-to-hip ratio; BMI: Body mass index; WC: Waist circumference; WHtR: Waist-to-height ratio; ABSI: Body shape index; BRI: Body roundness index; LAP: Lipid accumulation product.

in population studies and in clinical practice to assess central obesity, better reflecting the content of visceral fat, in addition to having a good relationship with total body fat[14,23]. However, it presents important limitations when used alone as a predictor of visceral fat[24].

WC and BMI indicators are widely used in the evaluation of individuals with NAFLD[2,8,25]. However, it is known BMI limitations in the evaluation of the composition and distribution of body fat, considering that this indicator only evaluates total body mass[26]. In most studies, the WC was assessed by WHO[26] (midpoint between the iliac crest and the last rib)[9,14,17]. Only Ju *et al*[10] performed the measurement of WC in the umbilical line. Yoo *et al*[14], in an observational cohort study, investigated the WC in 456 adults and elderly Koreans, with BMI of 26.5 ± 2.5 kg/m^2 in men NAFLD group and 27.3 ± 3.3 kg/m^2 in women NAFLD group and found a cutoff point of 89.0 cm for men and 84.0 cm for women, with sensitivity above 74%, in the prediction of NAFLD. Ju *et al*[10] investigated the WC in 9159 adults Koreans in a cross-sectional study with BMI of 22.4 ± 2.5 kg/m^2 in Non-NAFLD group and 25.6 ± 2.61 kg/m^2 in NAFLD group and identified with better levels of sensitivity and specificity, the WC cutoff of 84.9 cm for men and 80.4 cm for women. They observed that WC and BMI showed similar predictive capacity in both sexes, showing that there is still controversy about the best anthropometric parameter to predict NAFLD. In the study conducted by Zheng *et al*[9] in a cross-sectional study with 490 patients Chinese, between 15-56 years, which included 96 patients with liver disease, 86 hepatitis, 19 hepatic hemangioma, and 39 autoimmune liver disease, with BMI 24.8 ± 10.1 kg/m^2 in Non-NAFLD group and 36.8 ± 10.1 kg/m^2 in NAFLD group, BMI and WC had AUC above 0.84 in the total sample.

In children, the WC AUC was 0.94, with the cutoff point at the 80th percentile for age, in the study by Zhang *et al*[17], a cross sectional study with 7229 students in China. Lee *et al*[15] evaluated white and black obese adolescents in the United States and found an optimal WC cutoff point for predicting NAFLD of 101.5 cm, AUC: 0.847, 93% sensitivity and 80% specificity in white obese women, and with no statistical difference between the values found between the blacks. The cross-sectional study by Monteiro *et al*[16], which evaluated 145 obese children and adolescents in Brazil, found higher WC (AUC: 0.720) when compared to trunk fat mass (AUC: 0.661), estimated by Dual-energy X-ray absorptiometry, however lower than the intra-abdominal adipose tissue (AUC: 0.741), measured by ultrasound examination. It should be noted that the authors did not present the cut-off points of the indicators. It is observed that most of these studies were developed in Asian populations[9,14,17], considering that WC cutoff points may differing between racial and ethnic groups more studies are needed for this identification.

WAIST-TO-HIP RATIO

The Waist-To-Hip Ratio (WHR) is determined by dividing

Table 2 Cut-offs and areas under the ROC curve, sensitivity and specificity of anthropometric indicators to determine non-alcoholic fatty liver disease

Ref.	Indicator	Total				Women				Men			
		AUC (95%CI)	Cut-offs point	Sens (%)	Spec (%)	AUC (95%CI)	Cut-offs point	Sens (%)	Spec (%)	AUC (95%CI)	Cut-offs point	Sens (%)	Spec (%)
Yoo *et al*[14]	WHtR					0.80 (0.74-0.86)	0.53	90	63	0.72 (0.63-0.81)	0.52	71	65
	WC					0.79 (0.73-0.86)	84	84	62	0.74 (0.65-0.83)	89	75	64
Zheng *et al*[9]	WHR	0.916 (0.86-0.97)	0.89	99	66								
	BMI	0.854 (0.78-0.93)	24.22	96	64								
	WC	0.876 (0.81-0.94)	82.5	95	68								
	WHtR	0.878 (0.82-0.94)	0.49	96	64								
Ju *et al*[10]	WC					0.821 (0.801-0.840)	80.395			0.759 (0.746-0.773)	84.945		
	BMI					0.83 (0.811-0.850)	22.715			0.76 (0.747-0.773)	24.465		
Cuthbertson *et al*[8]	LAP	0.78 (0.73-0.83)				0.77 (0.69-0.85)				0.74 (0.66-0.82)			
Monteiro *et al*[16]	WC	0.72 (0.636-0.804)		66.7	64			0.733	0.704			0.567	0.6
Zhang *et al*[17]	WHtR	0.95 (0.94-0.96)	0.47	85.2	92.5								
	WC	0.94 (0.92-0.95)	80[1]	86.2	87.4								
	BMI	0.93 (0.91-0.94)	80[1]	86.2	87.6								
Motamed *et al*[11]	WHtR					0.8566 (0.8419-0.8714)	0.58	83.3	71.7	0.8457 (0.8320-0.8593)	0.533	82.7	70.8
	BRI					0.8566 (0.8419-0.8714)	5	83.3	71.7	0.8457 (0.8320-0.8593)	4	82.7	70.8
	WHR					0.7673 (0.7487-0.7860)				0.8018 (0.7862-0.8173)			
	ABSI					0.6598 (0.6382-0.6814)				0.6539 (0.6351-0.6727)			
Özhan *et al*[18]	WHtR		0.62	48.4	73.8								
Lin *et al*[19]	WHtR	0.80 (0.76-0.83)	0.469	70.1	76.9								
	WHR	0.755 (0.714-0.795)											
[1]Lee *et al*[15]	WC	0.847	101.5	93	80								
Dai *et al*[20]	LAP					0.887 (0.882-0.892)	23	82	79	0.843 (0.837-0.849)	30.5	77	75

[1]Data shown for white obese adolescents only. AUC: Area under ROC curve; Sens: Sensitivity; Spec: Specificity; WHtR: Waist-to-height ratio; WC: Waist circumference; WHR: Waist-to-hip ratio; BMI: Body mass index; LAP: Lipid accumulation product; BRI: Body roundness index; ABSI: Body shape index.

the waist circumference by the circumference of the hip. This indicator is used to characterize how body fat is distributed, if it is concentrated in the central region or in body extremities. Sometimes the gain or loss of weight causes similar alterations in waist and hip circumferences, without altering the final ratio, so in these cases, WHR is not so useful to evaluate body mass changes[27].

Few studies have evaluated the WHR in predicting NAFLD. However, the WHR was the indicator with the highest predictive capacity (AUC: 0.91) for NAFLD in the study by Zheng *et al*[9], as a cutoff point of 0.89 for the total sample. Motamed *et al*[11], in a cross-sectional study with Iranian adults, reported an AUC above 0.70 of the WHR in both sexes to predict NAFLD, but cut points were not presented. Despite the limited amount of studies found in the literature that addressed the predictive capacity of WHR in NAFLD, the results found in both studies show that this indicator can be considered as a good predictor of NAFLD.

WAIST-TO-HEIGHT RATIO

The WHtR is an indicator of abdominal obesity that has been used in several studies to evaluate metabolic disorders[28-30]. The analysis of the WHtR suggests that a person WC should not exceed half the height value, presenting a better sensitivity in the evaluation of the health risk when compared to the measurement of isolated WC in different populations[31].

Yoo *et al*[14], Zheng *et al*[9] and Motamed *et al*[11] reported an AUC above 0.71 of the WHtR to detect NAFLD. Yoo *et al*[14] identified cutoff points of 0.53 (sens: sensitivity 90%, spec: specificity 63%) for women and 0.52 (sens: 71%, spec: 65%) for men. The cutoff points found in the study conducted by Motamed *et al*[11] with Iranians were similar for men 0.533 (sens: 82.7%, spec: 70.8%), but not for women which was 0.580 (sens: 83.3%, spec: 71.7%). Already Zheng *et al*[9] did not stratify the results by sex.

In children, the WHtR was the indicator that presented the highest AUC 0.95 and with cutoff points of 0.47 (sens: 95%, spec: 96%) in Zhang *et al*[17]. Lin *et al*[19] evaluated 1210 children aged 10-19 years in Taiwan and identified that WHtR had AUC higher than that of WHR. The cutoff point of WHtR to predict NAFLD in the study population was 0.499 (sens: 70.1%, spec: 76.9%). Özhan *et al*[18], evaluated 332 obese children with and without NAFLD, and the WHtR presented an optimal cutoff point for the prediction of NAFLD of 0.62, but with low sensitivity (48.5%) and high specificity (73.8%). In all this study WC was measured according to WHO[26].

In adults, the advantage of this indicator is related to the ability to neutralize the differences between the heights, allowing to individualize the interpretation of the fat concentration for different ages, since only a change in the value of the indicator occurs if the modification comes from the WC. In addition to be a low-cost indicator, it uses traditional and simple body measurements, and is a non-invasive method, being easily interpreted and reproducible[29,30].

LIPID ACCUMULATION PRODUCT

LAP is a proposed indicator for estimating lipid concentration in adults. Its formula includes data from WC and the serum concentration of triglycerides in the fasted state. The indicator proposes to investigate if the concentration of lipids exerts effect in the evaluation of the cardiovascular risks, better than the BMI[32].

Only two studies used LAP in the prediction of NAFLD[8,20]. Cuthbertson *et al*[8] evaluated 4 cohorts in Germany and England and although it has demonstrated an AUC of 0.78, the authors did not calculate cutoff points for NAFLD. Already a large cross-sectional study[20] that evaluated 40459 Chinese, the LAP showed high accuracy for diagnosing NAFLD in both men and women (AUC 0.843 and 0.887, respectively), with cutoff points of 30.5 (sens: 77%, spec: 75%) and 23.0 (sens: 82%, spec: 79%), respectively, and especially in the younger population between 18 and 44 years old.

Therefore, further studies are needed to confirm its usefulness in predicting this condition in other population groups since it is an excellent predictor of NAFLD in the Asian population. In addition, it is important to highlight a limitation in the clinical practice of this indicator, which requires application of complex formula and serum triglyceride values[33].

OTHER INDICATORS

Two new indicators of obesity, Ody Shape Index (ABSI) e Body Roundness Index (BRI) were described in literature. The ABSI was proposed by Krakauer *et al*[34] and it is calculated with the values of WC, BMI and height. Their analysis suggests that the ABSI allows to evaluate the excess risk of high WC. And Thomas *et al*[35] developed BRI to predict body fat and percentage of visceral adipose tissue, calculated with WC and height data.

Motamed *et al*[11] evaluated the ability of two new obesity indicators to predict NAFLD. In this study BRI and WHtR presented the same discriminatory power (AUC above 0.84) for NAFLD. However, ABSI was not a good indicator to predict NAFLD. Because BRI is an indicator of obesity that is still recent and little studied, the use in clinical practice to assess the discriminatory capacity of NAFLD deserves caution.

Of all the articles studied only one study, Ju *et al*[10], stratified the population in NAFLD and NASH. However, this study did not evaluate the predictive capacity of these indicators in NASH, only in NAFLD, besides NASH classification was evaluated by the presence of steatosis associated with elevation of alanine aminotransferase. Therefore, these studies that investigated the anthropometric indicators of visceral adiposity can only predict NAFLD. In addition, we can highlight in these studies the predictive capacity of the anthropometric clinical indicators for NAFLD that the main limitations were the different diagnostic methods used to detect NAFLD, the different ethnicities and different age groups, such as the inclusion of the elderly in some samples.

CONCLUSION

There are still few studies that evaluated anthropometric indicators of visceral obesity in the prediction of NAFLD, and these already have promising results. The identification of these indicators, with specific cutoff points considering the ethnic and racial groups, for use in the prediction of NAFLD, and especially NASH, may help in the early diagnosis, allowing a therapeutic and preventive approach to this population.

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MINIREVIEWS

Dental pulp cell bank as a possible future source of individual hepatocytes

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Abstract

Mesenchymal stem cells (MSCs) as a source for regenerative medicine are now the subject of much clinical attention. There are high expectations due to their safety, low tumorigenic risk, and low ethical concerns. MSC therapy has been approved for acute graft-versus host diseases since 2015. Tooth-derived MSCs are known to have a great potential in their proliferation and differentiation capacities, even when compared with bone-marrow-derived MSCs. In particular, stem cells from human exfoliated deciduous teeth (SHEDs) are the best candidates for personal cell banking (dental pulp cell bank), because they can be obtained less invasively in the natural process of individual growth. SHEDs are known to differentiate into hepatocytes. There have been several studies showing the effectiveness of SHEDs on the treatment of liver failure in animal models. They may exert their effects either by repopulation of cells in injured liver or by paracrine mechanisms due to their immune-regulatory functions. Moreover, it may be possible to use each individuals' dental pulp cells as a future source of tailor-made differentiated hepatocytes in the context of a bioartificial liver or liver-on-a-chip to screen for drug toxicity.

Key words: Mesenchymal stem cells; Stem cells from

human exfoliating teeth; Hepatocytes; Dental pulp cell bank; Liver diseases

Core tip: Dental pulp-origin mesenchymal stem cells have a remarkable potential for regenerative medicine in both differentiation and proliferation capacity. Dental pulp cell banks are currently under operation in several institutions in Japan, as they can be obtained easily and less invasively in the personal growth process. Recent findings that they can differentiate into hepatocytes suggest that they can be applied to refractory liver diseases as either auto or allogenic cell therapies. These hepatocytes can be used as tailor-made components for a bioartificial liver or liver-on-a-chip to screen for drug toxicities in preparation for future use.

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INTRODUCTION

Mesenchymal stem cells (MSCs), which reside in a variety of tissues, are able to differentiate into many cell types. They have a low risk of tumorigenesis because they do not need the introduction of foreign genes to differentiate, unlike induced pluripotent stem (iPS) cells. They also have a low risk of immune rejection. They can be obtained in a minimally invasive manner such as umbilical cord blood, providing a promising cell source for regenerative medicine. Application of MSCs in the treatment of refractory liver diseases is currently under great clinical scrutiny[1-3].

It was first reported in 2000 that MSCs were present in dental pulp tissues within the teeth[4]. Dental pulp-derived MSCs (DP-MSCs) are known to differentiate into many cell types like other MSCs, such as osteoblasts, adipocytes and neural cells[5]. DP-MSCs also have good potential for proliferation and differentiation similarly to other types of MSC. In particular, MSCs derived from exfoliated deciduous teeth (SHED) in childhood have been reported to have a pronounced potential of proliferation[6,7]. Because these are normally discarded in the process of personal growth, they are perfectly suited for cell banking in a manner similar to umbilical cord blood[8]. The dental pulp cell bank is a best fit for future tailor-made medicine, where people deposit their own tooth-derived MSCs, preparing for their future medical needs. In this review, we concisely review the current and future status of DP-MSCs, including SHED-based regenerative medicines, particularly focusing on their application for liver diseases and for the construction of bio-assay systems that are suitable for drug side-effect testing, with the aim of achieving tailor-made medicine.

Cells from teeth for regenerative medicine

Recent progress in regenerative medicine has been outstanding; it is now possible to remove one's own cells or tissues, differentiate them into many cell types, and use these to repair dysfunctional organs. The development of iPS cells has contributed greatly to this movement. In Japan, a clinical study for age-related macular degeneration using iPS cells started in 2014[9]. However, there remain some clinical concerns regarding iPS cell-based regenerative medicine. For instance, because autologous transplantation of self-iPS cells is costly, heterologous cells must be used in practical situations. Establishment of cell panels to cover all HLA types remains costly and laborious. In addition, because they are prepared with transfection by foreign genes, the risk of tumorigenesis cannot be ignored[10].

On the other hand, because MSCs do not need transfection of genes, they may have a lower risk of tumorigenesis. In addition, they induce immune tolerance in general, so rejection of cells is unlikely. MSCs might also increase the acceptance of regenerative medicine because they do not undergo any gene manipulation.

In the dental field, starting with their acquisition from wisdom and deciduous teeth, MSCs from dental pulp, periodontal ligament, apical papilla, and dental follicle have been reported[11-13]. These dental stem cells have variety of differentiation and active proliferation capacities. These are obtained in a less-invasive manner, and the concept of "waste material re-utilization" is the main rationale to promote a system of dental pulp cell banking.

Gronthos *et al*[4] first reported that dental pulp-derived cells from adults were clonogenic, rapidly-progressive and produced dentin/pulp-like complex under specific conditions. This study opened the way for the application of DP-MSCs to regenerative medicine. Subsequently, it was shown that DP-MSCs could differentiate into cells that were irrelevant to teeth, such as adipocytes or neural cells[5] and are known to be osteogenic, odontogenic, dentinogenic, cementogenic, adipogenic, chondrogenic and neurogenic[11,14]. Miura *et al*[6] showed that SHEDs had a higher potential of proliferation and differentiation, and would therefore be a hopeful source for regenerative medicine. This may be the beginning of the concept of dental bank reusing the exfoliated juvenile teeth that would be discarded otherwise. MSCs from an early age may be expected to be more capable of regeneration and differentiation, as was shown by study finding that SHEDs were more proliferative than other DP-MSCs[7]. There was also a report showing a superior differentiation capacity for SHED when compared with stem cells from adult dental pulp[15].

It is known that stem cells from bone marrow or blood are able to differentiate into cells like hepatocytes[16-18]. Ishkitiev *et al*[19] first reported that DP-MSCs

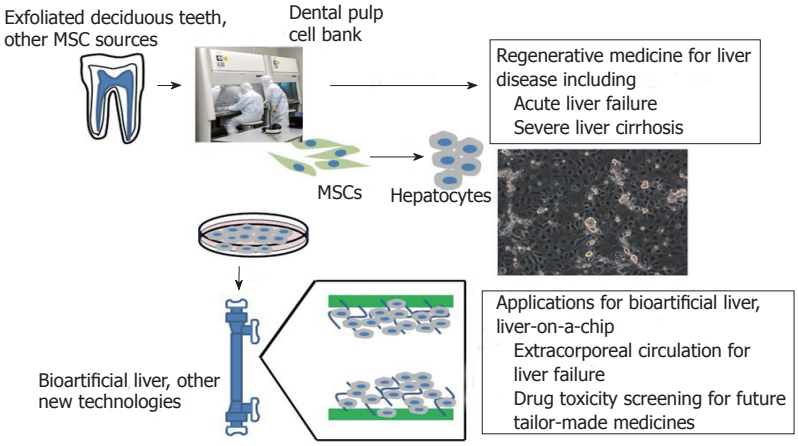

Figure 1 Schematic representation of processes that utilize dental pulp cell bank for future use as hepatocytes. They may be used as cellular sources for cytotherapies to treat refractory liver diseases or as a component of bioartificial liver aiming at tailor-made applications such as future drug-toxicity screening. A hepatocyte-like cell induced from dental pulp-derived-mesenchymal stem cells in our laboratory is shown (unpublished data).

could differentiate into hepatocytes. They cultured cells from deciduous teeth in medium containing HGF, Insulin-Transferrin-Selenium-X, and oncostatin M, and found that they differentiated into cells with an appearance of hepatocytes and produced albumin. These hepatocytes were able to metabolize ammonia to urea, suggesting the presence of a urea cycle. Purification of the cell fractions positive for CD117 enabled efficient induction of hepatocytes[20]. The level of hepatic differentiation in SHED when compared with bone marrow-derived MSC (BM-MSC)s was the same or higher[21]. A recent report also showed a higher expression of hepatocyte-markers in DP-MSCs than in BM-MSCs at both the genetic and protein levels[22]. We also succeeded in differentiating DP-MSC into cells with hepatocyte-morphology, by culturing them first under the presence of activin A, Na-butylate and fibroblast growth factor, and then insulin, dexamethasone, and hepatocyte growth factor (unpublished results, Figure 1).

CELL TRANSPLANTATION THERAPY WITH SHEDS FOR LIVER DISEASES

The effects of MSC-based therapy consist of two major mechanisms. The first is that MSCs transdifferentiate into the cells of damaged-tissues and compensate for organ dysfunction. The second is that, responding to cytokines from the inflamed tissues, MSCs exert paracrine functions including immunomodulation and tissue repair[23]. MSCs produce a variety of cytokines, chemokines and growth factors. The immunomodulatory effects may be one of the main mechanisms of MSC treatment for acute graft versus host disease that has been approved[24].

To date, about half of the papers describing cytotherapies with MSCs were those using bone-marrow-derived MSCs, followed by umbilical cord blood and adipose tissues, and very few were on DP-MSCs[23], despite their promising capabilities. This may partly be due to difficulties of collaboration between dental and medical departments.

Cytotherapies with MSCs have been applied for refractory liver diseases with severe dysfunctions and fibrosis[3]. Transdifferentiation of MSCs into hepatocytes and paracrine mechanisms have been considered to be the main effects. Shi *et al*[25] reported that 13/15 pigs with acute liver failure that were administered bone-marrow derived MSCs survived, while none of the controls did. They showed that 4.5% of cells in surviving liver were repopulated by MSC-derived hepatocytes, concluding that MSC paracrine mechanisms as well as repopulation of hepatocytes by transdifferentiated MSCs contributed to the effects of MSC treatment.

Paracrine mechanisms, including immunomodulation, have attracted the most clinical attention[26]. As the immune effects of MSCs are most likely caused by soluble factors, restriction by HLA in donor selection can be ignored[27]. Moreover, DP-MSCs might induce stronger immune tolerance than bone-marrow derived MSCs[28].

There have been several experimental reports that showed the application of DP-MSCs for liver diseases. Ishikiev *et al*[29] reported that transplantation of hepatocytes induced by SHEDs into the spleen of rats with acute and chronic liver failure improved hepatic functions *via* transdifferentiation and repopulation of the cells. Yamaza *et al*[30] also reported that trans-spleen administration of SHEDs into CCL4-induced cirrhotic mice significantly improved liver function, inflammation, and fibrosis. Both studies attributed the effects to the direct implantation of cells through their differentiation into hepatocytes. Ito *et al*[31] reported that only conditioned medium (CM) from SHEDs resulted in significant survival effects in rats with acute liver failure due to D-galactosamine. They reported that the survival effect of CM on liver failure was induced by anti-inflammatory M2 macrophages that suppressed hepatocyte apoptosis, and promoted hepatocyte proliferation. It is important

Table 1 Comparison of benefits and disadvantages among 3 types of cell sources, mesenchymal stem cells, induced pluripotent stem and embryogenic stem cells

	MSC	iPS cells	ES cells
Proliferation	Low	High	High
Differentiation	Limited	Pluripotent	Pluripotent
Gene transfer	No	Yes	No
Cancer risk	Low	Not neglected	Not neglected
Immune rejection	Low	Possible	High
Paracrine mechanism	Yes	Unknown	Unknown
Banking	Easy	Easy	Possible
Ethical hurdle	Low	Low	High

MSC: Mesenchymal stem cells; iPS: Induced pluripotent stem; ES: Embryogenic stem.

to know that only soluble factors, not the use of cells, induce significant clinical outcomes. Moreover, exosomes secreted by MSCs have been reported to be effective in the improvement of liver function and fibrosis[32,33]. Future studies should verify the effects of no-cell-therapy with conditioned medium or intracellular vesicles on liver diseases.

ESTABLISHMENT AND OPERATION OF DENTAL PULP CELL BANK

Three cellular resources, embryonic stem (ES) cells, iPS cells and MSCs, are currently the major candidates for the clinical application of regenerative medicine. A comparison of the benefits and disadvantages among these cellular resources is shown in Table 1. MSCs do not have higher potential of proliferation or differentiation than ES cells or iPS cells, some consider them to be a primary source for regenerative medicine because of the low possibility of tumorigenesis and the lack of ethical concerns.

SHEDs are an ideal resource in regenerative medicine because of their high capacity, low ethical concerns and cost, and re-use concept[8]. In addition, dental pulp is viable 5 d after extraction[34]. Not only could they be used as a low immunogenic source for allogenic transplantation therapy, but they can also be applied as a tailor-made self-source preparing for future needs[35].

Aiming at the future progress of regenerative medicine from ethical and technical aspects, new legislation was introduced in Japan in 2014. Regenerative medicine using tissue stem cells including MSCs is classified as medium risk, while those using iPS or ES cells are classified as high risk.

The dental pulp cell bank should be officially approved under investigation by the regenerative medicine committee, on the premise of acquisition of informed consent and act of protection of personal information. It must fulfill the requirement of Pharmaceuticals and Medical Devices Agency (PMDA). In Japan, two dental banks are currently under operation, including the Dental Cell Bank™ of The Nippon Dental University which started in 2016 after obtaining permission to operate as a cell processing facility (CPF) from the Japanese Government. Extracted teeth from registered dental clinics are stored in preservation solution and are sent to the Dental Cell Bank™. Dental pulp cells are propagated in culture and stored.

The merits of using dental pulp cells for regenerative medicines, in addition to the general benefits of MSC (Table 1), are follows: the stock cells are obtained when in good health and in a minimally-invasive manner, low cost, and low external radiation exposure because of their confinement in the enamel.

Although some difficulties remain to be overcome in order to achieve successful dental cell bank operations including cost barriers, restrictions imposed by current preservation technology, and the limitation of operation method, the promising capabilities of SHEDs and other tooth-derived sources are supporting the development of the dental pulp cell banking system.

APPLICATION OF HEPATOCYTES FROM DENTAL PULP CELL BANK TO TAILOR-MADE MEDICINE TO MEET FUTURE NEEDS

Fulminant hepatic failure is an aggressive disease that has an extremely poor prognosis. Liver transplantation may be the only medical method to rescue most patients. Because the keys of the success of liver transplantation depend on the acquisition of donor liver, medical bridging therapies while waiting for the appearance of donor liver are critical for life-saving. Extra-corporeal circulation using bioartificial livers that have hepatocytes in the column to reduce toxic substances such as ammonia that can affect consciousness levels have been developed[36]. Although primary hepatocytes or highly differentiated hepatoma cell lines were used for the column, significant survival elongation using bioartificial livers have not yet been confirmed. Recently, development of artificial livers using iPS cells has been reported. Takebe *et al*[37] cultured iPS cells with vascular endothelial cells and macrophages, and succeeded in the creation of an organ bud or mini-liver. Because DP-MSC-derived hepatocytes had high proliferation activity, express hepatocyte nuclear factor 4a (HNF-4a), and metabolize ammonia to urea (unpublished observation), they are expected to bear the function of bioartificial livers.

On the other hand, the liver is an organ involved in drug metabolism. In the era of new medicine development, there will certainly be a need to predict the adverse effects of drugs in a tailor-made manner. Because drug metabolism varies from individual to individual, it is necessary to use self-hepatocytes to screen for drug toxicity. Hepatocytes derived from dental pulp cell bank may suit this purpose. Cells lose differentiation levels in two dimension or spheroid cultures where diffusion of materials is the only way to feed the cells. Recently, microenvironments of the cells in tissues have been

simulated in the organ-on-a-chip system that reproduces the dynamic environments of real tissues[38]. Nakao *et al*[39] reported liver-on-a-chip that reproduced the cord-like structures of hepatocytes with bile -duct canalicular formations. Vernetti *et al*[40], succeeded in drug toxicity screening with construction of a culture system that had hepatocytes, vascular endothelial cells, immune and stellate cells. Hepatocytes derived from a dental pulp cell bank may be a good cellular source of such a three-dimensional culture system and may enable people who deposit their teeth to meet the future use of hepatocytes, such as in drug screening, while providing an allo-auto cellular source to cure liver diseases (Figure 1).

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MINIREVIEWS

Adiponectin as a novel biomarker for liver fibrosis

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Abstract

Adiponectin is known to play primary roles in the regulation of systemic glucose homeostasis and lipid metabolism. Interestingly, emerging evidence indicates beneficial effects of adiponectin on liver fibrosis; however, the exact mechanisms of this action remain unclear. Herein, we aimed to summarize the recent findings regarding the role of adiponectin in liver fibrogenesis and update the current comprehensive knowledge regarding usefulness of adiponectin-based treatments in liver fibrosis. Adiponectin has been demonstrated to have an anti-fibrotic action in the liver by blocking the activation of hepatic stellate cell-mediated adenosine monophosphate-activated protein kinase and peroxisome proliferator-activated receptor-alpha pathways, which in turn diminish the expression of pro-fibrotic genes. In addition, hyperadiponectinemia was noted in patients with various chronic liver diseases (CLDs)-related liver fibrosis. An increase in circulating adiponectin levels was also found to be associated with the development of liver fibrosis, indicating a role of adiponectin as a non-invasive biomarker for predicting the progression of liver fibrosis. It is therefore reasonable to speculate that adiponectin may be developed as a new therapeutic candidate for the treatment of liver fibrosis. Nonetheless, future observations are still necessary to fully elucidate the extent of the effects of adiponectin on

liver fibrotic outcomes, in order to modify adiponectin as an anti-fibrotic therapy that would speed up fibrosis reversal in patients with CLD.

Key words: Adiponectin; Hyperadiponectinemia; Liver fibrosis; Chronic liver disease; Biomarker

Core tip: Adiponectin plays a protective role against the development of liver fibrosis *via* inhibition of hepatic stellate cell activation, induced by specific signal transduction pathways. Among patients with chronic liver diseases (CLDs), hyperadiponectinemia is associated with the degree of liver fibrosis. The potential link between adiponectin and the limited progression of liver fibrosis has accelerated attraction in seeking adiponectin as a target for diagnostic detection tools and novel treatment methods. Nonetheless, additional current therapeutic and clinical trials of adiponectin in liver fibrosis are needed. In this context, we reviewed additional potential therapeutic applications of adiponectin in patients with various CLDs in the context of liver fibrosis.

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INTRODUCTION

Liver fibrosis is a leading cause of morbidity and mortality associated with end-stage liver complications in patients with chronic liver disease (CLD). It is presently recognized a reversible wound-healing reaction to chronic hepatic damage. The morphological characteristics of liver fibrosis include the excessive accumulation of extracellular matrix (ECM) components, mainly fibrillar collagens[1]. The complexity of liver fibrosis has hindered attempts to understand its pathology, which remains unclear. However, there is a wide spectrum of stimuli known to influence liver fibrogenesis, such as drugs, alcohol abuse, toxins, metabolic disorders, viral infections, and cholestasis[2]. If the underlying cause of hepatic fibrosis cannot be ameliorated, the majority of CLD patients with severe hepatic fibrosis will develop cirrhosis, hepatic failure, and hepatocellular carcinoma (HCC) and ultimately require hepatic transplantation. This has driven researchers toward the search and development of effective anti-fibrotic approaches focused on constraining the growth of fibrogenic cells and/or prohibiting the synthesis of ECM molecules, which would be helpful in improving the clinical outcomes of patients with CLD. Hepatic stellate cells (HSCs) are classically recognized as primary fibrogenic cells in the liver. Given that HSCs are the primary source of activated myofibroblasts and portal fibroblasts, HSCs unsurprisingly take part in enhancing the synthesis of ECM components and modulating matrix degradation in the injured liver. Generally, quiescent HSCs are dormant and their activity is to deposit retinoids. In response to liver injury, HSCs undergo an activation process, and become myofibroblast-like cells that secrete various cytokines/growth factors and produce ECM proteins[3]. Consequently, elucidating the molecular mechanisms of liver fibrosis and their relevance to HSCs is of paramount importance for the discovery of new therapeutic targets.

Adiponectin, a 28 kDa protein adipocytokine, is mainly produced and secreted into the circulation by white adipose tissue. The primary function of adiponectin is the regulation of carbohydrate and lipid metabolism. However, the full extent of its biological action remains to be elucidated, with a variety of effects on different cell and tissue types, including its immune modulatory, anti-inflammatory[4], and anti-fibrotic properties[5,6]. Regarding its protective effects, an experimental study demonstrated the extensive development of liver fibrosis in adiponectin-knockout mice[6]. Acting *via* transmembrane receptors, adiponectin regulates HSC proliferation, as well as migration, and induces their apoptosis through the activation of adenosine monophosphate-activated protein kinase (AMPK)[7]. Moreover, adiponectin can attenuate HSC activation and suppress the expression of pro-fibrogenic genes, including collagen Ⅰ, transforming growth factor-beta 1 (TGF-β1), and alpha-smooth muscle actin (α-SMA)[8], leading to the inhibition of liver fibrogenesis. With such potent effects on HSCs against liver fibrosis, adiponectin may be developed as a novel therapeutic agent in liver fibrosis. As adiponectin can be detected in the circulation and exerts its effects on various cells, it may have prognostic and diagnostic value for several human diseases. Interestingly, hyperadiponectinemia has been documented as highly prevalent in patients with CLD and liver fibrosis[9], thereby establishing the possible influence of adiponectin levels in the development and progression of liver fibrosis. Nevertheless, the underlying mechanisms of association between hyperadiponectinemia and liver fibrosis have yet to be completely elucidated. Therefore, the purpose of this minireview is to summarize an update on experimental and clinical studies that have been focused on the promising beneficial impacts of adiponectin on the treatment of liver fibrosis associated with many aspects of CLDs.

The articles published between 2000-2018 were searched manually from the PubMed and Scopus using the following keywords or combination of keywords: "Adiponectin", "Adiponectin levels", "Liver disease", and "Liver fibrosis". Initially, titles and abstracts related to the keywords were screened, and further full articles were evaluated for inclusion. Human clinical studies of any design providing circulating adiponectin levels associated with the severity of liver fibrosis in patients with various

CLDs were eligible for this review. Articles not written in English-language, letters to the editor, case reports/series, and editorials were excluded from this review. No restrictions on gender, ethnic background, number of study subjects, or publishing year were applied.

ADIPONECTIN BIOLOGY

Protein structure of adiponectin

Human adiponectin encoded by the *Adipo Q* gene spanning 17 kb on chromosome locus 3q27 is a multimeric protein hormone and exerts diverse biological functions. The encoded protein comprises an N-terminal signal sequence being a collagenous domain and a C-terminal globular domain maintaining biological properties after cleavage[10]. Pre-secretion, post-translational mechanisms (*e.g.*, hydroxylation and glycosylation) occurring in the collagenous domain of adiponectin at the four lysines have been shown to improve the activity of sub-physiological levels of insulin, which leads to the inhibition of gluconeogenesis in liver cells[11]. The globular domains of adiponectin form three major complexes including trimers, hexamers, and high-molecular-weight (HMW) multimers, classically existing in the circulation[12]. It seems clear that accurate adiponectin folding and assembly are an important step in regulating its complex distribution in the circulation. Although the different oligomeric complexes distributed in the circulation have distinct downstream biological effects on specific target tissues, HMW adiponectin, the dominant form in the circulation, is considered a marker for disease-associated adipocyte dysfunctions[13]. Among adipocytokines, circulating levels of adiponectin have been observed at high levels in healthy individuals, with approximately 0.01% of the total circulating protein ranging from 5 to 30 μg/mL[14]. On the other hand, hypoadiponectinemia has been previously associated with metabolic alterations, including insulin resistance, dyslipidemia, and atherosclerosis[15-17]. It is noteworthy that a physiological level of circulating adiponectin is important for defense against metabolic disorders and may be related to other chronic diseases including chronic obstructive pulmonary disease[18], chronic kidney disease[19], and knee osteoarthritis[20]. Notwithstanding, the precise mechanisms regulating adiponectin levels in the human body remain poorly understood. It has been suggested that there are multiple factors with important roles in regulating adiponectin levels in the human body, including genetics, mechanisms affecting its clearance, and post-translational modifications associated with controlling adiponectin gene expression[21-23]. Moreover, the regulation of adiponectin receptors is thought to be important for facilitating essential physiological functions of adiponectin.

Adiponectin receptors

The adiponectin receptors, through which adiponectin acts, include seven-transmembrane domains, consisting of two predominant isoforms: adiponectin receptor type 1 (adipo R1) and adiponectin receptor type 2 (adipo R2). These 2 major receptors have been identified in numerous tissues. In human tissues, both of them are observed in the brain and peripheral tissues. However, adipo R1 is ubiquitously detected, most abundantly in the skeletal muscle. The major expression of adipo R2 is in the liver[24]. The adiponectin receptors bind globular and full-length adiponectin with different affinities. The adipoR1 has a greater affinity for globular adiponectin, whereas adipo R2 has an intermediate affinity for both isoforms. Even though the adiponectin receptors comprise seven-transmembrane domains, their structure and functions are distinct from those of G protein-coupled receptors. Physiologically, the engagement of adiponectin with adipo R1 stimulates the phosphorylation of AMPK, which results in a concomitant suppression of energy-consuming biosynthetic pathways, including lipid synthesis and gluconeogenesis. A second common pathway activated by the adiponectin-adipo R2 axis is the peroxisome proliferator-activated receptor-alpha (PPAR-α) signaling pathway, which regulates fatty acid beta-oxidation[25]. Collectively, the aforementioned pathways boost catabolic processes to renew cellular energy under conditions of energy stress. From a classical perspective, adiponectin takes part in controlling glucose and lipid metabolism, through which it exerts a multitude of beneficial effects such as controlling insulin sensitivity. It is conceivable that adiponectin interacting with its distinct receptors influences the stimulation of an appropriate signaling pathway that becomes different in liver pathology.

MULTIFACETED ROLES OF ADIPONECTIN IN THE LIVER

It is commonly recognized that adiponectin regulates the metabolism of both glucose and lipid in the liver, and has been implicated in inhibiting gluconeogenesis, as well as activating fatty acid oxidation and glycolytic pathways. These metabolic impacts of adiponectin are activated by the stimulation of adipo R1 and adipo R2. Following the binding of adiponectin to its receptors, the downstream signaling effects of these receptors are associated with the activation of AMPK and the PPAR-α cascade. As a crucial downstream effector of Adipo R1, AMPK is essentially an energy-sensing gauge that is stimulated by AMP and inhibited by ATP. Activation of AMPK inhibits the transcriptional activity of glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK), which in turn decreases gluconeogenesis. Through the phosphorylation of acetyl-CoA carboxylase (ACC) converted into an inactive form and malonyl-CoA catalysis, AMPK also induces fatty acid degradation. Malonyl-CoA, the product of the carboxylase reaction, plays an essential role in inhibiting carnitine palmitoyl transferase-1 (CPT-1), which prevents the transport of long-chain fatty acyl-CoA into the mitochondria, re-

sulting in reduced fatty acid biosynthesis. In addition to its effects mediated by the inactivation of PEPCK, G-6-Pase, and ACC signaling, AMPK phosphorylation can limit the activity of sterol regulatory element binding protein-1c (SREBP-1c), which is a transcription factor regulating lipid combustion in the liver[26,27]. This action can cause a reduction in hepatic triglyceride content. It is comprehensible that adiponectin-AMPK signaling can promote fatty acid catabolism and inhibit gluconeogenesis and triglyceride production in the hepatocytes[28]. In parallel with its stimulation of AMPK, adiponectin-adipo R2 axis activates the PPAR-α signaling cascade that increases fatty acid combustion and energy consumption. This mechanism leads to reduced triglyceride accumulation in the liver and thus, increased insulin sensitivity[29].

Although the triggering of adipo R1 and adipo R2 reportedly controls glucose and lipid metabolism by the stimulation of AMPK and PPAR-α signaling cascades, there are additional signaling molecules related to a wide range of beneficial systemic effects of adiponectin in the liver. The pleiotropic actions of adiponectin have been reportedly associated with the activation of its cognate receptor-mediated stimulation of ceramidase activity[30]. Ceramidase is known to take a primary part in the control of ceramide and sphingosine metabolism, which results in the breakdown of ceramide to produce sphingosine for phosphorylation to sphingosine-1-phosphate. Ceramide and sphingosine comprise an important class of bioactive lipids associated with changes in insulin sensitivity, inflammation, and survival[31]. In 2017, Holland *et al*[32] also demonstrated the significant involvement of adiponectin in declining intracellular ceramide levels, which was accompanied by increased ceramidase activity. This peculiar effect of adiponectin-induced ceramidase signaling was supported in transgenic mice exhibiting an overexpression of adiponectin receptors. Overexpression of adiponectin receptors ameliorated ceramidase activity and induced metabolic improvements in glucose and lipid homeostasis, in addition to insulin sensitivity[32]. These observations provide further evidence regarding the beneficial effects of adiponectin, especially its insulin-sensitizing action, in which adiponectin is activated by its own receptor-mediated stimulation of ceramidase activity.

Besides its primary roles in regulating insulin sensitivity and fatty acid metabolism, adiponectin has been shown to possess anti-inflammatory effects. In particular, adiponectin expands its hepatoprotective effects by decreasing inflammation through blocking the activation of nuclear factor-kappa B (NF-κB)[33] and suppressing the release of tumor necrosis factor-alpha (TNF-α), which is an inflammatory cytokine[4]. The TNF-α that is predominantly secreted by HSCs and Kupffer cells in the liver has a critical effect on hepatic damage, given its capability to promote inflammation and apoptosis in liver cells under the appropriate circumstances of oxidative stress[34]. Adiponectin also limits the production of interleukin-1β, which is a pro-inflammatory cytokine that is responsible for liver injury and initiates the secretion of interleukin-10[35]. The anti-inflammatory actions of adiponectin have also been implicated in macrophage dysfunction[36], through which it suppresses the growth and movement of vascular smooth muscle cells[37] and modulates lymphopoiesis[38]. The pleiotropic biological actions of adiponectin in the liver are illustrated in Figure 1.

Anti-fibrotic action of adiponectin in the liver

One of the critical mechanisms by which adiponectin exerts an anti-fibrogenic effect on the liver is characterized by the induction of the activated phenotype of HSCs. It has been well established that activated HSCs constitutively express both types of adiponectin receptors[39,40], suggesting a potential physiologic action for adiponectin receptor-mediated liver fibrosis in HSCs. As described above, the alteration of HSCs into myofibroblasts is the keystone event of the pathogenesis of liver fibrosis during liver injury. Adiponectin was shown to repress the growth and movement of mouse HSCs stimulated by platelet-derived growth factor (PDGF)[7], which is considered a marker for activated HSCs. An experimental study further demonstrated that adiponectin diminished the effect of TGF-β1-induced expression of connective tissue growth factor (CTGF, also known as fibrogenic gene) on HSCs *via* suppression of the nuclear translocation of mothers against decapentaplegic homolog 2 (SMAD2)[8]. This is important evidence that supports the hypothesis that adiponectin may have anti-fibrogenic effects that are independent of metabolic actions. In this context, overexpression of adipoR2 in mice was found to have a protective role against the progression of liver fibrosis *via* the enhancement of PPAR-α signaling with reduced expression of TGF-β1[41]. Moreover, in cultured HSCs, the enhancement of adiponectin expression remarkably worsened HSC proliferation, as well as the expression of α-SMA, and prevented liver fibrosis through the modulation of caspase-mediated HSC apoptosis[8]. Adiponectin expression is considerably downregulated in activated HSCs, but it is abundantly present in the quiescent phenotype, suggesting a permissive role of adiponectin that favors the quiescent state of HSCs in the physiology of liver fibrosis. In this regard, adiponectin diminishes the development of liver fibrosis by modulating the activity of inhibitor of cytokine signaling-3 (SOCS-3) mediated by the long-form of the leptin receptor (Ob-Rb) and inducing the expression and activation of protein tyrosine phosphatase 1B (PTP1B). As negative regulators of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling, SOCS-3 and PTP1B inhibit JAK-2/STAT-3, which in turn regulate the formation of extracellular components, including tissue inhibitor of metalloproteinases-1 (TIMP-1) and matrix metalloproteinase-1 (MMP-1)[42].

ANTI-FIBROTIC ROLE OF ADIPONECTIN IN CLINICAL PRACTICE

Indeed, adiponectin protects liver injury and reverses

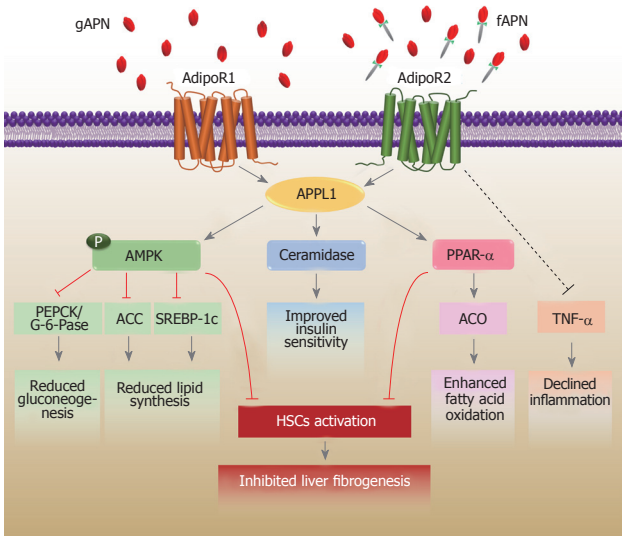

Figure 1 The biological effects of adiponectin on the liver. Adiponectin interacts with adiponectin receptors to prompt a number of signaling pathways. AdipoR1 and R2 dependent signaling is mediated *via* adaptor protein phosphotyrosine interaction (APPL) 1. The signaling activates AMP-activated protein kinase (AMPK), ceramidase activity, and peroxisome proliferator-activated receptor-alpha (PPAR-α) to suppress the accumulation of lipids and regulate glucose homeostasis. The adiponectin-adipoR2 axis can reduce inflammation by inhibiting tumor necrosis factor-alpha (TNF-α) activity. Importantly, activated adiponectin also limits the activation of hepatic stellate cells (HSCs) *via* both AMPK and PPAR-α activation, leading to the inhibition of liver fibrogenesis. ACC: Acetyl-CoA carboxylase; ACO: Acyl-CoA oxidase; AdipoR: Adiponectin receptor; fAPN: Full-length adiponectin; gAPN: Globular adiponectin; PEPCK: Phosphoenolpyruvate carboxykinase; SREBP-1c: Sterol regulatory element binding protein-1c.

the activation of HSCs in animal models. These effects support the possibility of its role against liver damage and fibrogenesis in humans. In pursuit of developing the clinical advantage of adiponectin as a potential predictor for the progression of liver fibrosis, a number of studies have assessed circulating adiponectin levels in a wide range of CLDs.

Adiponectin as a biological marker for liver fibrosis

Generally, liver fibrosis is a reversible physiologic and pathologic event in response to chronic liver injury that leads to liver cirrhosis and eventually end-stage liver disease. The reversal and prevention of these conditions have come to be critical endpoints in clinical trials with novel anti-fibrotic therapies. A liver biopsy has long remained a definitive diagnostic approach for the staging of fibrosis; however, highly efficient and specific non-invasive tools to assess liver fibrosis will be necessary for monitoring the development and progression of disease. Taking into account the risks for complications with respect to sampling error from a needle liver biopsy, there has been enormous interest in the search and development of noninvasive biological markers. Physical characteristics of adiponectin, *e.g.*, high stability in the circulation, little diurnal variation, and great abundance in the human body have made it a promising biomarker in medical contexts for clinical investigation, prognosis, and therapy of liver fibrosis in patients with CLD.

Given that adiponectin has been shown to have predominantly hepatoprotective and anti-fibrogenic effects in conditions of liver injury, whether circulating adiponectin levels are associated with an increased risk of development of liver fibrosis in CLD remains to be determined. Several studies have been focused on investigating the possible associations between adiponectin concentration and the stage of liver fibrosis in various CLDs, including, liver cirrhosis, biliary atresia (BA), hepatitis C viral infection (HCV), hepatitis B viral infection (HBV), non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH), as summarized in Table 1. First, in a cross-sectional study of 232 fasting patients with CLD, serum adiponectin levels were significantly elevated in patients with cirrhosis, as compared to those patients with other liver diseases. Serum adiponectin concentrations were also positively associated with surrogate biomarkers of liver fibrogenesis, including transient elastography, fasting serum bile acids, and hyaluronate in patients with CLD[9]. This study supports a recent report in which adiponectin levels were significantly elevated in patients with cirrhosis compared to controls and also associated with the severity of hepatic dysfunction in those patients[43]. The possible relevance of adiponectin in CLD has been attested by our previous studies, which evaluated circulating adiponectin concentrations in patients with cholestatic BA. A case-control study of 106 patients with BA and 40 healthy controls by Udomsinprasert *et al*[44] demonstrated that patients with BA exhibited considerably greater levels of serum adiponectin than healthy controls. Notably, serum adiponectin levels were positively correlated with the degree of liver fibrosis in patients with BA[44]. When assessing the relationship between circulating adiponectin levels and clinical parameters in patients with BA - particularly liver stiffness scores, serum adiponectin concentrations were also observed to be remarkably higher in patients with significant liver fibrosis than those with insignificant fibrosis, and a direct correlation of serum adiponectin and the severity of liver fibrogenesis was reported[45].

In addition to its significant involvement in the clinical outcome of BA with respect to the degree of liver fibrosis, a number of studies have reported the relationships between hyperadiponectinemia and the clinical parameters of liver fibrosis in cohorts with hepatitis viral infection. A more recent study by Carvalho *et al*[46] found that patients with HCV infection had significantly greater adiponectin levels than healthy controls. In 2013, Korah *et al*[47] examined serum adiponectin levels in patients with chronic HCV genotype 4 associated with steatosis and fibrosis. They also found that 45 men with chronic HCV genotype 4 and advanced fibrosis had significantly increased adiponectin levels, whereas these levels were remarkably reduced in patients with steatosis. Sumie *et al*[48] added another piece of supporting data, revealing that serum adiponectin levels were predictors of liver fibrosis in patients with HCC, in response to chronic HCV infection. Likewise, when Corbetta *et al*[49] analyzed

Table 1 Summary of studies on the association between circulating adiponectin levels and liver fibrosis in various types of chronic liver diseases

Reference	Yr	Diagnosis	Study design	Subjects	Significant results
Hyperadiponectinemia					
Balmer *et al*[9]	2010	CLD	Cross-sectional study	232 fasting patients with CLD, 64 with NAFLD, 71 patients with viral hepatitis, 18 patients with autoimmune disease, 3 patients with alcohol-induced liver disease, 31 patients with elevated liver enzyme of unknown origin, and 45 patients with cirrhosis	Adiponectin levels were substantially increased in cases of cirrhosis Adiponectin levels were positively correlated with surrogate markers of hepatic fibrosis, including transient elastography, fasting serum bile acids, and hyaluronate
da Silva *et al*[43]	2018	Cirrhosis	Case-control study	122 patients with cirrhosis and 30 healthy controls	Patients with CLD had higher adiponectin levels than controls Adiponectin levels were also associated with the severity of liver dysfunction and worse prognosis in those patients
Udomsinprasert *et al*[44]	2012	BA	Case-control study	106 patients with BA and 40 healthy controls	Serum adiponectin levels were significantly higher in BA patients than in healthy controls Adiponectin levels were associated with the severity of fibrosis in BA patients.
Honsawek *et al*[45]	2011	BA	Case-control study	60 patients with BA and 20 healthy controls	BA patients with significant liver fibrosis exhibited remarkably greater serum adiponectin than insignificant fibrosis Serum adiponectin was positively correlated with the degree of fibrosis.
Carvalho *et al*[46]	2018	HCV	Case-control study	33 patients with untreated HCV infection and 30 healthy controls	Patients with HCV infection had higher adiponectin levels, especially those with women
Korah *et al*[47]	2013	HCV	Case-control study	45 untreated men with chronic HCV genotype 4, and 15 healthy men	Serum adiponectin levels were significantly elevated in hepatic fibrosis, but decreased in steatosis
Sumie *et al*[48]	2011	HCV	Case-control study	97 patients with HCC and chronic HCV infection, and 97 patients (controls) with underlying disease	Serum total and HMW adiponectin levels were predictors of liver fibrosis in HCC patients, in response to chronic HCV infection
Corbetta *et al*[49]	2011	HCV	Case-control study	54 patients with chronic HCV hepatitis and healthy controls	Serum adiponectin levels were higher in patients with chronic HCV hepatitis Adiponectin levels were significantly related to the severity of fibrosis in patients with chronic HCV hepatitis
Derbala *et al*[50]	2009	HCV	Case-control study	92 patients with chronic HCV genotype 4 and 66 healthy controls	Adiponectin levels were associated with hepatic fibrosis and inflammation
Hsu *et al*[51]	2015	HBV	Case-control study	187 patients with chronic HBV infection and 187 without chronic HBV infection	Serum adiponectin levels were remarkably correlated with advanced liver fibrosis in elder male HBeAg-negative patients
Hui *et al*[52]	2007	HBV	Cross-sectional study	100 patients with HBV	Patients with fibrosis reduction had a marked decline in serum adiponectin levels after antiviral therapy Adiponectin levels were significantly correlated with fibrosis stage
Hypoadiponectinemia					
Lucero *et al*[54]	2017	NAFLD	Cross-sectional study	36 patients with NAFLD associated with metabolic syndrome and 24 metabolic syndrome patients without NAFLD	Adiponectin levels were significantly lower in NAFLD patients with metabolic syndrome than those patients without metabolic syndrome Adiponectin levels were associated with metabolic parameters and the degree of liver fibrosis
Nazal *et al*[55]	2010	NAFLD	Case-control study	70 patients with NAFLD and 69 normal controls	NAFLD patients had significantly lower serum adiponectin levels than controls Adiponectin levels were independently associated with liver fibrosis
Savvidou *et al*[56]	2009	NAFLD	Cross-sectional study	42 patients with NAFLD	Adiponectin levels were negatively associated with higher stages of fibrosis Adiponectin levels were independent predictors of advanced fibrosis
Yoneda *et al*[57]	2007	NAFLD	Cross-sectional study	248 patients with NAFLD and type 2 diabetes	A reduction in levels of serum adiponectin was independently associated with the severity of hepatic fibrosis in NAFLD patients with type 2 diabetes
Musso *et al*[58]	2005	NASH	Case-control study	20 patients with biopsy-proven NASH and 45 healthy controls	Serum adiponectin levels were significantly reduced in the NASH group, as compared to control groups Adiponectin levels correlated with the severity of hepatic steatosis and fibrosis

CLD: Chronic liver diseases; BA: Biliary atresia; HCV: Hepatitis C virus; HBV: Hepatitis B virus; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

circulating adiponectin levels in 54 patients with chronic HCV, they reported increased adiponectin levels. The investigators also reported that serum adiponectin levels were further correlated with the severity of liver fibrosis in those patients. The aforementioned findings suggest that monitoring adiponectin levels may be employed to improve care for liver fibrosis in patients with HCV infection. In support of this hypothesis, a study of Derbala *et al*[50] also examined circulating adiponectin levels in patients with chronic HCV. They observed that adiponectin concentration was directly associated with hepatic fibrosis and inflammation in the chronic HCV subjects. In addition, strong evidence for a possible correlation between adiponectin levels and progressive liver fibrosis in patients with chronic HBV was recently described by Hsu *et al*[51]. The authors showed that serum adiponectin levels were independently associated with the development of liver fibrosis in patients with HBV. This information supports the observations of Hui and coworkers, which showed that serum adiponectin levels were significantly reduced in patients with reduced fibrosis after antiviral therapy. In particular, adiponectin levels were also positively associated with the stage of fibrosis[52]. All of these findings support the notion that adiponectin has non-classical metabolic actions, including a potential capacity to suppress fibrosis and therefore, may link its role to the development of hepatic fibrogenesis.

Even though former clinical studies have demonstrated a direct relationship of adiponectin and liver fibrogenesis in patients with various CLDs, the exact mechanism responsible for an increase in circulating adiponectin in liver fibrosis remains uncertain. The possible explanation for these findings might be attributed to a reduction in its clearance. In CLD patients with liver fibrosis, declined adiponectin clearance could result from reduced uptake of adiponectin by liver sinusoidal endothelial cells (LSECs), which may lead to elevated adiponectin levels in the circulation. It is widely known that dysfunction of LSECs is one of pathologic events in liver fibrogenesis. In the healthy liver, LSECs generally promote HSCs quiescence. During the process of liver fibrosis, LSECs undergo phenotypic changes with the loss of several receptors and LSECs fenestration, leading to the capillarization of liver sinusoids and the abnormality of various substances uptake[3]. It has been shown that adiponectin levels and adipoR2 expression are decreased in the LSECs response to liver injury[53]. These phenomena may help explain why hyperadiponectinemia has been observed in CLDs patients with liver fibrosis.

In apparent contrast to the aforementioned studies, circulating adiponectin levels have been shown to be reduced in patients with steatosis and steatohepatitis, such as those with NAFLD and NASH. Most recently, Lucero *et al*[54] investigated systemic adiponectin levels in 36 patients with NAFLD related to metabolic syndrome and 24 metabolic syndrome patients without NAFLD. They reported that adiponectin levels were significantly elevated in NAFLD patients with metabolic syndrome when compared to those patients without metabolic syndrome. Besides, circulating adiponectin levels were correlated with metabolic parameters and the degree of liver fibrosis in NAFLD patients. Furthermore, a case-control study of 70 patients with NAFLD and 69 healthy controls conducted by Nazal *et al*[55], explored a possible correlation between adiponectin levels and NAFLD pathology. The authors reported that plasma adiponectin levels were markedly lower in patients with NAFLD than in controls, and they were inversely related to the presence of liver fibrosis. The findings of Savvidou *et al*[56] provide support to a negative association between adiponectin concentrations and higher stages of fibrosis in patients with NAFLD, suggesting that adiponectin levels can be used as predictors of advanced fibrosis. Supporting this finding, a large cohort study of 248 patients with NAFLD and type 2 diabetes found that reduced adiponectin levels were independently associated with the degree of liver fibrosis[57]. Finally, in a case-control study of 60 patients with NAFLD and 60 healthy controls, patients with NAFLD had markedly reduced plasma adiponectin levels, when compared to controls. Notably, a decline in adiponectin levels was found to be closely associated with the degree of liver fibrosis. Similarly, when Musso *et al*[58] determined adiponectin levels in 20 patients with biopsy-proven NASH and 45 healthy controls, they observed that low adiponectin levels were associated with the severity of hepatic steatosis and fibrosis in patients with NASH. Based on these findings, hypoadiponectinemia has been proposed as a contributory factor to the development of metabolic dysfunction in NAFLD and NASH. The reasons for these conflicting findings remain unexplained. These are likely due to differences in populations, disease advancement, or measurements applied, or to incomplete control of confounding variables.

The contrasting results regarding hypoadiponectinemia in patients with NAFLD and those with NASH compared to those with other CLDs could be partly attributed to the differences in pathophysiology of the diseases. Indeed, hypoadiponectinemia has been suggested to play a pathogenic role in the pancreatic-cell dysfunction observed in both NAFLD and NASH[59], and accumulated visceral fat can cause a decline in levels of circulating adiponectin[60]. Regardless of the role of environmental and genetic factors, adiponectin appears to be strongly associated with the hepatic phenotype, which is a major cause of morbidity in NAFLD. It has also been discovered that the adiponectin promoter polymorphism rs266729 was associated with the susceptibility of NAFLD, and the subjects with the GG genotype of rs266729 exhibited substantially lesser adiponectin values than those subjects with the GC or CC genotypes[61]. It is tempting to speculate that genetic variation of adiponecin and lifestyles choices causing visceral fat deposition/obesity may lead to reduced circulating adiponetin levels in patients with NAFLD and NASH. From these reports regarding its significant association with liver fibrosis, it is apparent that adiponectin levels in the circulation may be

a prognosticator for the development of liver fibrosis in various CLDs.

ADIPONECTIN-TARGETED TREATMENT FOR LIVER FIBROSIS

Given that accumulating data correlate the hepatoprotective functions of adiponectin with restricting HSC proliferation and myofibroblast restoration, which are both pivotal mechanisms of liver fibrogenesis, increases in adiponectin levels and its agonists could be an alternative treatment option for the protection and therapy of liver fibrosis. Currently, there are two prime approaches to increase adiponectin concentrations in the body.

Firstly, recombinant adiponectin, or drugs that bypass adiponectin by directly stimulating AMPK, have been extensively utilized in preclinical models. For example, trials utilizing ADP355, which is an adiponectin-like small synthetic peptide agonist, show promise in inhibiting tumorigenesis in a mouse xenograft breast cancer model[62]. Regarding its protective effects on liver fibrosis, an adiponectin-mimetic peptide analog (ADP355) that docks into the adiponectin receptors binding pocket, possesses potency in modulating multiple anti-fibrogenic mechanisms, in particular, AMPK phosphorylation, and has been shown to eliminate hepatic fibrosis in mice with carbon tetrachloride (CCl_4)-induced liver fibrosis. These mechanisms are mediated by the diminishing expression of pro-fibrogenic genes, including *α-SMA*, desmin, *CTGF*, *TGF-β1*, and *TIMP-1*, but enhanced expression of matrix metalloproteinase-13 (*MMP-13*) as a marker for restructuring of the collagen matrix[63]. In the light of these considerations, ADP355 may represent an alternative anti-fibrogenic agent in the treatment of liver fibrosis. However, due to the lack of clinical data concerning liver toxicity, further studies assessing the toxic and off-target effects of this agent will be necessary to determine the practicability and clinical efficiency of adiponectin for practical applications

In contrast, one additional method would be to characterize the classes of substances that could bring about an increase in the secretion or expression of adiponectin. Recently, a few studies have suggested that activation of PPAR-γ can enhance the expression and circulating concentrations of adiponectin *via* regulation of gene transcription[64]. The PPAR-γ cascade is therefore interesting, as it may be associated with direct targets that can modify adiponectin for potential clinical applications. Among the drugs presently in clinical use that are PPAR-γ agonists, the thiazolidinediones (TZDs) exert beneficial anti-proliferative and anti-inflammatory effects *via* PPAR-γ activation[65], and have been found to induce adipose tissue to release adiponectin into the circulation[66]. As TZDs have been broadly applied to improve insulin sensitivity in patients with diabetes, we cannot discern the specific conditions under which adiponectin has defensive effects against liver fibrosis. Accordingly, the rigorous identification of new drugs that target adiponectin in the treatment of liver fibrosis is presently even more important. However, further research efforts will be needed to validate the safety and efficacy of adiponectin before a standardized treatment regimen can be established.

CONCLUSION

The close relationships between the pathology of liver fibrogenesis and clinical appearance of the disease affirm the significance of extended basic and translational studies into the pathogenesis of liver fibrosis. To date, the development of anti-fibrotic therapies in fibrosis and/or cirrhosis has been largely unsuccessful. Considerable research conducted over the past several years has demonstrated the multifaceted and potentially contradicting effects of adiponectin as a novel regulator of liver fibrogenesis. The possible relevance of adiponectin has emerged from studies in cell cultures and animal models, in addition to clinical investigations. Indeed, experimental studies document the multiple effects of adiponectin on limiting HSC proliferation and suppressing the expression of pro-fibrogenic genes through specific signal transduction pathways. The possible effect of adiponectin against liver fibrosis is supported by clinical studies that link hyperadiponectinemia to the severity of liver fibrosis in many liver diseases, including BA, HCV, HBV, and liver cirrhosis; whereas reduced adiponectin levels have been reported to be a key factor in the development of metabolic disorders contributing to NAFLD and NASH. Based on these observations, adiponectin could be a plausible noninvasive biochemical marker identifying the severity of liver fibrosis in patients with CLDs. However, additional research is warranted to better comprehend the precise aspect of adiponectin in the pathogenesis of liver fibrosis, which will help to develop adiponectin as circulating indicator for distinct CLD patients who are at risk of developing liver fibrosis.

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ORIGINAL ARTICLE

Basic Study

Experimental bio-artificial liver: Importance of the architectural design on ammonia detoxification performance

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Abstract

AIM

To determine the influence of the construction design over the biological component's performance in an experimental bio-artificial liver (BAL) device.

METHODS

Two BAL models for liver microorgans (LMOs) were constructed. First, we constructed a cylindrical BAL and tested it without the biological component to establish its correct functioning. Samples of blood and biological compartment (BC) fluid were taken after 0, 60, and 120 min of perfusion. Osmolality, hematocrit, ammonia and glucose concentrations, lactate dehydrogenase (LDH) release (as a LMO viability parameter), and oxygen consumption and ammonia metabolizing capacity (as LMO functionality parameters) were determined. CPSI and OTC gene expression and function were measured. The second BAL, a "flat bottom" model, was constructed using a 25 cm^2 culture flask while maintaining all other components between the models. The BC of both BALs had the same capacity (approximately 50 cm^3) and both were manipulated with the same perfusion system. The performances of the two BALs were compared to show the influence of architecture.

RESULTS

The cylindrical BAL showed a good exchange of fluids and metabolites between blood and the BC, reflected by the matching of osmolalities, and glucose and ammonia concentration ratios after 120 min of perfusion. No hemoconcentration was detected, the hematocrit levels remained stable during the whole study, and the minimal percentage of hemolysis (0.65% ± 0.10%) observed was due to the action of the peristaltic pump. When LMOs were used as biological component of this BAL they showed similar values to the ones obtained in a Normothermic Reoxygenation System (NRS) for almost all the parameters assayed. After 120 min, the results obtained were: LDH release (%): 14.7 ± 3.1 in the BAL and 15.5 ± 3.2 in the NRS (n = 6); oxygen consumption (μmol/min·g wet tissue): 1.16 ± 0.21 in the BAL and 0.84 ± 0.15 in the NRS (n = 6); relative expression of *Cps1* and *Otc*: 0.63 ± 0.12 and 0.67 ± 0.20, respectively, in the BAL, and 0.86 ± 0.10 and 0.82 ± 0.07, respectively, in the NRS (n = 3); enzymatic activity of CPSI and OTC (U/g wet tissue): 3.03 ± 0.86 and 222.0 ± 23.5, respectively, in the BAL, and 3.12 ± 0.73 and 228.8 ± 32.8, respectively, in the NRS (n = 3). In spite of these similarities, LMOs as a biological component of the cylindrical BAL were not able to detoxify ammonia at a significant level (not detected *vs* 35.1% ± 7.0% of the initial 1 mM NH_4^+ dose in NRS, n = 6). Therefore, we built a second BAL with an entirely different design that offers a flat base BC. When LMOs were placed in this "flat bottom" device they were able to detoxify 49.3% ± 8.8% of the initial ammonia overload after 120 min of perfusion (n = 6), with a detoxification capacity of 13.2 ± 2.2 μmol/g wet tissue.

CONCLUSION

In this work, we demonstrate the importance of adapting the BAL architecture to the biological component characteristics to obtain an adequate BAL performance.

Key words: Bio-artificial liver; Ammonia detoxification; Device design; Ornithine Transcarbamylase; Rat liver microorgans; Carbamyl Phosphate Synthetase I

Core tip: This work describes the adaptation of a simplified bio-artificial liver (BAL) prototype to make it suitable to house rat liver microorgans (LMOs) as a biological component, and the evaluation of the performance in this new model. We demonstrate that the modification in the design of the artificial parts employed allows a good performance of LMOs, thus showing the importance of architecture and model configuration on the design of these devices. Besides its application as BAL, this mini bioreactor could serve as a suitable laboratory tool to evaluate the behavior and functionality of LMOs subjected to different incubation conditions due to its simple design and the utilization of standard materials.

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INTRODUCTION

Bio-artificial liver (BAL) devices are extracorporeal systems that contain functional hepatic tissue or cells (the biological component) seeded into a man-made bioreactor (the artificial component) and separated from blood flow by semipermeable membranes[1]. The aim of these devices is to fulfill the necessary hepatic functions to keep patients with hepatic failure stabilized until the regeneration of their own livers or until the appearance of a compatible donor[1,2].

Though the majority of liver functions are performed by hepatocytes, the other hepatic cellular types (Kupffer, Pit, stellate or Ito, and sinusoidal epithelial cells) are also involved and exert some influence over the different functions *in vivo*[2]. Therefore, we became interested in studying a biological component possessing all these different liver constituent cells in search of a better performance of our BAL prototype with respect to a

previous one designed in our laboratory to house isolated hepatocytes[3]. In this sense, liver microorgans (LMOs) are an appropriate choice mostly because they are thin slices of tissue that keep the liver structure and its physiological characteristics and allow a good exchange of substances with the surrounding liquid and gaseous environment[4,5].

Ammonia elimination from the blood of patients with liver failure is a key metabolic reaction that any biological component of an efficient BAL should accomplish in light of the correlation existing between this metabolite accumulation and the progression of hepatic failure[3,6]. Nonetheless, etiology of hyperammonemia can also be from non-hepatic situations, including sepsis[7,8], urea cycle inborn disorders[9], complications in a hepatic transplantation setting[10], complications from cirrhosis[11,12], and urinary tract infections[13]. Hyperammonemia should be quickly treated to avoid brain damage or even death, and simple hemofiltration is sometimes not sufficient to cope with it[10,11]. In this context, a device capable of efficiently detoxifying ammonia could be useful to ameliorate the condition of the patient. Also, it could be utilized in the non-hepatic situations until the origin of the complication can be determined and controlled[6].

When studying ammonia detoxification function displayed by LMOs, we found that these tissue slices had excellent performance when incubated in suspension in a shaker. This simple system was called the Normothermic Reoxygenation System (NRS)[14]. Surprisingly, they completely lost this detoxifying capacity in the BAL device when the blood was overloaded with ammonia.

In our laboratory we had previously designed a BAL consisting of a cylindrical shaped device to house isolated rat hepatocytes as the biological component[4]. This system showed a good performance and allowed the maintenance of adequate viability and functionality of fresh hepatocyte suspensions, and successfully fulfilling different *in vitro* tests that constitute the first step in the development of any BAL system[4]. In this work, we present the results obtained when we enlarged this original prototype to allow accommodation to the bulkier LMOs, hypothesizing that this was a straightforward path in the design. However, we showed that the LMOs were unable to detoxify NH_4^+, and therefore had to redesign the artificial component receptacle to make it suitable to this biological component. To the best of our knowledge, this is the first report that evaluates such an essential issue and is valuable information for scientists studying this field of research.

MATERIALS AND METHODS

Experimental design

Figure 1 summarizes the path followed to develop a BAL prototype suitable for using LMOs as the biological component. Step 1: Evaluation of LMO inherent performance, mainly regarding ammonia detoxification capacity in the NRS[5] (Figure 2A); Step 2: Adaptation of the BAL, originally designed for hepatocytes[3], to house LMOs on its biological compartment (BC) (Figure 2B). Testing of this prototype without any biological component establishes its correct functioning. Testing of this prototype with LMOs as biological component to establish its performance as BAL, especially in relation to ammonia detoxification. Comparison with the results obtained in the NRS; Step 3: Due to the failure of LMOs to detoxify ammonia in the cylindrical BAL (in comparison with NRS): development of a "flat bottom" BAL (Figure 2C). Testing with LMOs as a biological component to determine the influence of the BAL configuration and design over the LMO ammonia detoxification capacity. Other parameters remained similar to the values encountered for the cylindrical BAL and were already published[15].

LMO obtainment

LMOs were obtained from livers of 60-day-old male Wistar rats with a weight of 250-300 g. Animals were given a fourteen day adaptation period to get used to the experimental laboratory environment, during which they could access standard rodent laboratory food and water *ad libitum*. Rats received care according to the principles and recommendations given by the National Academy of Sciences (Argentina). The School of Biochemical and Pharmaceutical Sciences Institutional Animal Care and Use Committee (Universidad Nacional de Rosario, Res No. 139/2011) approved all experimental procedures.

Rats were anesthetized (chloral hydrate, 500 mg/kg body weight, i.p.) and the liver was surgically removed. The right medial lobe was cut into blocks and LMOs were manually obtained from these blocks as thin slices (thickness: 338 ± 27 μm, n = 25), using a disposable microtome blade. We did all the manipulations over an ice-cooled cutting device to reduce liver deterioration, and on top of a piece of filter paper to avoid tissue slippage and ensure the accurate cutting of LMOs[14]. After slicing, LMOs were placed in KH-Base (KHB) solution (KHB, Table 1) and were pre-incubated for 15 min before loading into the NRS or the BAL prototype. The different KH solution compositions are shown in Table 1. KHB media was used for the LMO obtaining procedure and during the pre-incubation period.

LMOs have been characterized regarding histology, water content, and viability (published results[5,14]). They were weighted for normalization of results and their weights were 0.070 ± 0.020 g (n = 25), showing size consistency.

NRS

To evaluate the biological component inherent performance before introducing it into our BAL, we

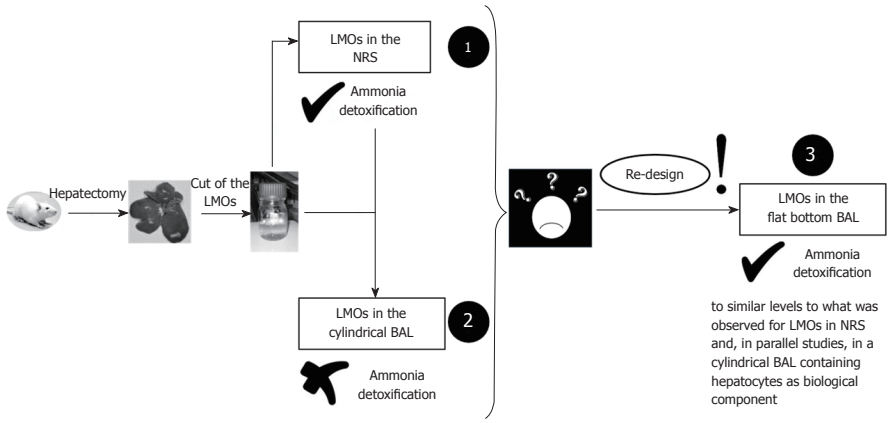

Figure 1 Schematic representation of the steps followed to arrive to the design of a "flat -bottom" bio-artificial liver device suitable to support the appropriate performance of liver microorgans as the biological component. Liver microorgans (LMOs) were obtained from Wistar rat livers: (1) Evaluation of LMO performance parameters in Normothermic Reoxygenation System; (2) Evaluation of LMO performance in cylindrical bio-artificial liver (BAL) and finding that they were unable to detoxify an ammonia overload in the test blood; (3) Evaluation of LMO performance in the newly designed "flat -bottom" BAL that proved suitable to support ammonia detoxification function. LMO: Liver microorgan; NRS: Normothermic Reoxygenation System; BAL: Bio-artificial liver.

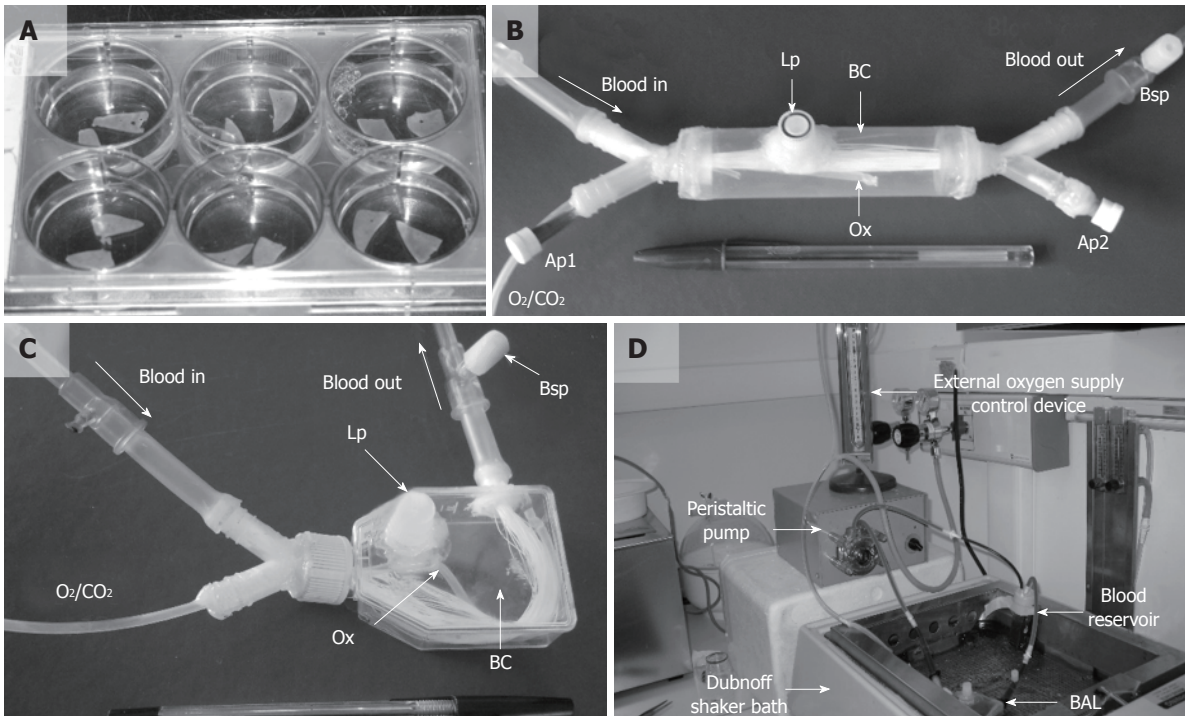

Figure 2 Devices used to analyze liver microorgan performance. A: Liver microorgan (LMO) disposition in the Normothermic Reoxygenation System (NRS).; B: Cylindrical shaped bio-artificial liver (BAL).; C: "Flat bottom" shaped BAL.; D: Perfusion system components. BC: Biological compartment; Lp: LMO loading port; Ap 1 and 2: Biological compartment access ports; Bsp: Blood sampling port; O_2/CO_2: Carbogen supply; Ox: Silicone oxygenating tube.

tested LMOs in a NRS (Figure 2A). In the NRS, they were maintained in Krebs-Henseleit (KH) solution at 37 ℃ under carbogen atmosphere (95% O_2: 5% CO_2) using a six-well culture plate for 120 min[5]. To determine LMO NH_4^+ metabolizing capability, we added an ammonia overload (1 mmol/L approximate NH_4^+ final concentration[16]) to KH-ammonia solution (KHA, Table 1) from a concentrated ammonium chloride solution (approximate concentration: 350 mmol/L). This ammonia overload is far in excess of the level of this metabolite encountered in plasma of patients with liver failure (approximately 0.2 mmol/L). We chose this condition of work to challenge LMOs because they are supposed to deal with continuously infused plasma when applied to animal models of hepatic failure or patients in the future[16–18]. The exact amount of ammonia in KHA solution was then determined, as explained later in this section.

LMO disposition in the NRS is shown in Figure 2A. Two LMOs were placed on each well with 5 mL

Table 1 Composition of the different Krebs-Henseleit solution used

Components	KHB solution	KHA solution
NaCl	114 mmol/L	114 mmol/L
KH_2PO_4	1.2 mmol/L	1.2 mmol/L
KCl	4.8 mmol/L	4.8 mmol/L
$MgSO_4$	1.2 mmol/L	1.2 mmol/L
$CaCl_2$	1.5 mmol/L	1.5 mmol/L
HEPES	10 mmol/L	10 mmol/L
$NaHCO_3$	25 mmol/L	25 mmol/L
Glucose	25 mmol/L	25 mmol/L
Allopurinol	1 mmol/L	1 mmol/L
Fructose	5 mmol/L	5 mmol/L
Glycine	-	3 mmol/L
Adenosine	-	10 μmol/L
Ornithine	-	6 mmol/L
Sodium lactate	-	10 mmol/L
pH	7.4	7.4
mOsm/kg H_2O	293 ± 6 (n = 10)	328 ± 5 (n = 10)

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mOsm/kg H_2O: Osmolality; KHB: KH-Base solution; KHA: KH-ammonia solution.

of KHA solution plus the ammonia overload, and the plate was then introduced in a Dubnoff metabolic shaker maintained at 37 ℃ and stirred at 60 cycles/min. Samples of tissue and the bathing solution were taken after 0, 60, and 120 min to evaluate lactate dehydrogenase (LDH) release, oxygen consumption, and ammonia metabolism[5]. This system is intended to evaluate the biological component performance and condition *per se* in a simpler and less stressing manner than in the BAL device.

The cylindrical shaped BAL designed to house LMOs as biological component

The device designed for hepatocytes[4] was modified to use LMOs as biological component (Figure 2B). It was constructed with a cylindrical plastic cartridge that contained 120 to 140 hollow fibers (Polyamix™, GAMBRO®) assembled to the ends of the cartridge by two Y-connectors (Nalgene, # 6152–0375) connected to S/P silicone tubes (6.4 mm i.d., 11.2 mm o.d., and 2.4 mm wall). Two Teflon large catheters (14 gauge, 2 mm i.d.) and an oxygenator (Ox) made of oxygen permeable tube (silicone tubing, 0.078 in. i.d., 0.125 in. o.d.; cat. No. T5715-9, Baxter Healthcare Corp., Deerfield, IL, United States) were assembled through the Y-connectors. LMOs were seeded through an access port (Lp, Figure 2B) in the space outside the fibers (BC capacity: approximately 50 cm^3), while ram blood (obtained from animals in the animal house facility of our school) circulated along the inner space of the fibers (the blood compartment).

The "flat bottom" BAL to house LMOs as biological component

We constructed a device with a flat base BC using a standard 25 cm^2 culture flask[15] as shown in Figure 2C. The culture flask was adapted introducing a Y-shape connector (Nalgene, # 6152–0375) onto its tap and a plain connector (S/P silicone tube, 6.4 mm i.d., 11.2 mm o.d., and 2.4 mm wall) on one side, to assemble the 140 hollow fibers (Polyamix™, GAMBRO®). The loading port (Lp) was introduced on its top surface and the oxygenating tube (Ox) (silicone tubing, 0.078 in. i.d., 0.125 in. o.d.; cat. no. T5715-9, Baxter Healthcare Corp., Deerfield, IL, United States) access the BC through the free branch of the Y-connector. Both prototypes of BALs were tested using the perfusion system (Figure 2D). The peristaltic pump ensured ram blood recirculation from its reservoir passing through a clot filter/bubble trap before entering the hollow fibers. The perfusion system was kept at 37 ℃ by the thermostatic bath.

System operation protocol

First of all, a sample of ram blood (basal blood) was taken before adding the ammonia overload and filling the blood perfusion system by the aid of a peristaltic pump (blood volume: approximately 35 mL). The BC was then filled with KHA alone (to test the bioreactor performance) or with KHA plus 1 g of LMO. Samples of blood and BC fluid were taken after 0, 60, and 120 min of perfusion to assay the different parameters mentioned below. Blood samples were centrifuged at 12000 × g for 3 min in a mini spin centrifuge.

During the experiments (BAL runs), the device was operated in horizontal position, immersed in the Dubnoff shaker bath at 37 ℃ stirred at 60 cycles/min. Carbogen gas pressure was kept at 85 mm Hg to introduce oxygen in the BC media *via* the silicone tube oxygenator, and in this way pH was maintained at 7.40 ± 0.10, which was controlled throughout the run. The blood maintained proper oxygenation, and its recirculation flow rate was maintained at 9 mL/min.

All the devices designed were subjected to two different types of studies: (1) without any biological component, to establish the adequate functioning of the device and the perfusion system[4]; and (2) with biological component, to determine if the chosen biological component could accomplish ammonia detoxification.

Study of system performance

In experiments without LMOs, we first checked the correct system functioning by evaluating the following parameters: (1) hematocrit of blood samples after centrifugation (10000 × g for 3 min, Rolco CH24 centrifuge), as the percentage of volume that red cells represent from the total (blood cells + plasma). It was used to evaluate fluid exchange and the occurrence of hemolysis during perfusion; (2) osmolality of plasma and extra-fiber fluid was determined with a freezing point osmometer (Osmomat 030 Gonotec, GmbH, Berlin, Germany) to check the proper passage of fluids through the fiber walls; and (3) glucose and ammonia concentrations were assessed as described below

Table 2 Sequences of the primers employed

Primers	Fragment size	Genbank access number
Cps1 5′-ATCTGAGGAAGGAGCTGTCT-3′ (sense) *Cps1* 5′-AAAACCACTTGTCAATGGAT-3′ (anti-sense)	120 bp	NM_017072
Otc 5′-ATGACAGATGCAGTGTTAGC-3′ (sense) *Otc* 5′-CAGGATCTGGATAGGATGAT-3′ (anti-sense)	120 bp	NM_013078
Actb 5′-CAACCTTCTTGCCAGCTCCTC-3′ (sense) *Actb* 5′-GACGAGCGCAGCGATATC-3′ (anti-sense)	79 bp	NM_031144.2
Rn18S 5′-TAACCCGTTGAACCCCATT-3′ (sense) *Rn18S* 5′-CCATCCAATCGGTAGTAGCG-3′ (anti-sense)	150 bp	X01117
Gapdh 5′-CCATCACCATCTTCCAGGAG-3′ (sense) *Gapdh* 5′-CCTGCTTCACCACCTTCTTG-3′ (anti-sense)	576 bp	NM_017008.4

and their mass balance was calculated to monitor the adequate exchange of metabolites between blood and the BC in the device.

Hemolysis assessment

The quantity of hemoglobin in plasma samples obtained after 0, 60, and 120 min of perfusion was assessed using the oxyhemoglobin method as previously described[19]. The equation proposed by Arnaud *et al*[20] was then used to calculate hemolysis percentage as follows:

$$\text{Hemolysis (\%)} = 100 \times [(Hb_S \times (1 - Ht))/Hb_T]$$

where Hb_S represents the sample hemoglobin contents, in g/100 mL; Hb_T is the total hemoglobin content determined in whole blood, and Ht represents the respective hematocrit value.

Glucose level determination

This parameter was assessed with a commercially available kit ("Glicemia Enzimática AA", Wiener Laboratories, Rosario, Argentina), as instructed in the information leaflet.

LDH release quantification

We used LDH release as a LMO viability parameter. This enzyme activity was determined in the KH incubation solution and in the liver slices as previously described[21]. Results are shown as the percentage of the total enzyme activity released to the bathing media.

Ammonia level assessment

Samples were stored in liquid nitrogen until ammonia quantification was done using the enzymatic method described by van Anken[22]. The reaction media (0.8 mL) was composed of 66.7 mmol/L phosphate buffer, pH = 8.30, 0.14 mmol/L NADPH, 6.5 mmol/L sodium α-ketoglutarate, 2.5 mmol/L ADP, and 120 UI/mL glutamate dehydrogenase (cat. #G2626, Sigma Aldrich, St. Louis, MO, United States).

In the BAL, ammonia mass balance was calculated using the equations described next:

$$Q_{B,t} = ([A]_{B,t} \times V_{B,t}) - ([A]_{B,Bas} \times V_{B,t})$$

$$Q_{BC,t} = ([A]_{BC,t} \times V_{BC,t}) - ([A]_{BC,Bas} \times V_{BC,t})$$

$$Q_{T,t} = Q_{B,t} + Q_{BC,t}$$

where: $Q_{B,t}$ and $Q_{BC,t}$ are the ammonia mass in blood and the BC solution at time t, respectively; $[A]_{B,t}$ and $[A]_{BC,t}$ represent respective ammonia concentrations in blood and BC fluid; $V_{B,t}$ and $V_{BC,t}$ are, respectively, the volumes of blood and the BC fluid at each time, and $Q_{T,t}$ is the total ammonia mass at the different assayed times.

Then, we calculated the percentage of ammonia initial dose metabolized at each time with the subsequent equation:

$$\text{Initial Dose Detoxified (\%)} = 100 - [Q_{T,t} \times 100/ Q_{T,0}]$$

Also, we estimated the μmol of ammonia detoxified per gram of LMO as follows:

$$\text{Ammonia Detoxification (μmol/g wet tissue)} = (Q_{T,0} - Q_{T,t})/ P_T$$

where P_T represents the mass of LMOs in grams.

In the case of the NRS, ammonia concentration was determined in the LMO bathing solution and the equations applied were:

$$\text{Initial Dose Detoxified (\%)} = 100 - [(Q_t \times 100)/ Q_i]$$

$$\text{Ammonia Detoxification (μmol/g wet tissue)} = (Q_i - Q_t)/ P_T$$

where Q_i and Q_t represent the amount of ammonia measured at the beginning of the experiments and after t minutes, respectively, and P_T is the total weight of LMOs in grams.

Cps1 and Otc expression analysis

As part of the LMO ammonia metabolism study, we determined the mRNA and activity levels of Carbamyl Phosphate Synthetase Ⅰ (CPSI) and Ornithine Transcarbamylase (OTC) that catalyze the first and second steps of the urea cycle, respectively.

Total RNA extractions were performed using TriReagent™ (Sigma Chem. Co., St. Louis, United State) and following the instructions provided by the manufacturer. We carried out reverse transcription and semi-quantitative PCR as previously described[23]. In Table 2, the base sequences of the primers used for the study of each gene expression are listed. We applied the $2^{-\Delta\Delta CT}$ method to obtain relative expression, using Glyceraldehyde Phosphate Dehydrogenase (*Gapdh*), β-Actin (*Actb*) and rRNA 18S (*Rn18S*) as reporter mRNAs to normalize the values. Each sample was analyzed in triplicate, and its template cDNA initial

quantity was expressed relative to a reference sample, considered 1X. This reference sample was taken at time 0.

CPSI and OTC enzymatic activity determination

To determine CPSI activity we performed the Pierson's colorimetric test[24] that involves the reaction of carbamyl phosphate and hydroxylamine to obtain hydroxyurea. An enhanced colorimetric test for ureido compounds allows the quantification of the hydroxyurea produced due to the chromophore absorbance measurement at 458 nm. CPSI activity is expressed as U/g of wet tissue, being U the μmoles of carbamyl phosphate produced per minute at 37 ℃.

OTC activity was measured with the method described by Ceriotti[25] as the rate of citrulline formation from ornithine and carbamyl phosphate is catalyzed by OTC. The quantity of citrulline produced was determined by the diacetyl monoxime-antipyrine reaction and OTC activity was also expressed as U/g of wet tissue, where U represents the μmoles of citrulline synthesized per minute at 37 ℃.

Oxygen consumption

Samples of LMOs were taken at different times (0, 60, and 120 min) from the NRS or the BAL prototype and were put into a thermostized oxygen electrode chamber constructed in our laboratory, filled with respiration media (KH plus 10 mmol/L HEPES and 2 mmol/Lm pyruvate, pH = 7.40 at 36 ℃)[14]. The oxygen levels in the incubation media were measured using a Clark-type oxygen electrode (YSI 5300, Yellow Spring, OH, United States). After a 2 min stabilization period, the endogenous respiration rate was recorded and calculated over a 5 min period. Results are expressed as μmol O_2/min/g wet tissue[14].

Statistical analysis

Results are expressed as mean ± SD. Data was analyzed using one-way or multifactor analysis of variance with Scheffe's multiple range tests as post-test. Differences between means were considered statistically significant when $P \leqslant 0.05$. Dr. Lucas D Daurelio (Estadística, Facultad de Cs. Bioquímicas y Farmacéuticas, UNR) has designed and supervised the statistical analysis performed in this work.

RESULTS

Cylindrical shaped BAL for LMOs: Performance without biological component

As was formerly explained, the first BAL constructed in our laboratory was designed to house hepatocyte suspensions as the biological component and showed a good performance[4]. In order to evaluate the use of LMOs as alternative biocomponent of our BAL prototype, we first studied their viability and functionality in a simpler model, the NRS, as shown in Figure 2A[5]. Once was established, their ability to metabolize ammonia (detoxification of 35.1% ± 7.0 %, or 14.3 ± 3.6 μmol/g wet tissue, after 120 min incubation, $n = 6$), we modified the cylindrical BAL, originally designed to contain hepatocytes, in order to accommodate LMOs on its BC.

The cylindrical shaped BAL for LMOs is shown in Figure 2B. The hollow fiber cartridge configuration is maintained: blood circulates inside the fibers and LMOs are placed in the compartment delimited between the plastic receptacle and the fiber outer surfaces, but this BC has a larger capacity (50 cm^3 *vs* 9 cm^3 in the previous model). Also, a bigger loading port (Lp in Figure 2B) was assembled to the plastic cartridge. Both, Lp and BC capacity are larger than in the original BAL[4] to allow easy LMO loading and retrieval, and to accommodate the biological material, respectively. In both designs, approximately 140 hollow fibers are aligned inside the cartridge connected to two Y-shaped connectors.

We studied the functioning of the device and the perfusion system in experiments without a biological component. The values obtained for the different parameters assayed can be seen in Table 3 ($n = 6$ independent runs). Hematocrit values and osmolality ratios remained stable during the 120 min of perfusion and, though there was an increment in the percentage of hemolysis, it was minimal. Ammonia and glucose could readily cross the fiber walls because their concentrations in both compartments evened out after 120 min. Total ammonia mass (Q_{NH4+}) did not change during experiments.

Cylindrical shaped BAL: performance with LMOs as a biological component

After determining that the BAL was functioning correctly without a biological component, the modified device was tested with LMOs to establish its capacity to keep these tissue slices functional and viable for 120 min of perfusion.

As a viability parameter, we assayed the release of the cytosolic enzyme LDH. The values measured for LMOs in the BAL prototype are shown in Table 4 ($n = 6$ independent LMO preparations in separate runs), compared to the values obtained in the NRS[5] ($n = 6$ different LMO preparations). In our BAL, the amount of LDH release increased with perfusion time. A similar pattern was observed in the NRS and no difference was detected when both systems were compared, reaching almost the same percentages after 120 min (14.7% ± 3.1% for the BAL prototype and 15.5% ± 3.2% for the NRS).

Respiratory activity determination constitutes a very specific metabolic test to evaluate tissue functionality and notice the existence of hidden damages[26]. LMOs were taken out at different times and put into an oxygen chamber to test their oxygen consumption capacity (Table 4, $n = 6$ LMOs taken at each time,

Table 3 Functional parameters of the cylindrical shaped bio-artificial liver[1] (*n* = 6 independent runs)

Perfusion time	Osm_B/Osm_{BC}	Ht (%)	Hemolysis (%)	$[Glucose]_B/[Glucose]_{BC}$	$[NH_4^+]_B/[NH_4^+]_{BC}$	Q_{NH4+} (μmol)
0 min	0.95 ± 0.05	43 ± 3	0.12 ± 0.06	0.12 ± 0.01	23.2 ± 1.2	31.1 ± 3.9
60 min	0.99 ± 0.01	42 ± 6	0.30 ± 0.08	0.81 ± 0.03	1.4 ± 0.4	31.1 ± 4.2
120 min	1.00 ± 0.01	38 ± 2	0.65 ± 0.10	0.94 ± 0.02	1.2 ± 0.2	31.3 ± 3.8

[1]The cylindrical bio-artificial liver designed for liver microorgans functioning without any biological component. B: Blood; BC: Biological compartment; Ht: Hematocrit.

Table 4 Viability and functional parameters evaluated for liver microorgans in the cylindrical shaped bio-artificial liver and the Normothermic Reoxygenation System (*n* = 6 different liver microorgan preparations in independent bio-artificial liver runs/Normothermic Reoxygenation System incubations)

Evaluated parameters		LDH release (%)			Oxygen Consumption (μmol/min·g wet tissue)		
Time (min)		0	60	120	0	60	120
Model	Cylindrical BAL	1.9 ± 0.9	9.4 ± 3.0[a]	14.7 ± 3.1[a]	1.21 ± 0.24	1.15 ± 0.20	1.16 ± 0.21
	NRS	2.6 ± 0.1	8.3 ± 1.2[a]	15.5 ± 3.2[a]	1.13 ± 0.11	0.85 ± 0.15[c]	0.84 ± 0.15[c]

[a]$P < 0.05$, different from other times; [c]$P < 0.05$, different from time 0. BAL: Bio-artificial liver; NRS: Normothermic Reoxygenation System; LDH: Lactate dehydrogenase.

Table 5 Relative gene expression of *Cps1* and *Otc* for liver microorgans in the cylindrical shaped bio-artificial liver and the Normothermic Reoxygenation System (*n* = 3 different liver microorgan preparations in independent bio-artificial liver runs/Normothermic Reoxygenation System incubations)

Relative gene expression		*Cps1*			*Otc*		
Time (min)		0	60	120	0	60	120
Model	Cylindrical BAL	1.05 ± 0.17	0.84 ± 0.09	0.63 ± 0.12	1.02 ± 0.26	0.87 ± 0.21	0.67 ± 0.20
	NRS	1.00 ± 0.24	0.96 ± 0.12	0.86 ± 0.10	1.00 ± 0.20	0.87 ± 0.16	0.82 ± 0.07

BAL: Bio-artificial liver; NRS: Normothermic Reoxygenation System; *Cps1*: Carbamyl phosphate synthetase Ⅰ; *Otc*: Ornithine transcarbamylase.

Table 6 Carbamyl phosphate synthetase Ⅰ and ornithine transcarbamylase enzymatic activity in the cylindrical shaped bio-artificial liver and the Normothermic Reoxygenation System (*n* = 3 different liver microorgan preparations in independent bio-artificial liver runs/Normothermic Reoxygenation System incubations)

Enzymatic activity		CPSI (U/g wet tissue)			OTC (U/g wet tissue)		
Time (min)		0	60	120	0	60	120
Model	Cylindrical BAL	2.48 ± 0.62	2.72 ± 0.61	3.03 ± 0.86	241.7 ± 12.6	235.1 ± 11.9	222.0 ± 23.5
	NRS	2.47 ± 0.60	2.82 ± 0.76	3.12 ± 0.73	209.7 ± 33.2	251.8 ± 29.4	228.8 ± 32.8

CPSI: Carbamyl phosphate synthetase Ⅰ; OTC: Ornithine transcarbamylase; BAL: Bio-artificial liver; NRS: Normothermic Reoxygenation System.

coming from different preparations in independent BAL-runs/NRS-incubations). This parameter remained stable during the 120 min of perfusion in the BAL and again no difference was observed compared to the NRS, even though in this system there was a significant decrease at 60 min and 120 min compared to the initial value.

Any biological component employed in a BAL must face and deal with the high levels of ammonia in the blood of the patients to be treated because the accumulation of this metabolite is associated with the progression of hepatic failure[3]. Therefore, we analyzed different parameters related to ammonia metabolism in LMOs. Relative gene expression and activity of CPSI and OTC, two key enzymes of the urea cycle, were studied and the results obtained are shown in Tables 5 and 6 (*n* = 3 different LMO preparations in independent BAL-runs/NRS-incubations). The levels of mRNA and activity for both enzymes did not significantly change after 120 min of perfusion in the BAL prototype. In addition, there were no significant differences in the levels measured for LMOs in the NRS[5], indicating proper enzyme quantities to perform ammonia detoxification. However, despite all these similarities, when the LMOs were used as the biological component of the cylindrical shaped BAL they were unable to metabolize ammonia (Table 7), while in the NRS they detoxified 22.2% ± 5.5% and 35.1% ± 7.0% of the initial dose after 60 min and 120 min, respectively (*n* = 6 different LMO preparations).

Table 7 Ammonia detoxification capacity (μmol/g wet tissue) for fresh liver microorgans and hepatocyte suspensions used as biological component of the "flat bottom" and cylindrical bio-artificial livers (*n* = 6 different liver microorgan/hepatocyte preparations in independent runs)

Time (min)	Ammonia detoxification capacity (μmol/g wet tissue)		
	Flat bottom BAL	Cylindrical BAL	
	LMOs	LMOs	Hepatocytes
60	8.1 ± 1.2[a]	ND	12.5 ± 1.8
120	13.2 ± 2.2	ND	18.6 ± 4.9

[a]$P < 0.05$, different from hepatocytes at time 60 min. 128×10^6 hepatocytes = 1 g of liver. ND: Not detectable, means that the total ammonia mass remained equivalent to the 100% of initial dose at the indicated times; LMO: Liver microorgan; BAL: Bio-artificial liver.

Cylindrical vs "flat bottom" shaped BAL prototypes: The importance of architecture

We hypothesized that the LMOs were unable to metabolize ammonia in the cylindrical shaped device was not related to the biological component because all the other functionality and viability parameters were similar to the NRS. Therefore, the problem could be due to the artificial part of the device, specifically the BC. To test this hypothesis, we decided to change the BC architecture of our BAL and mimic the configuration and disposition of the LMOs in the NRS (Figure 2A). The result was the design of a BAL prototype with a completely different shaped BC: The "flat bottom" BAL displayed in Figure 2C. Instead of using a cylindrical plastic cartridge, we utilized a 25 cm^2 culture flask that offers a flat base BC where LMOs adopt a similar distribution as the one in the NRS. All the other components and materials utilized were the same in both devices, including the perfusion system. This "flat bottom" BAL performed well in experiments without a biological component[15].

Ammonia detoxification was the key task that LMOs could not perform in the cylindrical shape BAL. When they were evaluated in the "flat bottom" BAL, fresh LMOs were able to metabolize 32.1% ± 2.2% and 49.3% ± 8.8% of the initial NH_4^+ dose at 60 min and 120 min, respectively (n = 6 different LMO preparations in independent runs)[15], results that support the importance of architecture.

According to the results, the devices designed in our laboratory could be successfully incorporated into BAL systems depending on the chosen biological component. In Table 7, we compare both ammonia detoxification capacities between LMOs in the "flat bottom" and cylindrical BAL prototypes as well as with hepatocyte suspensions in our previous cylindrical BAL (n = 6 different LMO/hepatocyte preparations in independent runs). For the purpose of comparison between the different biological components (LMOs *vs* hepatocytes), we performed calculations using the following equivalence: 128×10^6 hepatocytes = 1 g of liver[27-29]. After 60 min of perfusion, LMOs in the "flat bottom" BAL showed a significantly decreased level of ammonia detoxification compared to hepatocytes in the cylindrical model (Table 7). However, after 120 min both models had similar metabolizing capacities. Rat hepatocyte isolation and incubation in the cylindrical BAL were performed as already reported by our group[4] (routine protocol for hepatocyte isolation is shown in Figure S1; and cylindrical BAL operation in Figure S2).

DISCUSSION

The first step before using LMOs in our BAL device was the adaptation of the prototype designed for hepatocytes, and testing it without any biological components. The results obtained (Table 3) demonstrated a proper exchange of fluids between the blood and the BC since the relationship between osmolality and the hematocrit values remained stable during the 120 min of perfusion. Also, it can be appreciated that solute transport functioned properly in both directions: ammonia diffused from blood to the extra-fiber fluid, while glucose flowed the opposite way, almost matching their concentrations during the initial hour (Table 3). We did not detect unspecific loss of ammonia or interaction with any part of the device (Q_{NH4+} remained constant) and the small percentage of hemolysis measured can be attributed to the action of the peristaltic pump. Taken together, the results showed that the cylindrical shaped device made in our laboratory, with its simple design, could be easily adapted and scaled up, which is a very important issue in the construction of a BAL intended for clinical application.

After corroborating the proper functioning of the device, we performed several experiments with LMOs as its biological component. The results of these tests were unexpected: LMOs showed satisfactory viability levels (assayed by LDH release), oxygen consumption capacity did not change during the perfusion, and conserved levels of transcripts and adequate enzymatic activities of CPSI and OTC, but they were not able to metabolize ammonia in a significant amount. Even more astonishing was the fact that all the assayed parameters showed similar values to the ones determined in the NRS[5], where LMOs could detoxify 35.1% ± 7.0% of the initial 1 mmol/L NH_4^+ dose after 120 min.

When adapting the cylindrical BAL model to house LMOs as a biological component, we found that, in spite of the shaking applied, LMOs piled up over each other at the bottom of the cartridge in an array that could prevent the correct exchange of nutrients, oxygen, and metabolites. Furthermore, the experiments in the NRS were accomplished in flat wells in 6-well multi dishes. Then, we came up with the idea of mimicking the NRS configuration, and developed a BAL prototype with a flat surface BC. We reasoned that this geometrical disposition would allow LMOs to adopt a looser, not

stacked distribution that would benefit the exchange mechanisms between LMOs and the bathing media. In this way, provided that the volume of the BC is equivalent to that of the previous prototype (50 cm^3), this flat bottomed device offers a surface that allows an enhanced bathing and shaking of plane LMOs due to their better distribution.

When we tested fresh LMO ammonia detoxification efficiency in this new "flat bottom" BAL, the results obtained supported our hypothesis. They were able to detoxify 49.3% ± 8.8% of the initial ammonia overload after 120 min of perfusion[15], contrary to the LMOs applied to the cylindrical BAL (Table 7). This observation demonstrates the importance of adjusting the architecture and design of the artificial compartment to a given biological component in order to obtain an optimal BAL performance. Although we cannot rule out other factors influencing LMO detoxification activities that remained unnoticed, the only change between the cylindrical and flat bottom BALs was the shape of the BC. The BC volume, shaking speed, blood flow velocity, oxygen tension, and the brand of fibers used in their construction were identical.

Both systems published, *i.e.*, the cylindrical one operating with isolated hepatocytes[4] and the "flat bottom" one operating with LMOs[15], function satisfactorily in relation to ammonia detoxification (Table 7) and, according to the needs of the patient, could be optionally selected in the future. The device could also be used as a suitable mini-bioreactor to analyze drug toxicity or other parameters of interest. We are performing further studies to establish the synthetic capacity of the biological components in our prototypes.

At present no BAL is in use to clinically treat liver failure. Some clinical trials have been conducted (for an updated review consult the work of Sakiyama *et al*[30] and references therein) with some success. All of these devices use isolated liver-derived cells. Four of them use porcine primary hepatocytes either fresh (named LSS, Excorp Medical BLSS and AMC-BAL) or cryopreserved and microcarrier attached (HepatAssit), one uses human primary hepatocytes (MELS), and one uses HepG2/C3A cell line (Vitagen ELAD). These devices have not become mainstream in clinical settings yet because of the complexity of isolating hepatocytes and the difficulties for maintaining the viability of these cells viable for prolonged periods of time. This limits the assembly and transport of the devices and confines its use to the centers where they were developed. An exception to this could be the ELAD system that uses a cell line, but the costs of producing the required amount of cellular material makes it expensive. It is also limited to places with the facilities and expertise to conduct cell culture at a large scale. In this sense our BAL prototype, although it needs further testing, requires hand-cut LMOs, which are obtained by a low-cost technique that is easily learned. Also, the required biological material could be obtained from donor livers not acceptable for transplantation but meeting the criteria to obtain adequate pieces to feed this kind of BAL and possibly used in multiple treatments. Therefore, pre-assembled BALs could be filled with LMOs obtained in the same center where they will be immediately applied. The prototype we are presenting in this work is constructed with standard laboratory and medical supplies, and we envisage that they could be constructed at reasonable costs and, consequently, commercialized at reasonable prices.

After publishing our previous results[15], we became aware that it is difficult to predict the conditions needed for good performance of the devices based on rational design and, instead, much of the experience we have accumulated over the years mostly originated by trial and error. Especially in this field of BAL research, every laboratory seems to apply different strategies to achieve the objective of extracorporeal treatment of liver failure. Although some basic principles and features are followed by all of us, there seems to really exist one different BAL for each group of work[31,32]. As far as we know, this is the first time that a comparison of the same biological component applied to different configurations of BALs is reported. In the literature, we have always found comparisons of different BALs only in review articles that use data from different research groups, which renders the analysis incomplete and inadequate. However, we have made comparisons in the same set of experiments using cells and tissues from the same brood of experimental animals, same batches of solutions, same laboratory instruments, *etc*. These conditions render our results coherent and valid, and strongly demonstrate that architecture of BALs can determine the success of this kind of device.

Further investigations must be done in order to bring these types of devices to the clinic. Very few clinical trials have been performed, and they are typically carried out on patients with advanced deterioration[32-35]. In conclusion, we demonstrate the importance of adapting the artificial component architecture to the biological component characteristics to obtain an adequate BAL performance regarding blood ammonia detoxification.

ARTICLE HIGHLIGHTS

Research background

Liver failure is a condition that usually requires liver transplantation, but in some cases acute liver failure resolves spontaneously due to the viable hepatic mass remaining after the cause of the damage has disappeared. If the amount of this functional tissue has the sufficient capacity to handle the detoxification of harmful metabolites produced by the insult and to provide the needed essential hepatic molecules and factors, then the regeneration capacity of the organ allows the recovery. This is why many attempts have been pursued to help the patient´s liver to pass through this acute failure and either recover or extend the time frame for a liver transplantation. Artificial livers, either dialysis based or incorporating hepatic cells and tissues (these later referred to as bio-

artificial livers or BALs), are extracorporeal devices intended to aid the failing livers to overcome failure or at least to permit the patient to improve to undergo transplantation. In this sense, BALs are considered the choice to accomplish this job but until now they have been applied only by medical care teams that are able to obtain the biological component and to assemble the device at the same location making the practice limited to very few centers in the world.

Research motivation

The BAL research field is several decades old but still no successful device has been developed. Several prototypes have been submitted to clinical trials but none are routinely used in clinical settings or commercially available. These prototypes use isolated cells of hepatic origin (isolated primary human or pig hepatocytes and HepG2/C3A cell line), which is a biological material that requires expertise and money to be obtained and/or maintained. Additionally, cryopreservation of primary hepatocytes is not a very successful technique and recovery after thawing is poor. Among other researchers in the field, we propose and are testing the use of liver microorgans (LMOs) as the biological component for BAL devices, which are promising in terms of bearing all hepatic cellular types and microarchitecture and involve a simple method to obtain. These characteristics are appealing because they bring the possibility of using procured organs not suitable for transplantation to get the material necessary to feed pre-assembled cartridges at the same centers were the BAL would be needed. Our experience in the field has taught us that changes in the artificial part of these devices can have an impact on the biological component function. The finding that these changes in design, that can be minor or significant, have an influence on performance with effects ranging from subtle to massive, address the importance of finely tuning the interplay between the components of the device to optimize BAL operation.

Research objectives

LMOs failed to detoxify ammonia in a scaled-up BAL configuration that was previously successful. We set out to solve this problem and analyze the possible reasons of the phenomenon we were observing.

Research methods

The methodology used is the standard in our laboratory and has been previously published. The novelty is to perform and report the comparison of different BAL designs using the same biological component. This is the first report making such a comparison in the same set of experiments using cells and tissues from the same brood of experimental animals, same batches of solutions, same laboratory instruments, same materials to construct the devices, and so forth.

Research results

The main result we achieved in this work is that LMOs were totally incompetent to detoxify ammonia when placed in a cylindrical shaped BAL while they were fully able to detoxify ammonia inside a flat bottomed BAL.

Research conclusions

The accumulation of high levels of ammonia in the blood is an important issue in patients presenting liver failure, and is of the highest interest when studying this kind of device. In the literature, we have always found comparisons of different BALs only in review articles that use data from different research groups, which renders the comparative analysis incomplete. Our experimental design makes our comparison coherent and valid, and strongly demonstrates that the architecture of BALs can determine the success of this kind of device.

Research perspectives

We consider that this is an especially important finding, particularly in the light of the results presented, compelling future research to put an effort to finely tune the interplay between the artificial and biological components of BALs in order to achieve optimal performance and finally reach the clinical setting.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Spleen stiffness mirrors changes in portal hypertension after successful interferon-free therapy in chronic-hepatitis C virus patients

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Abstract

AIM

To investigate changes in spleen stiffness measurements (SSMs) and other non-invasive tests (NITs) after treatment with direct-acting antivirals (DAAs) and identify predictors of SSM change after sustained

virological response (SVR).

METHODS

We retrospectively analysed 146 advanced-chronic liver disease (ACLD) patients treated with DAA with available paired SSM at baseline and SVR24. Liver stiffness (LSM), spleen diameter (SD), platelet count (PLT) and liver stiffness-spleen diameter to platelet ratio score(LSPS) were also investigated. LSM $\geqslant$ 21 kPa was used as a cut-off to rule-in clinically significant portal hypertension (CSPH). SSM reduction > 20% from baseline was defined as significant.

RESULTS

SSM significantly decreased at SVR24, in both patients with and without CSPH; in 44.8% of cases, SSM reduction was > 20%. LSPS significantly improved in the entire cohort at SVR24; SD and PLT changed significantly only in patients without CSPH. LSM significantly decreased in 65.7% of patients and also in 2/3 patients in whom SSM did not decrease. The independent predictor of decreased SSM was median relative change of LSM. CSPH persisted in 54.4% patients after SVR. Delta LSM and baseline SSM were independent factors associated with CSPH persistence.

CONCLUSION

SSM and other NITs significantly decrease after SVR, although differently according to the patient's clinical condition. SSM faithfully reflects changes in portal hypertension and could represent a useful NIT for the follow-up of these patients.

Key words: Clinically significant portal hypertension; Spleen stiffness measurement; Advanced chronic liver disease; Direct-acting antivirals; Portal hypertension; Hepatitis C; Non-invasive test

Core tip: Liver stiffness measurement (LSM) and spleen stiffness measurement (SSM) are widely validated surrogates of portal hypertension (PH) and its complications. Their role in the assessment of therapy response, such as treatment with direct-acting antivirals (DAAs) of hepatitis C virus patients, is still under investigation. We demonstrated in a large cohort that not only LSM, but also SSM, is reduced six months after successful DAA therapy. As opposed to LSM, SSM directly reflects PH and is less influenced by the immediate reduction of liver necro-inflammation. We believe that SSM could represent a helpful tool for the clinician in the follow-up of these patients.

Ravaioli F, Colecchia A, Dajti E, Marasco G, Alemanni LV, Tamè M, Azzaroli F, Brillanti S, Mazzella G, Festi D. Spleen stiffness mirrors changes in portal hypertension after successful interferon-free therapy in chronic-hepatitis C virus patients. *World J Hepatol* 2018; 10(10): 731-742 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i10/731.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i10.731

INTRODUCTION

Chronic hepatitis C virus (HCV) infection represents one of the major causes of liver disease and is a leading cause of liver transplantation[1,2]. Recently, the introduction of the highly effective interferon-free direct-acting antivirals (DAAs) has enormously increased the number of patients who have achieved sustained virological response (SVR), even in patients with liver cirrhosis[3–5].

Studies, mostly from the interferon era, have shown that achieving SVR improves liver function[6,7], liver histology[8] and overall clinical outcomes[9]. However, the real impact of SVR in the DAA era, in terms of changes in portal hypertension (PH) and risk of decompensation on immediate follow-up, is not completely known, especially in patients with advanced chronic liver diseases (ACLD). PH is a progressive condition that represents a key point in the natural history of liver diseases[10]; therefore, its assessment by the hepatic venous pressure gradient (HVPG) measurement is fundamental in ACLD patients[11–14]. Indeed, the development of clinically significant portal hypertension (CSPH) in patients with compensated ACLD (cACLD)[11] is highly associated with the risk of clinical decompensation events (ascites, variceal bleeding, jaundice and hepatic encephalopathy)[10].

To date, several studies have demonstrated a significant reduction in HVPG (> 10%-20%) after achieving SVR, both after interferon-based[15–17] and DAA-based regimens[18–21]. Although the HVPG measurement is the gold standard to assess PH[11], it remains an invasive method[22] and its use is still limited only to highly specialized centres[12]; thus, its repeated measurements during the follow-up would hardly be applicable.

Consequently, many non-invasive tests (NITs) in the last decade, including liver and spleen stiffness measurements (LSM and SSM) as well as liver stiffness-spleen diameter to platelet ratio score (LSPS), have been developed and validated to accurately assess PH degree and its complications[11,22–29]. In fact, the Baveno Ⅵ Consensus recently recommended that LSM values of 10 kPa should rule out cACLD patients, and values of 20-25 kPa should accurately identify CSPH in patients with viral hepatitis[11]. However, to date, few studies have evaluated the role of NITs in the PH assessment of SVR patients after DAA treatment, and their role in the follow-up. Even if most studies agree on the fact that LSM rapidly decreases after virus eradication[18,19,30–32], controversial data have emerged regarding the changes of SSM after SVR[30–32].

MATERIALS AND METHODS

Aims of the study

We aimed to: (1) investigate the possible effect of HCV-

DAA treatment on PH, evaluated by spleen stiffness changes as a mirror of PH; (2) as well as those of other NITs, after HCV-DAA treatment; moreover, we aimed to (3) identify the presence of predictors of the SSM changes in SVR patients after DAA therapy.

Study design and population

This is a retrospective analysis of prospectively collected data of HCV-related cACLD patients treated with DAAs between January 2015 and September 2017 at our department, with valid measurements of LSM and SSM by transient elastography (TE) at baseline (BL) and at six mo after the end of DAA treatment (SVR24).

According to the Baveno Ⅵ Criteria[11], values of LSM > 10 kPa at TE were considered suggestive of having cACLD; LSM cut-off ⩾ 21 kPa was used to rule-in CSPH, as previously described[33,34]. At baseline, laboratory values, Model for end-stage liver disease (MELD) and Child-Turcotte Pugh (CTP) scores were also reported for each patient.

We excluded patients who (1) had incomplete response to surgical resection or loco-regional ablation of previous HCC; (2) developed HCC during antiviral treatment; (3) developed variceal bleeding and/or endoscopic banding legation (EBL) during the study period; and (4) initiated or changed the dosage of non-selective beta-blockers (NSBB) or had portal vein thrombosis, transjugular intrahepatic portosystemic shunt (TIPS) and non-cirrhotic PH. A subgroup of the patients who did not achieve SVR were separately investigated.

Antiviral treatment

Eligibility for treatment of HCV patients with DAAs was assessed following the priority criteria established in the protocol approved by the Italian Medicines Agency committee. The choice of DAA and treatment duration (12 or 24 wk) was based on viral genotype and stage of disease, according to the guidelines available at the time of enrollment[35]. SVR was defined as undetectable HCV-RNA using real-time PCR, with a detection limit of 15 IU/mL at the 12-wk post-treatment follow-up visit.

NITs for PH assessments

LSM values were assessed by expert operators using the FibroScan® apparatus with "M" probe (Echosens®, Paris, France) after overnight fasting and after a complete abdominal US examination. LSM values were obtained as previously reported[16], and the reliability criteria considered were according to the last EFSUMB Guidelines and Recommendations on the Clinical Use of Ultrasound Elastography[36]. SSM was assessed on the same day as LSM assessment, with the same probe utilized to perform LSM using the FibroScan® apparatus, as previously described[24]. Since no specific literature is present, we translated data from HVPG experience[11] and defined significant SSM as a reduction > 20% from BL. LSPS was calculated as liver stiffness × (spleen diameter (SD)/platelet count)[37]. SD was considered to be the bipolar diameter of the spleen as assessed by ultrasound.

Statistical analysis

Categorical data are expressed as numbers (percentages) and continuous variables as medians (IQR or range). For group comparison, the Mann-Whitney *U* test was used for continuous variables and the chi^2 test for categorical variables. Group comparisons among NITs at BL and SVR24 were evaluated with Friedman's non-parametric test, and Bonferroni-corrected alphas were used for post hoc pairwise comparison. Demographic, clinical, functional and elastometric variables were evaluated with univariate and multivariate Logistic Regression models in order to assess the predictive factors associated with PH improvement as assessed by SSM. After evaluation of multicollinearity, variables with a *P*-value < 0.10 upon univariate analysis were included in several multivariate Logistic Regression models with stepwise backward procedures. Prevalence of esophageal varices (EV) was not included in the multivariate analysis due to the limited number of patients with available EGD data (within 6 mo from TE assessment). The estimated odds ratios with their 95% confidence intervals, LR chi^2 and Area under ROC Curve were presented. For each multivariable logistic regression, the model discrimination and calibration were reported together with Akaike information criterion and Bayesian information criterion measures for comparing maximum likelihood models. Only *P*-values < 0.05 were considered statistically significant. The statistical analysis was conducted using Stata/SE (Version 14.0; Stata Corp, Texas, United States).

Ethics

The DAAs treatment protocol was approved by the National Institutional Review Board (IRB) of the Italian Medicines Agency committee. Local IRB [Institutional Ethics Committee of Sant'Orsola-Malpighi University Hospital (Bologna, Italy)] approval was authorized.

RESULTS

Patients characteristics

One hundred-ninety-seven cACLD patients treated with DAAs and with available valid baseline LS and SS measurements were evaluated. The following patients were excluded: two (1%) had HCC occurrence and three (1.5%) presented with active HCC, one (0.5%) underwent EBL during the study period, four (2%) had previous EBL, two (1%) patients presented with complete portal vein thrombosis, one (0.5%) required an increase in NSBB dosage and one (0.5%) had previous TIPS placement. An additional 37 (18.8%) patients were excluded: 22 (out of 197, 11.2%) due to lack of follow-up and 15 (out of 197, 7.6%) due to unfeasible SSM at follow-up. Accordingly, a total of 134

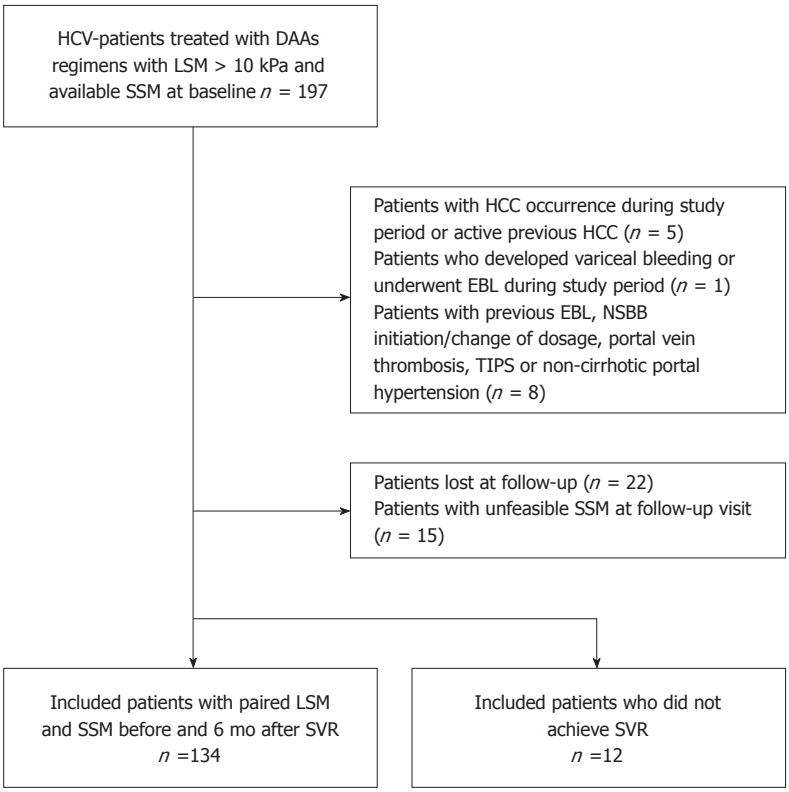


Figure 1 Flowchart of study design. DAA: Direct-acting antiviral; EBL: Endoscopic band ligation; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; LSM: Liver stiffness measurement; NSBB: Non-selective beta-blocker; SSM: Spleen stiffness measurement; SVR: Sustained virological response; TIPS: Transjugular intrahepatic portosystemic shunt.

patients with paired LSM and SSM at BL and SVR24 were included in the final analysis; 12 (6%) patients who did not achieve SVR were analysed separately (Figure 1).

Table 1 depicts the baseline characteristics of the study cohort. Regarding main NITs, the median values at BL were LSM 19.3 kPa (14.1-27 kPa) and SSM 58.8 kPa (42.2-75 kPa). In a sub-analysis, patients with CSPH (LSM $\geqslant$ 21 kPa) differed significantly for MELD score, platelet count, total serum bilirubin, INR, SSM, LSM, SSM and LSPS.

Changes in SSM and LSM after SVR

In the patients who achieved SVR, the median SSM significantly decreased from 58.8 kPa to 38.2 kPa (P = 0.001), with a median delta change in SSM of − 12.3%. The decrease in SSM was statistically significant in both groups, CSPH and not (Figure 2A); the median delta SSM was higher in patients without CSPH at baseline when compared to patients with CSPH (-20.4% *vs* -4.7%), although this difference did not reach statistical significance. A decrease in SSM values was found in 92 (68.7%) patients, of whom 73 (54.5%) had a decrease > 10% and 60 (44.8%) > 20% (Table 2 and Figure 3A). LSM values also decreased after SVR, with respective median values of 19.3 kPa and 13.8 kPa at baseline and SVR24 (P < 0.0001). The median delta LSM was -30% with similar changes in both groups; LSM decreased in 114 (85.1%) patients, of whom 88 (65.7%) had a decrease of > 20% (Table 2 and Figure 3A).

A LSM decrease was found in almost all patients in whom SSM decreased (95.3%). On the other hand, LSM significantly decreased (P = 0.022) in 2/3 of the patients in whom SSM did not decrease, with a median delta LSM of -28.3%. (Figure 3B).

Changes in other NITs after SVR

The median spleen diameter (SD) at baseline and SVR24 were 14 cm and 13.2 cm, respectively. Although the reduction was not statistically significant in the overall population, it reached significance in the subgroup of patients without CSPH. The increase of PLT (from 110 10^9/L to 130 10^9/L) did not reach statistical significance in the entire cohort either, but only in patients without CSPH (Figure 2B). Moreover, median LSPS differed significantly between baseline (2.78) and SVR (1.34), and in both subgroups as well.

Non-SVR patients

Twelve patients did not achieve SVR in our cohort. Baseline characteristics did not statistically differ from the patients included in the final analysis. In particular, in non-SVR patients, an LSM decrease (23.2 kPa at BL *vs* 21.6 kPa at FU24), an SSM increase (45.6 kPa at BL *vs* 57.8 kPa at FU24) and a PLT decrease (128 ×

Table 1 Baseline characteristics of included patients

Variable	Overall (n = 134)	CSPH (LSM ⩾ 21 kPa) (n = 60)	No CSPH (LSM < 21 kPa) (n = 74)
Age (yr)	60 (51-69)	57 (50.5-65)	61.5 (51-70)
Male	92 (68.7)	42 (70)	50 (67.6)
HCV-genotype			
1	95 (70.9)	41 (68.3)	54 (72.5)
2	12 (8.9)	4 (6.7)	8 (10.8)
3	20 (14.9)	11 (18.3)	9 (12.2)
4	7 (5.3)	4 (6.7)	3 (4.5)
Treatment regimen			
SOF/RBV	33 (24.6)	10 (16.7)	23 (31.1)
SOF/SMV	29 (21.6)	15 (25)	14 (18.9)
SOF/DCV	38 (28.4)	19 (31.6)	19 (25.6)
SOF/LDV	16 (12)	7 (11.7)	9 (12.2)
Other	18 (13.4)	9 (15)	9 (12.2)
Child Pugh Score			
A	115 (85.8)	52 (86.7)	63 (85.1)
B	19 (14.2)	8 (13.3)	11 (14.9)
MELD Score	8 (7-10)	9 (8-10)	8 (7-10)
Spleen Diameter (cm)	14 (12.3-15.5)	14.7 (12.8-15.8)	13.9 (12.1-15)
Laboratory results			
Platelets (cells × 10^9/L)	110 (79-150)	102 (74-132)	134 (92-159)
ALT (U/L)	58 (39-95)	55 (39-84)	60 (38-105)
Bilirubin (mg/dL)	0.9 (0.67-1.29)	1 (0.84-1.52)	0.8 (0.6-1.1)
Albumin (g/dL)	3.8 (3.6-4.1)	3.8 (3.5-4.1)	3.8 (3.6-4.1)
Creatinine (mg/dL)	0.8 (0.7-0.98)	0.8 (0.70-0.96)	0.85 (0.71-1.08)
INR	1.1 (1.06-1.2)	1.17 (1.1-1.21)	1.08 (1.04-1.13)
NITs			
SSM (kPa)	58.8 (42.2-75)	69.9 (55.7-75)	46.2 (31.6-63.9)
LSM (kPa)	19.3 (14.1-27)	29.1 (23.9-39.7)	14.6 (12-17)
LSPS	2.78 (1.4-4.94)	5.1 (3.05-7.48)	1.58 (1.09-2.79)

Qualitative data were expressed as number and percentage (%); quantitative data were expressed as median (25%-75% quantiles). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CSPH: Clinically significant portal hypertension; DCV: Daclatasvir; HRV: High risk varices; INR: International normalized ratio; LDV: Ledipasvir; LSM: Liver stiffness measurement; LSPS: Liver stiffness to spleen/platelet score; MELD: Model for end-stage liver disease; NITs: Non-invasive tests; RBV: Ribavirin; SMV: Simeprevir; SOF: Sofosbuvir; SVR: Sustained virological response; SSM: Spleen stiffness measurement.

10^9/L at BL *vs* 100 × 10^9/L at FU24) were observed; none of these changes reached statistical significance (Supplementary Table 1).

Predictors of significant SSM Decrease (> 20%)

Table 3 shows the differences observed between patients who had an SSM decrease > 20% and those who did not. In the entire cohort, patients with significant SSM reduction differed in the prevalence of EV, MELD score, albumin levels, as well as baseline SSM, LSPS values and LSM-related variables. In multivariate analysis, relative LSM changes remained as the only independent predictor of an SSM decrease > 20%. Furthermore, predictors of an SSM decrease > 20% (Supplementary Table 2) were investigated among patients with CSPH at baseline. Once again, a higher prevalence of EV, higher creatinine levels, lower LSM values at SVR24 and higher delta LSM were observed among patients with an SSM decrease > 20%. In multivariate analysis, higher serum creatinine levels and delta LSM > 20% were the predictors of a significant SSM decrease.

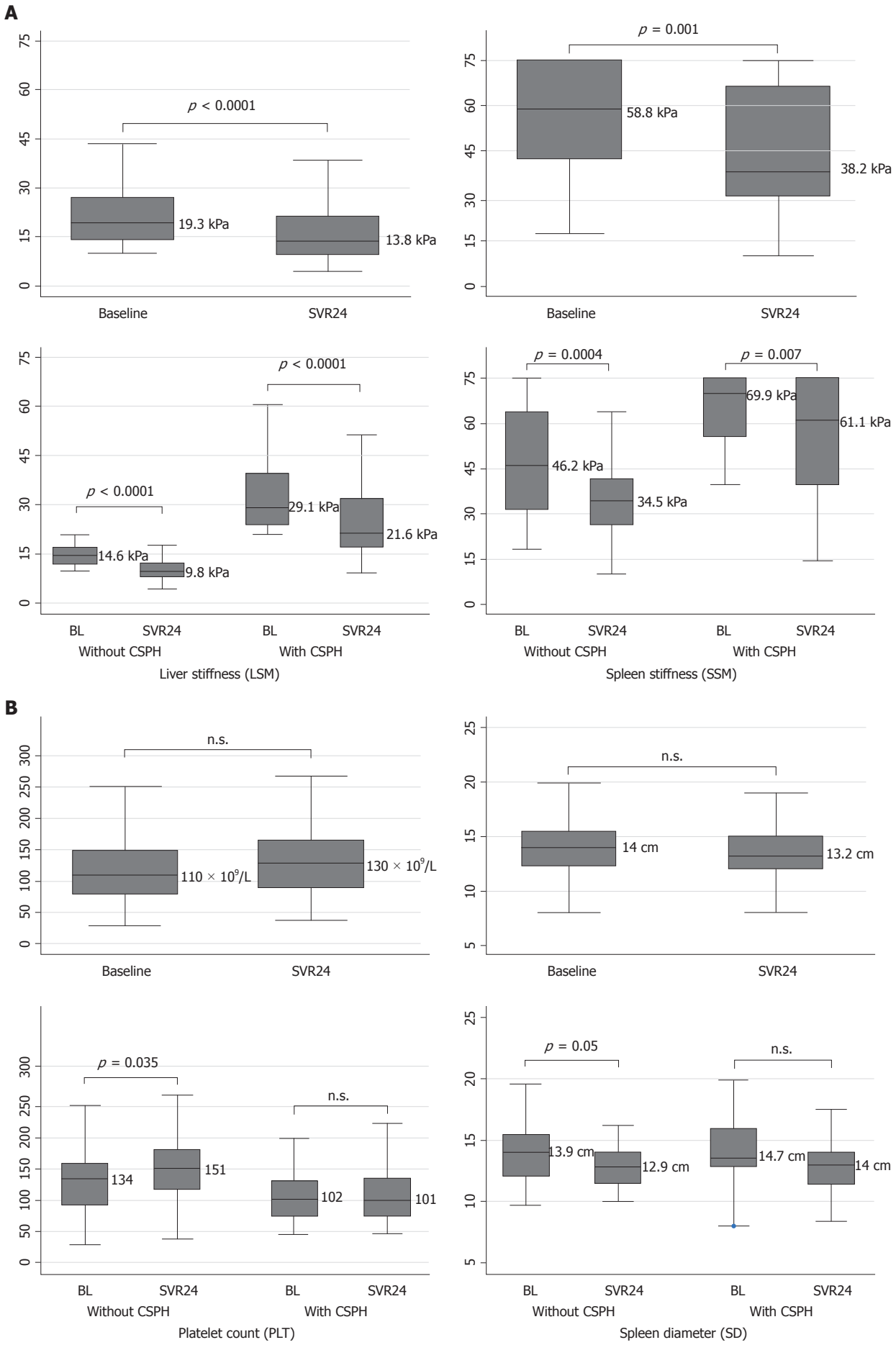
Changes of CSPH state after SVR

Figure 4 shows that 60 (44.8%) patients presented with CSPH at baseline, defined as an LSM ⩾ 21 kPa. After a 6 mo follow-up, none of the 74 patients without CSPH at baseline progressed to CSPH. In patients with CSPH, 46.7% of them had reduced LSM under the CSPH threshold after treatment. Supplementary Table 3 shows the predictors of CSPH persistence after DAA treatment.

DISCUSSION

The main aim of our study was to evaluate PH changes assessed by non-invasive methods after successful viral eradication in patients treated with DAAs. Our data show that SSM and LSM significantly decrease after SVR, according to the baseline clinical patient condition.

The IFN-free regimens are highly effective, allowing to treat and achieve SVR in patients who also have ACLD[4,38]. However, the individual clinical benefit in these patients is still under debate, especially in terms of changes in PH and CSPH-driven complications[39–41]. While results from the interferon era might not necessarily be translatable to DAA regimens[21], recent studies have also unanimously demonstrated that HVPG significantly decreases after SVR[18–21]. Although many


A
p < 0.0001
19.3 kPa
13.8 kPa
Baseline
SVR24
p = 0.001
58.8 kPa
38.2 kPa
Baseline
SVR24
p < 0.0001
p < 0.0001
14.6 kPa
9.8 kPa
29.1 kPa
21.6 kPa
BL
SVR24
BL
SVR24
Without CSPH
With CSPH
Liver stiffness (LSM)
p = 0.0004
p = 0.007
46.2 kPa
34.5 kPa
69.9 kPa
61.1 kPa
BL
SVR24
BL
SVR24
Without CSPH
With CSPH
Spleen stiffness (SSM)
B
n.s.
110 × 10⁹/L
130 × 10⁹/L
Baseline
SVR24
n.s.
14 cm
13.2 cm
Baseline
SVR24
p = 0.035
n.s.
134
151
102
101
BL
SVR24
BL
SVR24
Without CSPH
With CSPH
Platelet count (PLT)
p = 0.05
n.s.
13.9 cm
12.9 cm
14.7 cm
14 cm
BL
SVR24
BL
SVR24
Without CSPH
With CSPH
Spleen diameter (SD)

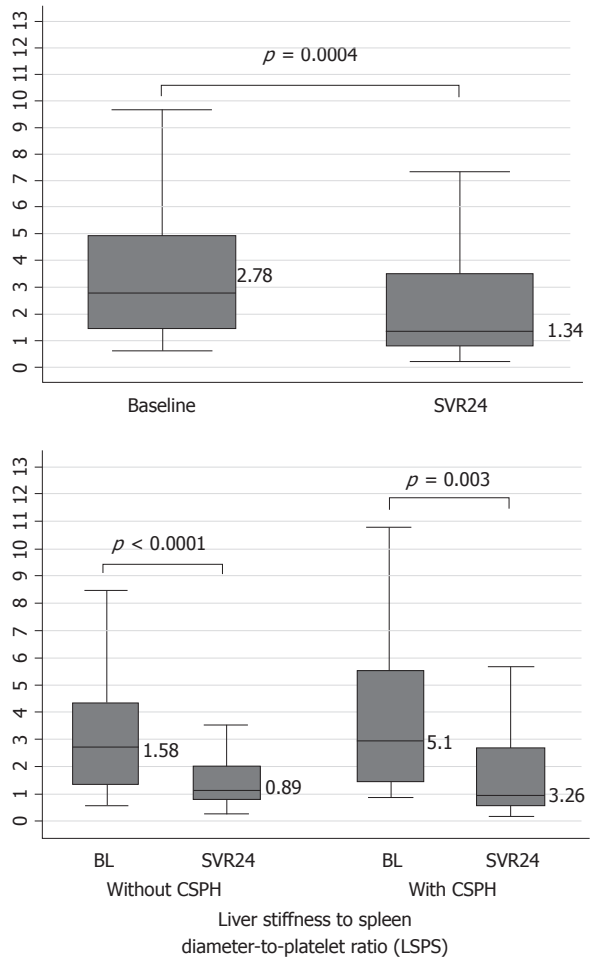


Figure 2 Non-invasive tests changes after sustained viral response by clinically significant portal hypertension presence. A: LSM and SSM changes; B: PLT, SD, LSPS changes. CSPH: Clinically significant portal hypertension; LSM: Liver stiffness measurement; SSM: Spleen stiffness measurement; PLT: Platelet count; SD: Spleen diameter; LSPS: Liver stiffness-to-spleen diameter-to-platelet count ratio score.

studies have shown that LSM rapidly decreases after DAA treatment[42,43], not much is known about the changes of PH surrogate NITs, such as SSM and LSPS, after viral eradication. In fact, NITs have yet to be validated in SVR patients, and their role in the clinical follow-up is still to be determined.

The main finding of this study is that SSM significantly changes after 24 wk of SVR in patients with cACLD, with a median relative change of -12.3% (Table 2). To our knowledge, only two complete papers[30,32] and one letter to the editor[39] have investigated the changes in SSM after SVR, with opposing results. In fact, only in the study by Pons *et al*[32] SSM was found to rapidly decrease at only 4 wk after therapy initiation in 41 patients, with no ulterior significant changes until 48 wk of follow-up; the other studies concluded that SSM did not significantly decrease at SVR24[30,32].

In our study that analyzed a large cohort of cACLD patients, we demonstrated that SSM significantly decreased after DAA treatment. These results confirm previous studies in which PH was assessed by paired HVPG measurements[18-21]. Moreover, our study is the first to assess and demonstrate the improvement of LSPS, another accurate surrogate of PH, after SVR24. Moreover, in the eight patients who did not achieve SVR, SSM and other NITs did not significantly differ during follow-up measurements (Supplementary Table 1).

We classified patients with and without CSPH according to a LSM cut-off of 21 kPa[33,34]. Interestingly, the relative changes in SSM and LSM performed differently in patients with and without CSPH. In fact, while the median delta LSM in patients with and without CSPH was very similar (-28.3% *vs* -30.8%), the reduction of SSM was much more evident in patients without CSPH (-20.4% *vs* -4.7%). This last result is consistent with the relative HVPG changes described by Mandorfer *et al*[18]. Moreover, the other surrogates of PH, including the platelet and spleen diameter**,** significantly changed only when split by CSPH presence. Regarding the different changes of NITs in patients with and without CSPH, we could speculate that this behaviour can reflect the different stages of underlying PH pathogenic mechanisms. Indeed, determinants of portal pressure affecting SSM, such as intrahepatic resistance and liver necro-inflammation[44], improve in both subgroups. However, in CSPH, other major actors of PH, such as

Table 2 Liver and Spleen stiffness measurement decreases after sustained viral response

Variable	Overall (n = 134)	CSPH (LSM ⩾ 21 kPa) (n = 60)	No CSPH (LSM < 21 kPa) (n = 74)
Relative SSM decrease (%)	12.3 (0-36.3)	4.7 (0-32.5)	20.4 (0-39.7)
Overall SSM decrease	92 (68.7)	40 (66.7)	52 (70.3)
> 10%	73 (54.5)	31 (51.7)	42 (56.8)
> 20%	60 (44.8)	23 (38.3)	37 (50)
Relative LSM decrease (%)	30 (13.5-42.4)	28.3 (11.4-41.9)	30.8 (13.9-42.4)
Overall LSM decrease	114 (85.1)	51 (85)	63 (85.1)
> 10%	108 (80.6)	48 (80)	60 (81.1)
> 20%	88 (65.7)	40 (66.7)	48 (64.9)
PLT Increase (%)	12.4 (-10.1 to 29.6)	5.5 (-15.6 to 25.9)	17.4 (-0.67 to 35.6)

CSPH: Clinically significant portal hypertension; LSM: Liver stiffness measurement; PLT: Platelet count; SSM: Spleen stiffness measurement.

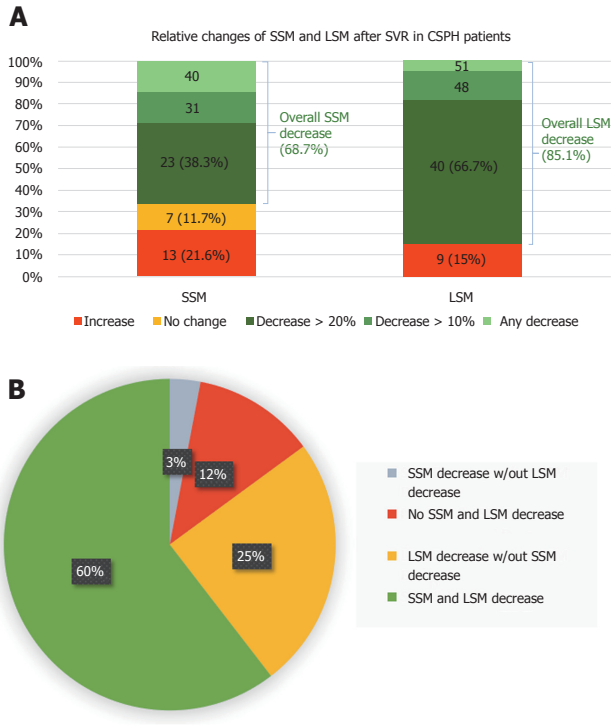


Figure 3 Spleen and liver stiffness measurement decreases after sustained viral response (A), and liver stiffness measurement decreases in patients without spleen stiffness measurement improvements (B). BL: Baseline; CSPH: Clinically significant portal hypertension; LSM: Liver stiffness measurement; SSM: Spleen stiffness measurement; SVR: Sustained virological response.

extra-hepatic hemodynamic factors[34] and spleen structural changes[45], might not ameliorate in the short-term follow-up (6 mo after SVR). This hypothesis could explain why we found a less prominent SSM decrease (-4.7% *vs* -20.4%), even when liver necro-inflammation reduction as assessed by delta LSM (-28.3% *vs* -30.8%) was the same.

SSM reduction was present in 68.7% of patients after 6 mo of follow-up. We found that the only independent predictor of a significant PH improvement, as reflected by a SSM decrease > 20%, was the relative change in LSM (Table 3), confirming previous studies with HVPG[18,21]. However, when we assessed PH improvement as reflected by SSM in our study, as PH surrogate, and by HVPG in the study by Lens *et al*[21], we noticed similar proportions of patients with a significant response (> 20%) when comparing SSM and HVPG (38.3% *vs* 39.8%, respectively), but not LSM and HVPG (66.7% *vs* 39.8%, respectively) (Figure 3A). Even if a correlation between HVPG and SSM changes after DAA treatment has not been demonstrated to date, our data may suggest that an SSM reduction > 20% could be a more accurate non-invasive predictor of a significant HVPG reduction[11].

A statement in the Baveno VI consensus was that the main therapeutic goal in patients with mild PH (6-9 mmHg) is to prevent CSPH development[11]. In our cohort, none of the patients who achieved SVR progressed to CSPH. More challenging, however, is the concept of assessment of CSPH presence/absence after SVR due to its clinical implications, since there is not sufficient evidence showing that the cut-offs after DAAs are the same as the ones used in the pre-treatment phase[23,46]. However, promising data documented that a LSM of 20-25 kPa could be an accurate cut-off to rule-in CSPH after DAA therapy[21]. Accordingly, we also investigated CSPH persistence after SVR (Figure 4). Using these cut-offs, we found that 53% of the patients with CSPH at baseline presented CSPH at SVR24. In multivariate analysis, higher baseline values of SSM (indicating a more severe PH) and lower LSM relative changes were found to be predictors of CSPH persistence (Supplementary Table 3). These results are in line with another study[21] in which higher BL HVPG and relative LSM changes were predictors of CSPH persistence after DAA treatment.

All of the above results seem to reflect the different dynamics in LSM and SSM changes after achieving SVR. LSM consensually decreased in almost all patients with SSM reduction (95.2%), while the opposite was not found to be true. In fact, LSM significantly decreased, with a median delta -28.3%, in 2/3 of the patients in whom no SSM reduction was found. This result emphasizes the fact that LSM is heavily influenced by the reduction of liver necro-inflammation[44] after SVR, and that changes in LSM might not be the most adequate predictors of PH changes in this context. On the other hand, a SSM decrease > 20% could identify patients who significantly clinically benefit from viral eradication.

Table 3 Univariate and multivariate analysis of factors associated with a SSM decrease > 20%

Variable	Entire Population (*n* = 134)					
	Univariate analysis				Multivariate analysis	
					LR Chi-2 = 16.48 AUROC = 0.6821	AIC = 171.8 BIC = 177.6
	SSM Decrease > 20% (*n* = 60)	No SSM Decrease > 20% (*n* = 74)	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age (yr)	62 (52-69)	56 (50-68)	1.005 (0.975-1.037)	0.727		
Sex (male)	21 (28.4)	21 (35)	1.359 (0.653-2.828)	0.412		
Presence of varices (*n* = 67) (yes)	9 (34.2)	24 (82.8)	0.110 (0.031-0.388)	0.001		
Spleen diameter (cm)	13.6 (11.65-15.15)	14.5 (13-16)	0.800 (0.660-0.970)	0.023		
Child Pugh Score	5 (5-6)	5 (5-6)	0.885 (0.601-1.303)	0.535		
Child Pugh Score B (yes)	8 (13.3)	11 (14.9)	0.881 (0.330-2.352)	0.801		
MELD score	8 (7-10)	9 (8-10)	0.786 (0.648-0.954)	0.015		
MELD > 10	12 (20)	30 (40.5)	0.367 (0.167-0.804)	0.012		
AST (U/L)	54.5 (38-85)	56 (35.5-87)	0.996 (0.987-1.004)	0.322		
ALT (U/L)	62 (37-105)	53 (40-90)	1.002 (0.995-1.008)	0.620		
ALT ⩾ 2 × ULN at BL	59.5 (37.5-101)	54 (40-91.5)	1.326 (0.597-2.944)	0.489		
INR	1.09 (1.05-1.17)	1.12 (1.09-1.21)	0.127 (0.001-0.551)	0.023		
Bilirubin (mg/dl)	0.85 (0.65-1.16)	1.02 (0.71-1.52)	0.903 (0.535-1.525)	0.703		
Albumin (g/dl)	3.8 (3.52-4.12)	3.78 (3.52-4.12)	2.096 (0.959-4.581)	0.063		
Creatinine (mg/d)	0.8 (0.7-1)	0.81 (0.69-0.93)	0.327 (0.674-1.585)	0.165		
Platelet count (10^9/L)	118 (92-154)	91 (74-137)	1.002 (0.996-1.007)	0.579		
LSM BL (kPa)	18 (14.6-25.7)	21.1 (14-38.5)	0.988 (0.962-1.015)	0.391		
LSM SVR24 (kPa)	12.4 (9.4-18)	17.5 (10.4-32.4)	0.944 (0.908-0.981)	0.004		
SSM BL (kPa)	60.4 (45.7-70.7)	53.2 (37.4-75)	1.012 (0.992-1.032)	0.225		
LSPS BL	2.17 (1.33-3.77)	4.15 (1.65-6.26)	0.817 (0.684-0.975)	0.025		
LSM decrease (Delta, %)	33 (18.1–44.6)	19.4 (0–31.3)	0.0332 (0.005-0.225)	< 0.0001	0.0332 (0.005-0.225)	< 0.0001
LSM decrease > 10% (yes)	54 (90)	54 (73)	3.333 (1.242-8.946)	0.017		
LSM decrease > 20% (yes)	47 (78.3)	41 (55.4)	2.910 (1.352-6.262)	0.006		

Qualitative data were expressed as number and percentage (%); quantitative data were expressed as median (25%-75% quantiles). AIC: Akaike information criterion; ALT: Alanine aminotransferase; AUROC: Area under curve ROC; AST: Aspartate aminotransferase; BIC: Bayesian information criterion; CSPH: Clinically significant portal hypertension; DCV: Daclatasvir; HRV: High risk varices; INR: International normalized ratio; LDV: Ledipasvir; LR: Like-hood ratio; LSM: Liver stiffness measurement; LSPS: Liver stiffness to spleen/platelet score; MELD: Model for end-stage liver disease; NITs: Non-invasive tests; RBV: Ribavirin; SMV: Simeprevir; SOF: Sofosbuvir; SVR: Sustained virological response; SSM: Spleen stiffness measurement.

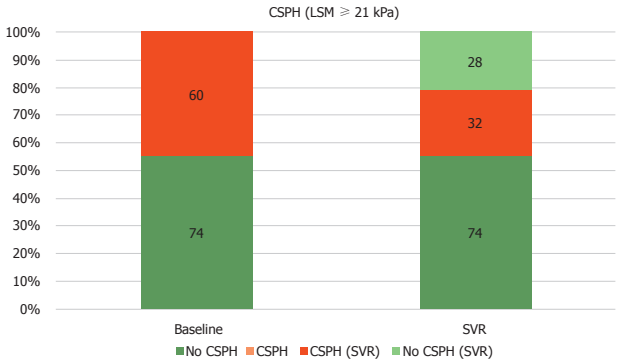


Figure 4 Clinically significant portal hypertension presence, according to Baveno Ⅵ (liver stiffness measurement ⩾ 21 kPa) at baseline and after SVR24. CSPH: Clinically significant portal hypertension; LSM: Liver stiffness measurement; SSM: Spleen stiffness measurement; SVR: Sustained virological response.

When looking at the bigger picture, SSM could represent a feasible tool to monitor therapy response and assess its benefit. This is also supported by a recent study by Buechter *et al*[47] that investigated LSM and SSM changes after TIPS placement.

The present study has some limitations: (1) its retrospective nature, even though SSM and LSM were prospectively collected according to the Italian Medicines Agency committee eligibility criteria for the treatment of HCV patients with DAAs, and (2) the absence of a gold-standard reference for PH assessment. However, according to the Baveno Ⅵ consensus[11], we could consider NITs, in addition to LSM, to be good surrogates of invasive methods, such as liver biopsy and HVPG. The time of follow-up was too short to fully correlate SSM changes with clinical outcomes after viral eradication, such as events of decompensation after SVR[48]. As in previous studies that include SSM, the upper limit of 75 kPa for SSM affects the possibility to detect changes in patients with severe PH[49,50]; in fact, both BL and SVR24 values were 75 kPa in seven (5.2%) patients.

In conclusion, SSM could be an accurate and useful NIT for the follow-up of patients after SVR, as it faithfully reflects changes in PH better than other NITs, including LSM. Further prospective studies are required in order to confirm the accuracy and usefulness of SSM and other NITs in the follow-up of patients with ACLD and its correlation with clinical outcomes.

ARTICLE HIGHLIGHTS

Research background

The long-term benefits of achieving sustained virological response (SVR) in cirrhotic patients are still to be established. Non-invasive tests (NITs), such

as liver (LSM) and especially spleen stiffness (SSM), are widely validated in hepatology as portal hypertension (PH) surrogates. However, their use in SVR patients and their changes after virus eradication is still under discussion.

Research motivation

Many studies have reported rapid LSM decrease after achieving SVR. However, only a few have investigated changes in SSM in such patients, with contrasting results. Given that there is a decrease in SSM after therapy, it means that SSM could be exploited to assess changes in PH and PH-driven complication after achieving SVR.

Research objectives

The main objective of the study was to investigate changes in PH after successful eradication of HCV infection, as reflected by its non-invasive assessment by SSM and other NITs.

Research methods

This is a retrospective study of prospectively collected data. Patients with available paired SSM assessment at baseline and 6 mo after end-of-therapy (SVR24) were included in the study.

Research results

Our main result is that a significant SSM decrease at SVR24 was demonstrated in a large cohort of 134 patients. This is the first study that also reveals a decrease in LSPS after SVR. SSM reduction differed according to the patient's clinical condition, especially when divided by the presence of clinically significant PH. An LSM decrease of > 20% was evident in the majority of patients, and also in patients in whom no SSM reduction was present. This finding likely reflects the reduction in liver necro-inflammation rather than PH improvement.

Research conclusions

PH, reflected by NITs, improves after achieving SVR in cirrhotic patients. SSM is a direct surrogate of PH and less influenced by liver necro-inflammation, as opposed to LSM. Its decrease (> 20%) could help the clinician to stratify the risk for PH-related complication after DAA therapy.

Research perspectives

Future prospective studies should investigate whether changes in SSM are predictive of clinical decompensation or other complications of cirrhosis after viral eradication. SSM could become a helpful and accurate method to assess therapy response and the risk of complications.

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EVIDENCE-BASED MEDICINE

Comparison of hepatitis C virus testing recommendations in high-income countries

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Abstract

AIM

To investigate hepatitis C virus (HCV) testing recommendations from the United States and other high-income countries.

METHODS

A comprehensive search for current HCV testing recommendations from the top quartile of United Nations Human Development Index (HDI) countries (very high HDI) was performed using Google and reviewed from May 1 - October 30, 2014 and re-reviewed April 1 - October 2, 2017.

RESULTS

Of the 51 countries identified, 16 had HCV testing recommendations from a government body or recommendations issued collaboratively between a government and a medical organization. Of these 16 countries, 15 had HCV testing recommendations that were primarily risk-based and highlight behaviors, exposures, and conditions that are associated with HCV transmission in that region. In addition to risk-based testing, the HCV Guidance Panel (United States) incorporates recommendations for a

one-time test for individuals born during 1945-1965 (the birth cohort) without prior ascertainment of risk into their guidance. In addition to the United States, six other countries either have an age-based testing recommendation or recommend one-time testing for all adults independent of risk factors typical of the region.

CONCLUSION

This review affirmed the similarities of the HCV Guidance Panel's guidance with those of recommendations from very high HDI countries.

Key words: Hepatitis C; Testing; Recommendations; Mass screening; Guidelines

Core tip: This report investigates hepatitis C virus (HCV) testing recommendations from the United States and other high-income countries to assess any risk-based or universal screening categories that should be considered for updates to HCV testing guidance from the HCV Guidance Panel (United States). This review affirmed the similarities of the HCV Panel guidance with those of very high-income countries. No significant gaps in the guidance were identified. HCV testing recommendations from very high-income countries will be continually reviewed and as new risk categories or universal screening recommendations are identified, they will be considered for incorporation into the HCV Panel guidance when peer-reviewed evidence is available to support the incorporation of the HCV testing practices in the United States.

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INTRODUCTION

Hepatitis C virus (HCV) infection, the leading cause of liver cancer and liver failure, is now curable with the recent emergence of short-duration, non-toxic, all-oral therapies[1-4]. This breakthrough in curative therapies for HCV infection has renewed interest in developing mechanisms to improve the HCV care continuum (testing, linkage to care, treatment initiation, cure). Notably, estimates suggest that 50% of HCV infected individuals remain undiagnosed[5]. Thus, it has become extremely important in infectious disease public health policy to identify the appropriate groups of individuals to test for HCV infection and to have this information readily available to healthcare professionals.

To provide healthcare professionals with a single web-based resource for evidence-based, expert-developed recommendations for hepatitis C testing and management, the American Association for the Study of Liver Diseases (AASLD) and the Infectious Diseases Society of America (IDSA) formed the HCV Guidance Panel[6]. The HCV Guidance Panel is also supported, in part, by the Centers for Disease Control and Prevention (CDC) and the International Antiviral Society United States (IAS-USA). The guidance published by the HCV Guidance Panel (the HCV Panel guidance) are updated periodically as new evidence is reviewed, including any updates to formal recommendations from the CDC and United States Preventive Services Task Force (USPSTF)[6-8]. In preparation for making updates to the HCV Panel guidance, HCV testing recommendations from the top quartile of United Nations Human Development Index (HDI) countries were evaluated for similarities and differences[9]. These data have been used periodically by the HCV Guidance Panel to explore HCV testing recommendations globally for comparison to the United States and for consideration in updating the HCV Panel guidance when additional peer-review data is available to support the inclusion of the category in the United States. For example, the periodic reviews of global HCV testing recommendations have served to inform discussion on categories that are not included in the HCV Panel guidance. Any new categories for consideration by panel members are reviewed and literature searches are conducted to ensure that the panel addresses all relevant data on the subject[6]. Each guidance statement is then rated in terms of the level and strength of evidence[6].

MATERIALS AND METHODS

The HDI is a summary measure of average achievement in health, education, and gross national income per capita[9]. Fifty-one countries including the United States were identified as having a very high HDI, defined as the top quartile of HDI countries[9]. A comprehensive search for current HCV testing recommendations from the top quartile of HDI countries was performed using a Google search with a combination of free text terms. Relevant terms included: country name, hepatitis C, HCV, screening, testing, recommendations, and guidelines. The Google results were then reviewed with experts in the field of hepatitis C (including email and in-person interviews) inquiring about any additional countries known to have HCV testing recommendations.

Testing recommendations were considered if they were from a government body or represented collaborative recommendations between a government and a medical organization. To be included in our analysis, recommendations needed to be available online May 1, 2014 - October 2, 2017. Non-English language documents were translated into English using Google Translate. When logical, testing categories were combined to make the tabulation of the results more manageable. Because in the United States both the CDC and USPSTF independently issue HCV testing recommendations, the HCV Guidance Panel's routine synthesis of these recom-

mendations into their guidance were used for the purpose of this report. From May 1 - October 30, 2014, two reviewers performed the initial searches and engaged consultants to identify HCV testing recommendations from very high HDI countries. Two reviewers re-reviewed HCV testing recommendations through Google searches and follow up with expert consults from April 1 - October 2, 2017 to identify and update any changes.

RESULTS

Of the 51 countries categorized as very high HDI, 16, including the United States were found to have HCV testing recommendations (Table 1)[6,10-26]. Of these 16 countries, 15 had HCV testing recommendations that were primarily risk-based and highlight behaviors, exposures, and conditions that are associated with HCV transmission in that region. In addition to risk-based testing, the HCV Panel guidance incorporates CDC and USPSTF recommendations for a one-time test for individuals born during 1945-1965 (the birth cohort) without prior ascertainment of risk[6-8]. Six additional countries have either an age-based testing recommendation or recommend one-time testing for all adults independent of risk factors. In the United States, individuals born from 1945-1965 are included in both CDC and USPSTF HCV testing recommendations as they account for 75% of all HCV infections and evidence confirmed that a risk-based strategy alone failed to identify more than 50% of HCV infections due to provider and patient barriers in correctly ascertaining risk[6,27].

The HCV Panel guidance recommends one-time testing for all HIV-positive individuals and for any persons about to start pre-exposure prophylaxis (PrEP) for HIV. The HCV Panel guidance also recommends annual HCV testing for persons who inject drugs and for HIV-infected men who have unprotected sex with men.

As of 2017, the HCV testing categories identified in other very high HDI countries not included in the HCV Panel guidance are: (1) Acute hepatitis or hepatitis symptoms; (2) Receiving an immunization or a medical procedure in a specified country or in a country where hepatitis C is common or where universal precautions are not in place; (3) Body piercing or tattoo history; (4) Hemophilia history; (5) Hepatitis A or B infection history; (6) Homeless persons; (7) Immigrants or visitors from countries where HCV is endemic; (8) Liver Cancer; (9) Living with, or sexual partner of, HCV-positive person; (10) Multiple sex partners, history of sexually transmitted infections (STIs), or high risk sexual behaviors; and (11) Attending STI clinic (± any risk factors)

DISCUSSION

The HCV Panel guidance is based on epidemiologic data and behaviors, exposures, and conditions associated with acquisition of HCV infection in the United States and are compiled by the expert panel and reviewed regularly[6]. As a result of this initial work in 2014, solid organ donors (deceased and living) were identified as a group not included in the guidance and were thus considered for review. After additional review of the available literature on the subject, donors were added to the HCV Panel guidance in 2014 and given an evidence rating of Class Ⅰ, Level B[6,28,29]. Prior to this, donors had not been explicitly named as a testing category but were discussed in the text below the testing guidance.

Currently, the HCV Panel guidance include the vast majority of HCV testing recommendation categories noted in other HDI countries and many of the people within risk categories not directly specified would likely receive testing nonetheless because of other associated risks or because of typical clinical care in the United States. For instance, acute hepatitis/hepatitis symptoms, hepatitis A or B history, and liver cancer would prompt a clinical evaluation for hepatitis C and might also be captured by the HCV Guidance Panel category of unexplained chronic liver disease and/or chronic hepatitis including elevated alanine aminotransferase levels[6]. Additionally, hemophilia alone is not recommended for testing by the HCV Guidance Panel; however, certain persons with hemophilia would be tested for HCV because the HCV Panel guidance incorporates recommendations to screen anyone who received blood components before 1992 or clotting factor concentrates produced before 1987 for HCV[6]. The remaining recommendation categories covered by other very high HDI countries but not included in the HCV Panel guidance include: body piercing or tattoo history; being homeless; living with, or the sexual partner of, an HCV-positive person; multiple sex partners, history of STIs, or high risk sexual behaviors; STI clinic populations; and immigrants/visitors from countries were HCV is endemic or those vaccinated or receiving medical procedures in those countries or where universal precautions are not in place. Although body piercings and tattoos are not specifically mentioned in the HCV Panel guidance, they do incorporate recommendations to test persons with percutaneous/parenteral exposures in an unregulated setting[6]. Hence, according to the HCV Guidance Panel, individuals with body piercings/tattoos obtained outside of licensed parlors should undergo HCV testing. Furthermore, this practice would also likely capture medical procedures where strict infection control may not have been followed, both domestically and internationally. While the higher prevalence of HCV in homeless populations is acknowledged by the HCV Guidance Panel, the homeless are not specifically named as a group for testing at this time. Although several studies have noted prevalence rates above 10% in several homeless populations in the United States, it is believed that the risk is often due to high rates of substance use disorders, which would be captured by the HCV Panel guidance to test injection and intranasal drug users[30]. Sexual transmission of HCV is generally considered inefficient except among HIV-infected MSM; therefore, guidance related to sexual transmission categories for the general United States population have not been included as an HCV testing

Table 1 Hepatitis C virus testing recommendations from the top quartile of human development index countries

Countries and approving bodies	Yr Recommendations approved	Abnormal Aminotransferases	Acute hepatitis or hepatitis symptoms	Being immunized/receiving a medical procedure in a specified country where hepatitis C is common or lack of universal precautions	Birth cohort/age recommendations or one-time testing for all	Blood/clotting factor transfusion Patients/ Transplant Patients	Body Piercing or Tattoo History	Children born to HCV-infected mothers (expectant mothers)
Argentina Dirección de Sida y ETS, Ministerio de Salud de la Nación	2016	X	X	X	X	X		X
Australia National HCV Testing Policy Expert Reference Committee	2016	X	X			X	X	X
Canada Canadian Task Force on Preventive Health Care	2017 and 2009	X	X	X		X	X	X
Chile Ministerio de Salud de Chile (Chilean Ministry of Health)	2015	X			X	X		X
Denmark Sundhedsst-yrelsen (Danish Health Authority)	2013	X				X		X
Finland Sosiaali- ja terveysministeriö (Social and Health Ministry)	2016			X	X	X	X	X
France ANRS/AFEF with Ministère des Affaires sociales et de la Santé (Ministry of Social Affairs and Health)	2014	X		X	X	X	X	X
Greece Υπουργειο ΥγειαΣ (Ministry of Health)	2017	X			X	X		X
Ireland Feidhmeannacht na Seirbhise Slainte	2012	X		X		X	X	X
Italy Istituto Superiore di Sanità	2005					X		
Japan Ministry of Health, Labour and Welfare	2011				X			
Russian Federation Министерство здравоохранения (Ministry of Health)	2017		X			X		X
Spain Ministeriode Sanidad, Servicios Socialese Igualdad (Ministry of Health, Social Services and Equality)	2015					X	X	X
Switzerland Bundesamt für Gesundheit (Swiss Federal Office of Public Health)	2013	X	X	X		X	X	X
United Kingdom NHS and National Inst for Health and Care Excellence	2015 and 2012	X		X		X	X	X
United States HCV Guidance Panel	2017	X			X	X		X

HCV: Hepatitis C virus; MSM: Men who have sex with men; X denotes that category is advised for testing.

Table 1 Hepatitis C virus testing recommendations from the top quartile of human development index countries

Countries and approving bodies	Chronic liver disease, cirrhosis, or fibrosis	Contact tracing /exposure to infected blood, shared hygiene materials with HCV + person	Donors - blood, blood product, tissue or organ	Drug users - injecting	BDrug users - intranasal	Healthcare workers (public safety) at risk and/or post-exposure	Hemodialysis history or repeated percutaneous injections	Hemophilia history	Hepatitis A or B history (or non-A/ non-B hepatitis)
Argentina Dirección de Sida y ETS, Ministerio de Salud de la Nación				X	X		X		X
Australia National HCV Testing Policy Expert Reference Committee	X	X	X	X		X	X		X
Canada Canadian Task Force on Preventive Health Care	X	X		X	X		X	X	X
Chile Ministerio de Salud de Chile (Chilean Ministry of Health)	X			X		X	X	X	
Denmark Sundhedsst-yrelsen (Danish Health Authority)	X			X	X	X	X	X	X
Finland Sosiaali- ja terveysministeriö (Social and Health Ministry)			X	X	X				
France ANRS/AFEF with Ministère des Affaires sociales et de la Santé (Ministry of Social Affairs and Health)		X	X	X	X		X		
Greece Υπουργειο ΥγειαΣ (Ministry of Health)				X			X		X
Ireland Feidhmeannacht na Seirbhise Slainte			X	X	X	X	X		X
Italy Istituto Superiore di Sanità				X			X		
Japan Ministry of Health, Labour and Welfare									
Russian Federation Министерство здравоохранения (Ministry of Health)	X	X	X	X		X	X		X
Spain Ministeriode Sanidad, Servicios Socialese Igualdad (Ministry of Health, Social Services and Equality)		X		X	X	X	X		X
Switzerland Bundesamt für Gesundheit (Swiss Federal Office of Public Health)	X			X	X	X	X		X
United Kingdom NHS and National Inst for Health and Care Excellence				X					
United States HCV Guidance Panel	X	X	X	X	X	X	X		

MSM: Men who have sex with men; X denotes that category is advised for testing.

Table 1 Hepatitis C virus testing recommendations from the top quartile of human development index countries

Countries and approving bodies	HIV-positive patients	Homeless	Immigrants or visitors from countries where HCV is endemic	Incarceration history alone or with additional risk factors	Liver cancer	Living with or sexual partner of HCV-positive person	MSM, limited to HIV + MSM (with or without unprotected sex), or sexually active starting PrEP	Multiple Sex Partners, history of STIs, or high risk sexual behaviors	Patient request, percutaneous-parenteral exposure, prior positive HCV test	STI clinic (+ /- any risk factors)
Argentina Dirección de Sida y ETS, Ministerio de Salud de la Nación						X		X	X	
Australia National HCV Testing Policy Expert Reference Committee	X		X	X	X	X	X		X	X
Canada Canadian Task Force on Preventive Health Care	X	X	X	X				X	X	
Chile Ministerio de Salud de Chile (Chilean Ministry of Health)	X				X	X				
Denmark Sundhedsst-yrelsen (Danish Health Authority)	X		X		X					
Finland Sosiaali- ja terveysministeriö (Social and Health Ministry)	X		X	X			X	X		
France ANRS/AFEF with Ministère des Affaires sociales et de la Santé (Ministry of Social Affairs and Health)	X		X	X		X	X			
Greece Υπουργειο ΥγειαΣ (Ministry of Health)	X		X	X		X		X	X	
Ireland Feidhmeannacht na Seirbhise Slainte	X		X	X		X	X		X	
Italy Istituto Superiore di Sanità						X		X	X	
Japan Ministry of Health, Labour and Welfare										
Russian Federation Министерство здравоохранения (Ministry of Health)				X		X	X	X		
Spain Ministeriode Sanidad, Servicios Socialese Igualdad (Ministry of Health, Social Services and Equality)	X			X		X	X		X	
Switzerland Bundesamt für Gesundheit (Swiss Federal Office of Public Health)	X		X	X	X	X	X		X	
United Kingdom NHS and National Inst for Health and Care Excellence		X	X	X		X	X		X	X
United States HCV Guidance Panel	X			X			X		X	

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; STI: Sexually transmitted infection; MSM: Men who have sex with men; X denotes that category is advised for testing.

category[31]. STI clinics are also not currently included in the HCV Panel guidance. Data suggest that STI clinic populations have higher prevalence rates of HCV infection than the general population due to overlapping risk factors of sexually transmitted infections and hepatitis C[32,33]. However, the higher prevalence of HCV infection in STI clinic populations is often attributed to the birth cohort (individuals born between 1945 and 1965) and injection drug use, risk groups captured elsewhere in the guidance[32-35]. Finally, while the HCV Guidance Panel continues to review evidence on immigrants/visitors from countries where HCV is endemic, any future guidance for HCV testing among foreign-born individuals would need to account for geographic disparities in HCV prevalence and practicalities in implementing this in clinical practice settings[36,37]. As an example of how this might be implemented, in 2008 the CDC recommended hepatitis B screening for persons born in regions of high and intermediate HBV endemicity (HBsAg prevalence > 2%)[38].

Our report has the following limitations. Our search methodology may have missed recommendations and/or classified recommendations incorrectly from some very high HDI countries due to search terms not capturing relevant information in different languages, recommendations not being accessible on the Internet at the time of the searches, or misinterpretation of the role of the government's involvement in development of the recommendations. Additionally, some governments may rely on international or national organizations for recommendations on hepatitis C testing without publishing their own recommendations.

This review affirmed the similarities of the HCV Panel guidance with those of very high HDI countries. No significant gaps in the guidance were identified. HCV testing recommendations from very high HDI countries will be continually reviewed and as new risk categories or universal screening recommendations are identified, they will be considered for incorporation into the HCV Panel guidance when peer-reviewed evidence is available to support the incorporation of the HCV testing practices in the United States.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) infection, the leading cause of liver cancer and liver failure, is now curable with the recent emergence of short-duration, non-toxic, all-oral therapies. This breakthrough in curative therapies for HCV infection has renewed interest in developing mechanisms to improve the HCV care continuum (testing, linkage to care, treatment initiation, cure). This renewed interest in HCV has led to many countries updating their HCV testing recommendations.

Research motivation

The United States HCV Guidance Panel provides healthcare professionals with a single web-based resource for evidence-based, expert-developed recommendations for hepatitis C testing and management. HCV recommendations in countries around the world have been recently updated due to advances in HCV treatment. However, this data is not compiled in a central location. This report investigates HCV testing recommendations from the United States and other high-income countries. In preparation for making updates to the HCV Panel guidance, HCV testing recommendations from the top quartile of United Nations Human Development Index (HDI) countries were evaluated for similarities and differences.

Research objectives

The main objective of this study was to identify HCV testing recommendations from the top quartile of United Nations HDI countries. The identified HCV recommendations were evaluated for similarities and differences. These data have been used periodically by the HCV Guidance Panel to explore HCV testing recommendations globally for comparison to the United States for consideration in updating the HCV Panel guidance when additional peer-review data is available to support inclusion of the category in the United States.

Research methods

A comprehensive search for current HCV testing recommendations from the top quartile of HDI countries was performed using a Google search with a combination of free text terms. Relevant terms included: country name, hepatitis C, HCV, screening, testing, recommendations, and guidelines. The Google results were then reviewed with experts in the field of hepatitis C (including email and in-person interviews) inquiring about any additional countries known to have HCV testing recommendations. Testing recommendations were considered if they were from a government body or represented collaborative recommendations between a government and a medical organization. To be included in our analysis, recommendations needed to be available online May 1, 2014-October 2, 2017. From May 1-October 30, 2014, two reviewers performed the initial searches and engaged consultants to identify HCV testing recommendations from very high HDI countries. Two reviewers re-reviewed HCV testing recommendations through Google searches and follow up with expert consults from April 1-October 2, 2017 to identify and update any changes.

Research results

Of the 51 countries identified, 16 had HCV testing recommendations from a government body or recommendations issued collaboratively between a government and a medical organization. Of these 16 countries, 15 had HCV testing recommendations that were primarily risk-based and highlight behaviors, exposures, and conditions that are associated with HCV transmission in that region. In addition to risk-based testing, the HCV Guidance Panel (United States) incorporates recommendations for a one-time test for individuals born during 1945-1965 (the birth cohort) without prior ascertainment of risk into their guidance. In addition to the United States, six other countries either have an age-based testing recommendation or recommend one-time testing for all adults independent of risk factors typical of the region.

Research conclusions

This review affirmed the similarities of the HCV Guidance Panel's guidance with those of recommendations from very high HDI countries. As a result of this initial work in 2014, solid organ donors (deceased and living) were identified as a group not included in the guidance and were thus considered for review. After additional review of the available literature on the subject, donors were added to the HCV Panel guidance in 2014 and given an evidence of rating of Class I, Level B. Prior to this, donors had not been explicitly named as a testing category but were discussed in the text below the testing guidance.

Research perspectives

HCV testing recommendations from very high HDI countries will be continually reviewed and as new risk categories or universal screening recommendations are identified, they will be considered for incorporation into the HCV Panel guidance when peer-reviewed evidence is available to support the incorporation of the HCV testing practices in the United States.

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SYSTEMATIC REVIEW

Thrombosis prophylaxis in pediatric liver transplantation: A systematic review

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Abstract

AIM

To review current literature of thrombosis prophylaxis in pediatric liver transplantation (PLT) as thrombosis remains a critical complication.

METHODS

Studies were identified by electronic search of MEDLINE, EMBASE and Cochrane Library (CENTRAL) databases until March 2018. The search was supplemented by manually reviewing the references of included studies and the references of the main published systematic reviews on thrombosis and PLT. We excluded from this review case report, small case series, commentaries, conference abstracts, papers which describing less than 10 pediatric liver transplants/year and articles published before 1990. Two reviewers performed study selection independently, with disagreements solved through discussion and by the opinion of a third reviewer when necessary.

RESULTS

Nine retrospective studies were included in this review. The overall quality of studies was poor. A pooled analysis of results from studies was not possible due to the retrospective design and heterogeneity of included studies. We found an incidence of portal vein thrombosis (PVT) ranging from 2% to 10% in pediatric living donor

liver transplantation (LDLT) and from 4% to 33% in pediatric deceased donor liver transplantation (DDLT). Hepatic artery thrombosis (HAT) was observed mostly in mixed LDLT and DDLT pediatric population with an incidence ranging from 0% to 29%. In most of the studies Doppler ultrasonography was used as a first line diagnostic screening for thrombosis. Four different surgical techniques for portal vein anastomosis were reported with similar efficacy in terms of PVT reduction. Reduced size liver transplant was associated with a low risk of both PVT (incidence 4%) and HAT (incidence 0%, $P < 0.05$). Similarly, aortic arterial anastomosis without graft interposition and microsurgical hepatic arterial reconstruction were associated with a significant reduced HAT incidence (6% and 0%, respectively). According to our inclusion and exclusion criteria, we did not find eligible studies that evaluated pharmacological prevention of thrombosis.

CONCLUSION

Poor quality retrospective studies show the use of tailored surgical strategies might be useful to reduce HAT and PVT after PLT; prospective studies are urgently needed.

Key words: Pediatric liver transplantation; Prophylaxis; Hepatic artery thrombosis; Surgical technique; Portal vein thrombosis

Core tip: Graft loss and patient death after pediatric liver transplantation (PLT) are most frequently caused by hepatic artery thrombosis and portal vein thrombosis. For this reason, the prevention of hepatic artery and vein thrombosis represents a primary interest for clinicians and researchers, considering the scarcity of hepatic allografts. In our systematic review, we found only nine poor quality retrospective studies showing that tailored surgical strategies might be useful to reduce thrombosis. We did not find eligible studies evaluating pharmacological prevention strategies. Prospective studies are urgently needed to standardize thrombosis prevention in PLT.

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INTRODUCTION

Vascular complications are relevant causes of poor outcome for patient and allograft after pediatric liver transplantation (PLT)[1-5]. Among vascular complications of PLT hepatic artery thrombosis (HAT) and portal vein thrombosis (PVT) are one of the most frequent[1-7] and serious causes of graft loss and also patient death[1,2,6-9]. In particular HAT is an extremely serious complication resulting in bile duct necrosis and often requiring retransplantation[10,11]. Thrombosis of other intra-abdominal vessels, such as the hepatic vein and inferior vena cava occurs less frequently[6,11,12]. In the first years of PLT the observed incidence of thrombosis was very high, up to 42%[13-15]. In the last years, an improvement of perioperative care has significantly decreased the thrombosis incidence[1-3]. More recently, an incidence rate of HAT ranging from 2% to 10% after liver transplantation in the pediatric population has been reported[1,6,7,11]; likewise, the incidence rate of PVT ranged from 2% to 10%[1,6,7,9].

In this context, the prevention of HAT and PVT remains very important for PLT outcome and it should be a matter of primary interest for clinicians and researchers, considering the ongoing scarcity of hepatic allografts[1,2,11,12,16-18]. In clinical practice, there is not a standardized approach for thrombosis prevention in PLT. Different surgical techniques and pharmacological prophylaxis have been purposed in several studies[1,5,6,12,14,19-27]. Therefore, we performed a systematic review of current literature about surgical and pharmacological prophylaxis for prevention of thrombosis after PLT to evaluate the current evidence available.

MATERIALS AND METHODS

Search strategy

The publications were selected through an electronic search of the MEDLINE and EMBASE and Cochrane Library (CENTRAL) databases up to March 2018. The search strategy used the following Medical Subject Headings (MeSH) and EMTREE terms and text words: ("liver transplantation"/exp OR "liver transplantation") AND ("thromboembolism"/exp OR "thromboembolism" OR "ischemia"/exp OR "ischemia" OR "vascular disease" /exp OR "vascular disease") AND ("prophylaxis"/exp OR "prophylaxis" OR "prevention"/exp OR "prevention") AND ([newborn]/lim OR [infant]/lim OR [child]/lim OR [preschool]/lim OR [school]/lim OR [adolescent]/lim). In addition the references of the selected studies and two systematic reviews on thrombosis and PLT[11,17] were screened to identify further relevant studies.

Two reviewers (Giulia Maria Ruggeri and Giovanna Colombo) performed an independent study selection, solving any disagreements through discussion and the opinion of a third reviewer (Mirco Nacoti). They obtained study characteristics like year of publication, design, study centre, patients' characteristics like number, mean age and gender, treatments and number of arterial and venous thrombotic complications.

The following criteria were needed in order to be considered potentially eligible for this systematic review: (1) phase Ⅲ randomized clinical trials or cohorts including case series with more than 10 patients undergoing

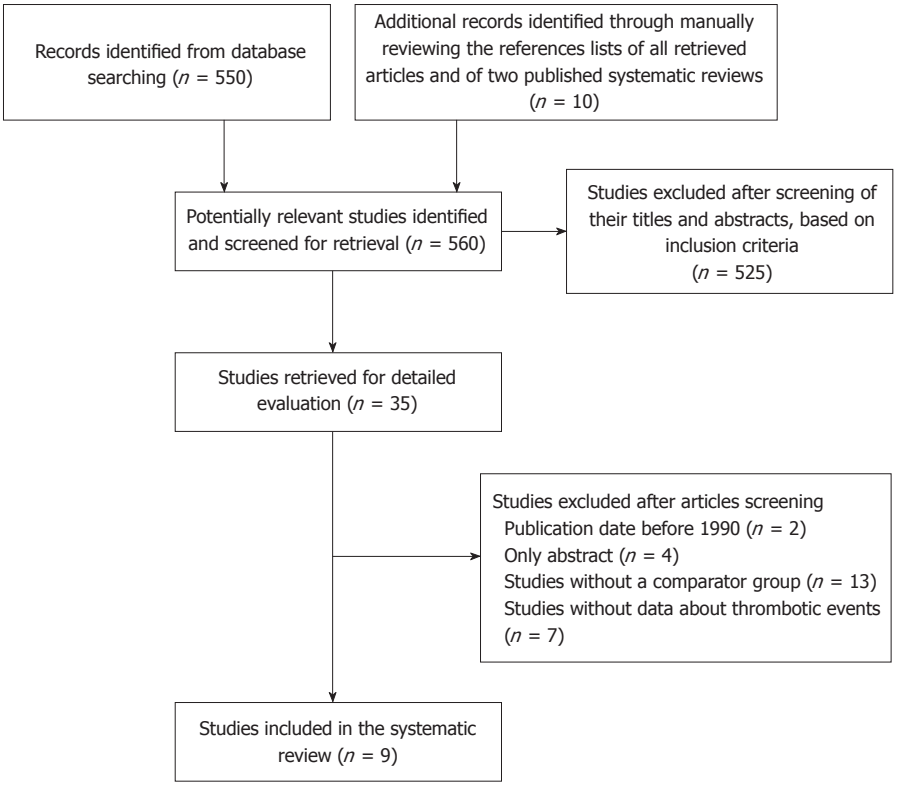

Figure 1 Flow-chart of study selection process.

elective PLT (age ranged from 0 to 18 years); and (2) reporting arterial and venous thrombosis as primary or secondary events in all different groups according to the used prophylaxis strategy. If data from a study were reported in several publications, data from the most recent paper were used. Studies–describing less than 10 PLT/year were excluded as significant high mortality is associated with low volume center[28,29]. Articles published before 1990 were also excluded because new developments in perioperative PLT have dramatically improved the survival[1-3].

Risk of bias assessment

Although the evaluation of quality for observational studies is controversial[30], Giulia Maria Ruggeri and Giovanna Colombo assessed the risk of bias using the following items for cohort studies: Type of study (prospective or retrospective); selection of the patients (consecutive or not); thrombosis as pre-specified outcome; quality of measurements (studies in which thrombotic events were measured in an objective way were considered higher quality than studies without these characteristics). A scoring system was put in place to identify the following two quality categories: Studies at low-risk of bias (4 points) and studies at high-risk of bias ($\leqslant$ 3 points).

RESULTS

Results of the search strategy

The study process is presented in Figure 1. A total of 560 publications (550 retrieved with the electronic search strategy and 10 by manually reviewing the reference lists of all retrieved articles) were identified. After reading their titles and abstracts 525 were excluded, according to inclusion and exclusion criteria. The remaining 35 publications were analyzed in full for detailed evaluation. Twenty-six papers were eliminated for the following reasons: Publication date before 1990 (n = 2), only abstract (n = 4), studies without a comparator group (n = 13) and studies (n = 7) which did not contain explicit data about pediatric thrombotic events.

Nine manuscripts[31-39], which included a total of 1034 PLT in 991 subjects, were included in this systematic review (Table 1). None of the all included studies were at high risk of bias according to pre-defined requirements (Table 2). In particular, no study were prospective, only 7 studies reported thrombosis as a pre-defined outcome, and 8 studies detailed how the diagnosis of thrombosis had been made. The poor quality and the heterogeneity of included studies did not allow us to perform a pooled analysis of results.

Incidence of the artery and PVT

Table 1 shows detailed incidence of artery and PVT as reported in the included studies. Most studies reported a total incidence (early and late) of thrombosis. The incidence of PVT varies from 2% to 10% in pediatric living donor liver transplantation (LDLT)[37,39] and from 33% in pediatric LDLT to 4% in pediatric deceased donor liver transplantation (DDLT) with reduced graft[33]. HAT is presented mostly in mixed LDLT and DDLT pedia-

Table 1 Summary of findings of the nine included studies

Reference	Country	Population (*n*)	Study design	Method used for diagnosis and follow-up duration	Intervention	Outcome	Main results
Sabra *et al*[37]	Japan	113 pediatric LDLT	Retrospective	Doppler US twice daily till 1st week. If any PV complications were found, specific tests such as angiography were performed 1 yr of follow-up	PV reconstruction with VG (31 pts) PV reconstruction with EEA (82 pts)	Preoperative recipient factors PVT incidence Pt survival Graft survival	Global incidence PVT (2.6%) in the first 3 mo after OLT 1 PVT in 31 VGs *vs* 2 PVT in 82 without VGs No significant difference for PVT, Pt survival, Graft survival In the two groups
Julka *et al*[38]	Taiwan	87 pediatric LDLT	Retrospective	Routine doppler US post LT; CT angiography for HAT confirmation 5 yr of follow-up	HA reconstruction with two arterial stumps. 2 HA stumps with 2 HA reconstruction = 20 pts 2 HA stumps with 1 HA reconstruction = 22 pts 1 HA stump with 1 HA reconstruction = 45 pts	HAT incidence BC incidence	Overall HAT incidence 6.9% The incidence of HA thrombosis and biliary complications was similar in the three groups
Saad *et al*[39]	Japan	110 LDLT in pediatric pts	Retrospective LDLT	Doppler US, performed routinely before, during and after surgery Follow-up not defined	Different types of portal vein reconstructions Type 1: End- to- end anastomosis = 36 pts Type 2: Branch patch anastomosis = 27 pts Type 3: Anastomosis to the confluence (superior mesenteric vein-splenic vein) = 16 pts Type 4: Vein graft = 32 pts Chosen according to the surgical evaluation	C TC SC Survival rate	Type 1: 1 SC / 36 pts Type 2: 2 TC / 27 pts Type 3: 0 / 16 pts Type 4: 1 TC / 32 pts Overall survival rate 86%
Shackleton *et al*[31]	California	194 pediatric OLT for biliary atresia (mixed LDLT and DDLT)	Retrospective	Clinical suspect confirmed by angiography and/or surgical exploration. 3 yr of follow-up	Gr 1: Conventional artery reconstruction (*n* = 166) Gr 2: MHR (*n* = 28)	Risk factors for HAT Impact of MHR on incidence of HAT, need of re-OLT, patient and graft survival	Impact of MHR HAT incidence: Gr 1 32/166 (19%) *vs* Gr 2 0/28 (0%), *P* = 0.006 Re-OLT: Gr 1 31/166 (19%) *vs* Gr 2 1/28 (4%), *P* = 0.05 1 yr actuarial survival: Gr 1 81% *vs* Gr 2 100%, *P* = 0.02 (univariate analysis) BUT *P* = 0.076 in step wise Cox regression for patient survival
López *et al*[32]	Spain	104 OLT in 82 pediatric pts (mixed LDLT and DDLT)	Retrospective	Doppler US routinely and selective arteriography for confirmation. 3 yr of follow-up	Arterial revascularization technique: Gr 1 (*n* = 48) AhG Gr 2 (*n* = 56) EEA Chosen according to the surgical evaluation	HAT incidence Survival rate	HAT incidence Gr 1. (AhG): 6.25% Gr 2. (EEA): 8.92% (*P* not significant) Graft Survival rate (1 yr) 61.5% (AhG) *vs* 60% (EEA) (*P* < 0.05) Graft survival rate (5 yr): 77.5% (AhG) *vs* 75.1% (EEA) (*P* < 0.05)
Millis *et al*[33]	Illinois	66 pediatric LDLT and 48 pediatric cadaveric RLT	Retrospective	Doppler US every day for the first 3 d and at 1, 3, 6, 12, 18, and 24 mo after transplantation + angiography for confirmation 5 yr of follow-up	Portal anastomosis with venous graft conduit in LDLT Gr 1 (*n* = 18): Native reconstructed vein Gr 2 (*n* = 37): Cryopreserved iliac vein; Gr 3 (*n* = 11):	Incidence of PVC Graft survival Patient survival	Incidence PVC LDLT 33/66 (50%) *vs* RLT 4/48 (8%) *P* < 0.0001 Early PVT LDLT Gr 1: 6 (33%)[a] LDLT Gr 2: 3 (8%) LDLT Gr 3: 1 (9%) RLT: 2 (4%)

					Cryopreserved femoral vein		[a]$P < 0.005$ *vs* RLT Late PVC LDLT Gr 1: 3 (16%) LDLT Gr 2: 19 (51%)[a] LDLT Gr 3: 1 (9%) RLT: 2 (4%) [a]$P < 0.005$ *vs* RLT; $P < 0.02$ *vs* Gr 1 and Gr 3 Graft survival PVC: 61% No PVC: 67%, P = NS Patient survival: PVC: 67% No PVC: 71%, P = NS
Jurim *et al*[34]	California	35 pediatric OLT Emergency transplants only (type of donor not specified)	Retrospective	Not reported. Follow-up not defined	Gr 1: RLT = 7 pts Gr 2: Whole graft = 18 pts	HAT incidence Incidence of other complications: Biliary; bleeding; chronic rejection	HAT: Gr 1:0 (0%)/Gr 2:5 (29%) ($P < 0.05$) The incidence of biliary complications, bleeding (requiring surgical exploration) and chronic rejection were similar between the groups
Yandza *et al*[35]	France	143 DDLT in 122 pediatric pts	Retrospective	Doppler US daily the first 15 d, twice/wk until discharge Follow-up not defined	Gr 1 (n = 41 pts, n = 50 grafts) children < 10 kg Gr 2 (n = 81 pts, n = 93 grafts) children > 10 kg Surgical technique: EEA *vs* AhG	Effect of the site of liver graft arterial inflow on HAT incidence according to the recipient weight	Overall HAT incidence: 14/143 (10%) HAT incidence between the 2 groups: Gr 1: 6/50 (12%) *vs* Gr 2: 8/93 (9%), P not significant; Gr 1 EEA 5/31 (16%) *vs* Gr 1 AhG 1/19 (5%); P not significant Gr 2 EEA 4/60 (6%) *vs* Gr 2 AhG 4/32 (12%) P not significant
Stevens *et al*[36]	Chicago	134 OLT in 100 pediatric pts < 2 yr : mixed LDLT and DDLT	Retrospective	Doppler US, frequency not defined Follow-up	60 standard whole liver *vs* 74 RLT (13 LDLT) Surgical technique: Arterial inflow with 83 hepatic artery *vs* 32 celiac artery *vs* 5 supraceliac aorta *vs* 27 infrarenal aorta *vs* 7 unusual reconstruction	Effect of the graft type and site of arterial inflow on the Incidence of HAT	HAT incidence in 25% whole liver transplant *vs* 23% in LDLT *vs* 15% RLT (P = 0.06) Aortic anastomosis (supraceliac and infrarenal) reduces incidence of HAT (6% *vs* 24%, P = 0.02)

BA: Biliary atresia; BW: Body weight; CTA: CT angiography; Gr: Group; GRWR: Graft-to-recipient weight ratio; HAG: Hepatic artery graft; LT: Liver transplantation; OLT: Orthotopic liver transplantation; Pt: Patient; RLT: Reduced size liver transplantation; re-OLT: Re-transplantation; SC: Stenotic complication; TC: Thrombosis complication; US: Ultrasonography; LDLT: Living donor liver transplantation; PV: Portal vein; VG: Vein graft; EEA: End-to-end anastomosis; PVT: Portal vein thrombosis; HAT: Hepatic artery thrombosis; HA: Hepatic artery; BC: Biliary complications; C: Complications; MHR: Microsurgical hepatic arterial reconstruction; AhG: Aortohepatic interposition graft; PVC: Portal vein complications; DDLT: Deceased donor liver transplantation.

tric population; incidence of HAT varies from 0% to 29%[31,32,34-36,38].

Screening protocol for thrombosis detection

Most of the studies used Doppler ultrasonography (US) as a first line diagnostic screening for thrombosis[32,33,35-39]; frequency and duration of the screening is quite variable. Confirmation of the thrombosis detected by a second level diagnostic test, such as computer CT angiography, surgery or other methods is rarely specified[31-33,37,38].

Intraoperative surgical prophylaxis

Table 1 summarizes the main results of the studies analyzed. In LDLT there are four different modalities to perform portal vein anastomosis: (1) standard reconstruction with end to end anastomosis[37,39]; (2) reconstruction with anastomosis to the bifurcation of the recipient left and right vein[39]; (3) reconstruction with anastomosis to the confluence of the recipient mesenteric vein[39]; and (4) reconstruction with an interposition of vein graft[33,37,39]. The overall results of different techniques were similar. The choice of the type of reconstruction depended on the size (length and diameter) and quality of the portal vein and size mismatch between donor and recipient portal vein[33,37,39]. Millis *et al*[33] showed a low PVT incidence with reduced size liver transplant (RLT) [4%

Table 2 Risk of bias and quality of the studies

Reference	Study design	Consecutive enrolment	Thrombosis as pre-defined outcome	Methods use to the diagnosis of thrombosis
Shackleton *et al*[31]	Retrospective	Yes	Yes, HAT	Clinical grounds and angiography and/or surgical exploration for confirmation
López *et al*[32]	Retrospective	Yes	No	Doppler US and angiography for confirmation, post mortem second confirmation
Millis *et al*[33]	Retrospective	Yes	Yes, PVT	Doppler US and angiography for confirmation
Jurim *et al*[34]	Retrospective	Yes	Yes, HAT	Not reported
Yandza *et al*[35]	Retrospective	Yes	Yes, HAT	Doppler US
Stevens *et al*[36]	Retrospective	Yes	Yes, HAT	Doppler US
Sabra *et al*[37]	Retrospective	Yes	Yes, PVT	Doppler US
Julka *et al*[38]	Retrospective	Yes	No	Doppler US and angiography for confirmation
Saad *et al*[39]	Retrospective	Yes	Yes, PVT	Doppler US

PVT: Portal vein thrombosis; HAT: Hepatic artery thrombosis; US: Ultrasonography.

vs 33% with whole liver transplant (WLT) and native reconstructed vein, $P < 0.005$]; RLT was developed in attempt to resolve the mismatch size liver between donor and recipient and was applied to split liver transplantation and LDLT.

Three different surgical procedures seemed to reduce HAT incidence: RLT with cadaveric left lobe [incidence 0% *vs* 29% with WLT, $P < 0.05$[34]]; aortic arterial anastomosis without graft interposition [incidence 6% *vs* 24% in celiac-hepatic artery anastomosis, $P = 0.02$[36]] and microsurgical hepatic arterial reconstruction (MHR) [incidence 0% *vs* conventional artery reconstruction, $P = 0.006$[31]].

López *et al*[32] and Yandza *et al*[35] did not find significant HAT difference between end-to-end anastomosis and aortohepatic interposition graft; Julka *et al*[38] showed that single hepatic artery reconstruction did not increase the HAT incidence in pediatric LDLT having dual hepatic arterial stump in the liver graft.

Post-operative pharmacological prophylaxis

No studies on pharmacological prophylaxis compared clinical outcomes according to different treatments used[1,9,11-13,17,22-24,27].

DISCUSSION

Vascular thrombotic complications were a serious life-threatening complication in the first year of PLT with an incidence up to 42% associated with mortality up to 50%[13–15]. Although factors causing thrombotic complications are not fully understood[15], a global improvement of perioperative care has significantly decreased the thrombosis incidence in the last 20 years[1-3,6,7,9,11]. Several retrospective studies without control group tried to identify factors for thrombosis; among them should be mentioned medical factors, such as administration of fresh frozen plasma, elevated hematocrit, protein C deficiency[1,14,40,41] and surgical factors, such as cold ischemia time, technique of anastomosis, small vessel diameter, the use of aortic grafts, donor arterial anatomy and reconstruction[1,7,14,31,36], but without any definitive conclusions.

Accordingly, the aim of this systematic review was to identify evidence based methods both surgical and pharmacological for the prevention of thrombosis after PLT. In this systematic review, we found no prospective studies and only 9 retrospective studies with a control group referred to surgical prevention.

RLT (with left lobe or segment of left lobe)[34] direct aortic anastomosis and MHR[31] seem the best surgical options for reducing thrombotic complications in PLT, but the impact of RLT and aortic anastomosis on HAT were not confirmed by Stevens[36] and López-Yandza[32,35] respectively. MHR is an arterial reconstruction performed with an operating microscope; it was introduced by the Kyoto group for the fine graft arteries (less than 2 mm in diameter) in LDLT[42]. The amazing results of MHR (0 HAT in 28 PLT)[31] need to be confirmed in a larger clinical trial. It is worth noting that one of the most extensive studies about incidence and risk factors for vascular complication in liver transplantation was excluded from this systematic review because it included a mixed adult and pediatric population, without an appropriate control group[1].

Pharmacological prophylaxis is a relevant topic in PLT. Several studies[1,9,11-13,17,22-24,26] reported their experience using different drugs, such as unfractionated heparin, low molecular weight heparin, vitamin K antagonist fresh frozen plasma, aspirin, dipyridamole, antithrombin concentrate, dextran 40, thrombin inhibitor, prostaglandin. Unfortunately, in these studies there was not a comparator group, necessary in order to achieve formal proof of efficacy and safety and according to the inclusion criteria of this systematic review. In this regard, for example, aspirin is one of the most extensive drugs used for HAT prevention[11,14,17], but without formal evidence derived from prospective clinical trials.

The careful search of the literature and the inclusion of different types of studies are the main strengths of this review. Nevertheless, our study presents some weaknesses. First, the risk of thrombosis might have been underestimated because we assumed not all

authors systematically reported thrombotic events. Second, the description of methods for preventing vascular thromboses may be incomplete because only studies reporting the outcome were considered.

Although, HAT and PVT incidence has decreased in the last decades[1-3,6,7,9,11], they remain one of the more frequent and serious complications causing a poor outcome after PLT[1,2]. Furthermore, the old question "thrombosis after PLT - a medical or surgical event?"[14] remains an unresolved issue. Concerning this, our systematic review of studies, in which different prophylaxis strategies were tested for the prevention of HAT and PVT failed to provide enough evidence for a definitive conclusion due to the poor quality of studies found[31-39]. However, our analysis emphasizes the need of developing well-designed clinical studies in order to correctly determine PLT-associated thrombosis risk and to define an evidence-based antithrombotic prophylactic strategy. The recent "single ventricle trial"[43] showed that randomized clinical trials are possible also in the pediatric surgery area.

ARTICLE HIGHLIGHTS

Research background

Hepatic artery thrombosis (HAT) and portal vein thrombosis (PVT) commonly occur after pediatric liver transplantation (PLT) that may cause graft loss and patient death. Different surgical techniques and pharmacological prophylaxis have been purposed in several studies; nevertheless, there is not a standardized approach for thrombosis prevention in PLT.

Research motivation

Prevention of HAT and PVT remains very important for PLT outcome and it should be a matter of primary interest for clinicians and researchers, considering the ongoing scarcity of hepatic allografts.

Research objective

We performed a systematic review of current literature about surgical and pharmacological prophylaxis for prevention of thrombosis after PLT to evaluate the current evidence available.

Research methods

Studies were identified by electronic search of MEDLINE, EMBASE and Cochrane Library (CENTRAL) databases until March 2018. We excluded from this review case report, small case series, commentaries, conference abstracts, papers which describe less than 10 pediatric liver transplants/year and articles published before 1990. Two reviewers performed an independent study selection, solving any disagreements through discussion and the opinion of a third reviewer.

Research results

Nine retrospective studies were included in this review. They showed the use of tailored surgical strategies might be useful to reduce thrombosis. We did not find eligible studies evaluating pharmacological prevention strategies. The overall quality of studies was poor. A pooled analysis of results from studies was not possible due to the retrospective design and heterogeneity of included studies.

Research conclusions

This systematic review in which different prophylaxis strategies were tested for the prevention of HAT and PVT failed to provide enough evidence for a definitive conclusion due to the poor quality of studies found.

Research perspective

This systematic review showed there is no evidence based strategy for thrombosis prevention in PLT. Prospective studies are urgently needed. The recent "single ventricle trial" showed that randomized clinical trials are possible also in the pediatric surgery area.

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META-ANALYSIS

Liver transplantation and atrial fibrillation: A meta-analysis

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Abstract

AIM

To assess prevalence of pre-existing atrial fibrillation (AF) and/or incidence of AF following liver transplantation, and the trends of patient's outcomes overtime; to evaluate impact of pre-existing AF and post-operative AF on patient outcomes following liver transplantation.

METHODS

A literature search was conducted utilizing MEDLINE, EMBASE and Cochrane Database from inception through

March 2018. We included studies that reported: (1) prevalence of pre-existing AF or incidence of AF following liver transplantation; or (2) outcomes of liver transplant recipients with AF. Effect estimates from the individual study were extracted and combined utilizing random-effect, generic inverse variance method of DerSimonian and Laird. The protocol for this meta-analysis is registered with PROSPERO (International Prospective Register of Systematic Reviews, No. CRD42018093644).

RESULTS

Twelve observational studies with a total of 38586 liver transplant patients were enrolled. Overall, the pooled estimated prevalence of pre-existing AF in patients undergoing liver transplantation was 5.4% (95%CI: 4.9%-5.9%) and pooled estimated incidence of AF following liver transplantation was 8.5% (95%CI: 5.2%-13.6%). Meta-regression analyses were performed and showed no significant correlations between year of study and either prevalence of pre-existing AF (P = 0.08) or post-operative AF after liver transplantation (P = 0.54). The pooled OR of mortality among liver transplant recipients with pre-existing AF was 2.34 (2 studies; 95%CI: 1.10-5.00). In addition, pre-existing AF is associated with postoperative cardiovascular complications among liver transplant recipients (3 studies; OR: 5.15, 95%CI: 2.67-9.92, I^2 = 64%). With limited studies, two studies suggested significant association between new-onset AF and poor clinical outcomes including mortality, cerebrovascular events, post-transplant acute kidney injury, and increased risk of graft failure among liver transplant recipients (P < 0.05).

CONCLUSION

The overall estimated prevalence of pre-existing AF and incidence of AF following liver transplantation are 5.4% and 8.5%, respectively. Incidence of AF following liver transplant does not seem to decrease overtime. Pre-existing AF and new-onset AF are potentially associated with poor clinical outcomes post liver transplantation.

Key words: Atrial fibrillation; Liver; Hepatic; Transplant; Transplantation; Systematic reviews; Meta-analysis

Core tip: Atrial fibrillation (AF) occurs in a substantial number of postoperative and post-transplantation patients. In addition, postoperative AF confers both short-term and long-term morbidity and mortality in liver transplant patients. However, the incidence of postoperative AF in patients undergoing liver transplantation and its impacts remain unclear. To further investigate, we conducted a meta-analysis to assess the rates of preexisting AF and AF following liver transplantation as well as the outcomes of liver transplant patients with AF. Incidence of AF following liver transplant does not seem to decrease overtime. Pre-existing AF and new-onset AF are potentially associated with poor clinical outcomes post liver transplantation.

Chokesuwattanaskul R, Thongprayoon C, Bathini T, Ungprasert P, Sharma K, Wijarnpreecha K, Pachariyanon P, Cheungpasitporn W. Liver transplantation and atrial fibrillation: A meta-analysis. *World J Hepatol* 2018; 10(10): 761-771 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i10/761.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i10.761

INTRODUCTION

Atrial fibrillation (AF) is one of the most common heart diseases, affecting 3 to 6 million populations in the United States, almost 30 million people worldwide, which is expected to reach 50 million peoples worldwide in 2050[1-4]. Patients with AF carry a higher risk of adverse cardiovascular events and reduced survival[5,6]. Incidence of AF increases with age. At the same time, aging population is likely to develop other chronic diseases and one of them is end-stage liver disease or cirrhosis[7-9]. This treatment of cirrhosis comprises of multidisciplinary approach ranging from very simple, symptomatic treatment with diuretic or treatment of primary cause, down the road to the most advanced treatment; liver transplantation[10-13].

Liver transplantation is the treatment of choice for end-stage liver diseases[10,13]. In 2017, around 8000 patients all over the United State suffered from end-stage liver disease receiving liver transplantation and the number trends to increase 3% to 5% annually in the past 20 years along with the excellent outcomes with almost 95% survival rate at 1-year post-procedure and some patients could live even more than 30 years after liver transplantation[14-17]. Recent advances in basic and clinical sciences, including surgical technique, immunosuppressive therapy and postoperative supportive care, have led to the substantial improvement in quality of life and survival after liver transplantation[18,19]. In addition, higher risk patients tend to receive transplantation in a higher proportion than they did before. In the view of higher risk patients, they tend to carry the risk factors that accompany with older age such as cardiovascular diseases.

In transplant centers, AF and liver transplantation are entities that we commonly encounter in the practice[20-23]. However, the occurrence rates of preexisting AF and AF following liver transplantation as well as clinical outcomes of liver transplant patients with AF remain unclear[20-31]. Thus, we conduct this meta-analysis: (1) to assess prevalence of pre-existing AF and/or incidence of AF following liver transplantation, and the trends of patient's outcomes overtime; and (2) to evaluate impact of pre-existing AF and post-operative AF on patient outcomes following liver transplantation.

MATERIALS AND METHODS

Search strategy and literature review

We registered this systematic review protocol with International Prospective Register of Systematic Reviews, No. CRD42018093644 (PROSPERO). We conducted a systematic literature search of EMBASE (between January 1988 and March 2018), Ovid MEDLINE (between January 1946 and March 2018), and the Cochrane Database of Systematic Reviews (from database inception to March 2018): (1) to estimate prevalence of pre-existing AF and/or incidence of AF following liver transplantation; and (2) to evaluate impact of pre-existing AF and post-operative AF on patient outcomes following liver transplantation. Ronpichai Chokesuwattanaskul and Charat Thongprayoon, two investigators, independently performed the systematic literature review using the search strategy that consolidated the terms of "liver" OR "hepatic" AND "transplant" OR "transplantation" AND "atrial fibrillation", described in online supplementary data 1. No language restriction was implemented. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE)[32] and the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement[33].

Study selection

Our inclusion criteria comprised: (1) clinical trials or observational studies such as cohort, cross-sectional, or case-control studies; (2) available data on prevalence of pre-existing AF or incidence of AF following liver transplantation or outcomes of liver transplant recipients with AF; and (3) available data on prevalence, incidence, odds ratios (OR), hazard ratios, or relative risks. Retrieved articles were individually reviewed for eligibility by the two investigators as mentioned prior. Inclusion was not restricted by the size of study. Contrarieties were discussed and solved through joint agreement. We used Newcastle-Ottawa quality assessment scale to assess the quality of study for cohort and case-control studies[34], as shown in Table 1.

Data items and data collection process

We used a structured information collecting form to collect the data from individual article including last name of the first investigator, title, year of publication, country that the research was carried out, baseline characteristics of liver transplant patients, processes utilized to diagnose AF, prevalence of pre-existing AF, incidence of post-operative AF, patient outcomes following liver transplantation.

Statistical analysis

We used Comprehensive Meta-Analysis Version 3.3.070 software (Biostat Inc., Englewood, NJ, United States) for all analyses. Estimated prevalence, incidence and estimated risks from each study were incorporated by the random-effect, generic inverse-variance approach of DerSimonian and Laird[35]. Given the possibility of between-study variance, we used a random-effect model rather than a fixed-effect model. Cochran's *Q* test and I^2 statistic were implemented to assess heterogeneity caused by between-study differences. I^2 values of 0%-25% indicate insignificant heterogeneity. I^2 values of 26%-50% indicate low heterogeneity. I^2 values of 51%-75% indicate moderate heterogeneity and I^2 value of 76%-100% indicate high heterogeneity[36]. Egger test was used to evaluate publication bias[37].

RESULTS

Study selection and characteristics

Applying our search strategy, 121 potential studies were selected. Following the elimination of 83 studies (title and abstract clearly not meeting inclusion criteria due to study design, type of study, patient population or reported outcomes), 38 studies were included for complete examination. After the complete review, twenty articles were omitted because the outcome of interest was not provided and six articles were excluded since they were descriptive studies without data of interest. Hence, we included 12 articles[20-31] into the final analysis including 9 cohort studies[20,22-24,26-30] and 3 case-control studies[21,25,31] with 38586 liver transplant recipients were enrolled, as demonstrated in Figure 1. Study characteristics and quality appraisal of studies are shown in Table 1[20-31].

Prevalence of pre-existing AF and incidence of AF following liver transplantation

Overall, the pooled estimated prevalence of pre-existing AF in patients undergoing liver transplantation was 5.4% [95% confidence intervals (CI): 4.9%-5.9%, I^2 = 66%, Figure 2]. The pooled estimated prevalence of pre-existing AF in patients undergoing liver transplantation was 5.4% (95%CI: 4.4%-6.5%, I^2 = 8%) in case-control studies and 5.4% (95%CI: 4.9%-6.0%, I^2 = 75) in cohort studies, respectively, when analysis was conducted based on type of study. The pooled estimated incidence of AF following liver transplantation was 8.5% (95%CI: 5.2%-13.6%, I^2 = 99%, Figure 3). When analysis was performed based on type of study, the pooled estimated incidence of AF following liver transplantation was 9.4% (95%CI: 5.5%-15.6%, I^2= 73%) in case-control studies and 5.3% (95%CI: 1.6%-16.3%, I^2= 99%) in cohort studies, respectively.

Meta-regression analyses were performed and showed no significant correlations between year of study and either prevalence of pre-existing AF (P = 0.08) or post-operative AF after liver transplantation (P = 0.54), as shown in Figures 4 and 5.

Outcomes of liver transplant recipients with AF

Data on the association between pre-existing AF and the risk of mortality were limited in two studies[20,21].

Table 1 Main characteristic of studies included in meta-analysis of atrial fibrillation and liver transplantation

	Fouad *et al*[24]	VanWagner *et al*[25]	Nicalau-Raducu *et al*[26]	Josefsson *et al*[27]
Country	Canada	United States	United States	Sweden
Study design	Retrospective Cohort	Case-Control	Retrospective Cohort	Retrospective Cohort
Yr	2009	2012	2014	2014
Total number	197	242	389	186
Mean age ± SD	56	55	55	52
Duration (yr)	6 mo	1 yr	3.4	4
Outcome definition	Cardiac complication after LTx	CV complication after LTx	Early (< 1 yr) and Late (> 1 yr) post LTx AF	Incident cardiac event post LTx
Outcome ascertainment	Review EKG in medical records	EKG, Echo, LHC, RHC, DSE as indicated	Review medical records	Review medical records
Incidence of pre-operative AF	NA	All 12/242 (5.0%) NASH 7/115 (6.1%) Alcohol 5/127 (3.9%)	NA	Atrial fibrillation/flutter 4/186 (2.2%)
Incidence of post-operative AF	Intraoperative 1/197 (0.5%) Early postoperative (0-30 d) 3/197 (1.5%) Late postoperative (1-6 mo) 2/197 (1.0%)	All 21/242 (8.7%) NASH 11/115 (9.6%) Alcohol 10/127 (7.9%)	All 12/389 (3.1%) Early (< 1 yr after transplant) 10/389 (2.6%) Late (> 1 yr after transplant) 2/389 (0.5%)	Arrhythmia (mainly AF or flutter) All 36/186 (19.4%) Peri-transplant 24/186 (12.9%) Late 12/186 (6.5%)
Outcomes	NA (study aim to identify predictor of cardiac complication 6 mo after LTX)	NA (study aim to compare CV event between liver disease before liver transplant)	NA (study demonstrated target DSE prior liver transplant associated with increased risk of AF)	NA (study aim to assess pretransplant EKG as a predictor of post liver transplant event)
Confounder adjustment	NA	NA	NA	NA
Newcastle-Ottawa scale	S3 C0 O3	S4 C2 O3	S3 C3 O3	S4 C2 O3
	Vannucci *et al*[20]	**Bargehr *et al*[21]**	**Xia *et al*[22]**	**Piazza *et al*[23]**
Country	United States	United States	United States	Italy
Study design	Retrospective Cohort	Case-Control study	Retrospective Cohort	Retrospective Cohort
Yr	2014	2015	2015	2016
Total number	757	717	1387	143
Mean age ± SD	57.9 ± 6.8	58	54	55
Duration (yr)	1 yr	NA	30 d	3
Outcome definition	30 d and 1-yr survival after Liver Tx.	Cardiac complication after LTX	POAF (postoperative AF in LTX)	Incident AF (also other CVE) in NASH and alcoholic s/p LTx
Outcome ascertainment	Medical records	Review Medical records	EKG, Holter and medical records	Review medical records
Incidence of pre-operative AF	19/757 (2.5%)	32/717 (4.5%)	77/1387 (5.6%)	Alcoholic cirrhosis 2/65 (3.1%) NASH cirrhosis 3/78 (3.8%)
Incidence of post-operative AF	NA	1/63 (1.6%)	New onset AF within 30 d after LT 102/1387 (7.4%)	2/143 (1.4%)
Outcomes	1-mo mortality 5.29 (1.73-16.18) 1-yr mortality 3.28 (1.63-6.59)	Intraoperative cardiac complications 7.83 (1.94-31.49) Mortality 1.50 (0.61-3.69)	Median Hospital stays 31 d (16-67) in POAF *vs* 20 d (12-37) AKI 2.5 (1.06-5.70) Mortality 2.36 (1.45-3.85) Graft failure 2.28 (1.44-3.59)	NA (study aim to compare outcome as CV event after liver transplant between patients with NASH and those with alcoholic cirrhosis who receive liver transplant)
Confounder adjustment	NA	Age, MELD, donor risk index, DM	Age, MELD, intraoperative blood transfusion	NA
Newcastle-Ottawa scale	S3 C0 O3	S4 C2 E3	S4 C2 O3	S4 C2 O3

	VanWagner *et al*[28]	VanWagner *et al*[31]	VanWagner *et al*[29]	Wange *et al*[30]
Country	United States	United States	United States	Sweden
Study design	Retrospective Cohort	Case Control	Retrospective Cohort	Retrospective Cohort
Yr	2016	2017	2018	2018
Total number	32810	1024	671	63
Mean age ± SD	55 ± 10	56	Various by renal disease classification group	45
Duration (yr)	90 d	1 yr	NA	10
Outcome definition	MACE after Liver transplantation	CVD complication *vs* No CVD complication group	1-yr CV complication	Incident AF post LTx who survive > 3 yr (LTx ATTRm amyloidosis)
Outcome ascertainment	Medical record in patient admitted by MACE	EKG, Holter and medical records	Medical record	Echo and Holter every visit
Incidence of pre-operative AF	1969/32810 (6.0%)	62/1024 (6.1%)	2145/37322 (5.7%)	1/63 (1.6%)
Incidence of post-operative AF	204/32810 (0.6%)	130/1024 (12.7%)	65/671 (9.7%)	Incident AF 20/63 (31.7%) All AF post-op 21/63 (33.3%) (Median diagnosis 2 yr)
Outcomes	Pre-transplant AF and 30-d MACE (MI, HF, AF, cardiac arrest, PE, stroke) 6.9 (5.0-9.6) Pre-transplant AF and 90-d MACE 6.1 (4.5-8.3)	Pre-transplant AF and CVD complication 8.96 (3.70-22.0)	NA (study aim to assess degree of renal disease to 1-yr CV outcome in liver transplant patient)	Cerebrovascular events (TIA, ischemic stroke, intracerebral hemorrhage, subarachnoid hemorrhage) 3.8 (1.1-9.5)
Confounder adjustment	Sex, age, history of stroke, type of cirrhosis, and pre-transplant creatinine	Age, sex, race, working status, education, respiratory failure on ventilator at transplant, pulmonary hypertension, HCC, hypertension, DM, heart failure	NA	Cardiomyopathy, ischemic heart disease
Newcastle-Ottawa scale	S3 C0 O3	S4 C2 E3	S4 C2 O3	S3 C0 O3

ATTRm: Amyloid-forming variant Transthyretin proteins; CVD: Cardiovascular disease; HCC: Hepatocellular carcinoma; HF: Heart failure; MACE: Major adverse cardiovascular event; MI: Myocardial infarction; AF: Atrial fibrillation; DSE: Dobutamine stress echocardiogram; EKG: Electrocardiogram; LTX: Liver transplantation; NASH: Nonalcoholic steatohepatitis; NA: Not available; RA: Right atrium; S: Selection; C: Comparability; O: Outcome; AKI: Acute kidney injury; POAF: Post-operative atrial fibrillation; MELD: Model for End Stage Liver Disease.

The pooled OR of mortality among liver transplant recipients was 2.34 (95%CI: 1.10-5.00, I^2 = 45%). In addition, pre-existing AF is associated with postoperative cardiovascular complications among liver transplant recipients (3 studies[21,28,31]; OR: 5.15, 95%CI: 2.67-9.92, I^2 = 64%). New onset AF is associated with poor outcomes after liver transplantation[22,30]. Wange *et al*[30] demonstrated a significant association between incident AF and cerebrovascular events in liver transplant patients with OR of 3.80 (95%CI: 1.10-9.50). In addition to increased mortality risk, Xia *et al*[22] demonstrated significant associations of new-onset AF with post-transplant acute kidney injury (OR: 2.50, 95%CI: 1.06-5.70), and increased risk of graft failure (OR: 2.28, 95%CI: 1.44-3.59) among liver transplant recipients.

Risk of bias across studies

Funnel plots, as demonstrated in Supplementary Figures 1 and 2, and Egger tests were conducted to assess for possibility of publication bias in analyses evaluating prevalence of pre-existing AF and incidence of postoperative AF in liver transplant patients, respectively. The graph is somewhat asymmetric and implies the possibility of publication bias towards negative studies in analysis of prevalence of pre-existing AF (P = 0.01). However, we found no significant publication bias in analysis evaluating incidence of postoperative AF in liver transplant patients, P = 0.32.

DISCUSSION

In this meta-analysis, we demonstrated that end stage liver disease patients who received liver transplantation had a prevalence of AF of 5.6%, which was higher than prevalence of AF in general patient population of 2.5%[38]. This number of higher prevalence may imply that patients who received liver transplantation appeared to carry the higher risk profiles. In addition, our study showed the pooled incidence of post-liver transplant AF of 8.5%, which is lower incidence, when compared to those patients who underwent heart transplantation (incidence of AF up to 40%)[39-44] or other open-heart surgeries (incidence of AF up to 50%)[5,45,46].

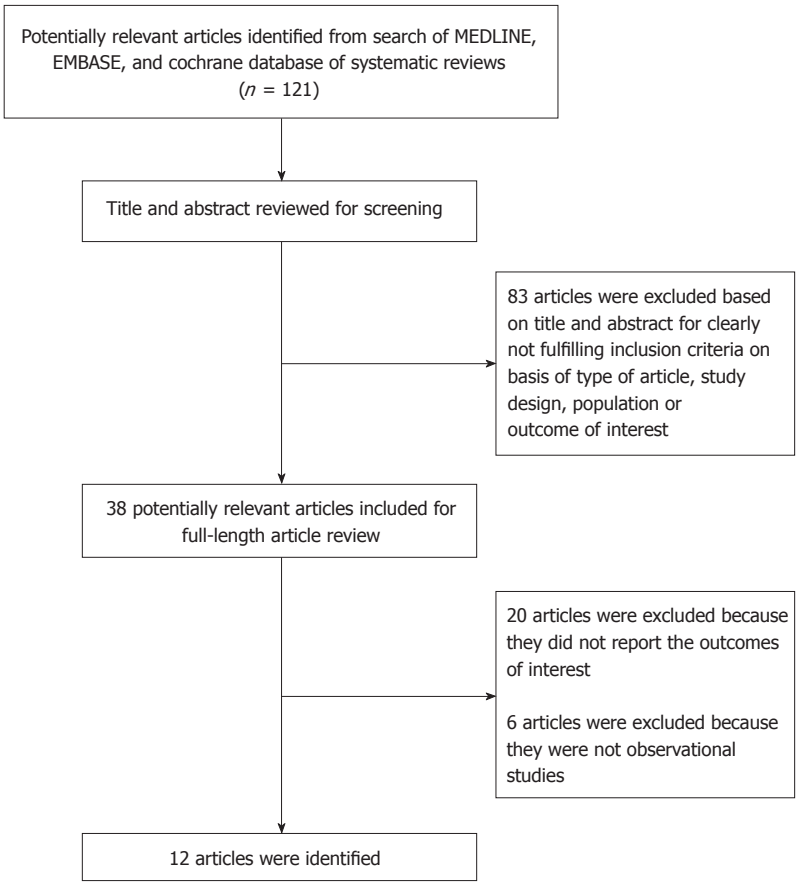

Figure 1 Outline of our search methodology.

Group by Type of study	Study name	Event rate	Lower limit	Upper limit	Z-Value	*P*-Value	Relative weight
Case-Control	Bargehr *et al*	0.045	0.032	0.062	-16.940	0.000	31.66
Case-Control	VanWagner *et al* (1)	0.050	0.028	0.085	-9.973	0.000	12.51
Case-Control	VanWagner *et al* (3)	0.061	0.047	0.077	-20.926	0.000	55.83
Case-Control		0.054	0.044	0.065	-26.917	0.000	
Cohort	Josefsson *et al*	0.022	0.008	0.056	-7.553	0.000	1.32
Cohort	Piazza	0.035	0.015	0.081	-7.288	0.000	1.61
Cohort	Vannucci	0.025	0.016	0.039	-15.750	0.000	5.55
Cohort	VanWagner *et al* (2)	0.060	0.057	0.063	-118.365	0.000	37.75
Cohort	VanWagner *et al* (4)	0.057	0.055	0.060	-125.774	0.000	37.94
Cohort	Wange *et al*	0.016	0.002	0.104	-4.094	0.000	0.34
Cohort	Xia *et al*	0.056	0.045	0.069	-24.168	0.000	15.50
Cohort		0.054	0.049	0.060	-48.512	0.000	
Overall		0.054	0.049	0.059	-55.479	0.000	

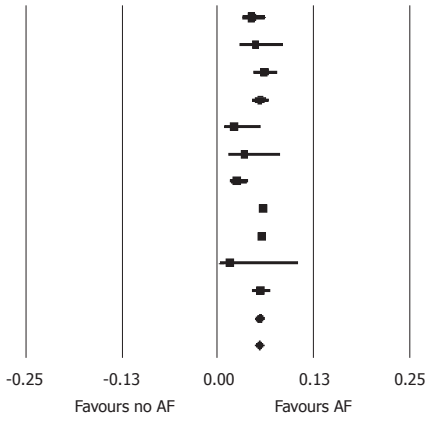

Figure 2 Forest plots of the included studies assessing prevalence of pre-existing atrial fibrillation in patients undergoing liver transplantation. AF: Atrial fibrillation.

This mitigated number of incidence of postoperative AF in liver transplantation could be explained by the use of intensive postoperative hemodynamic care and, immunosuppressive therapy, the surgical technique, and not physically direct impact to the heart[28-31].

In general population, AF can put the patients at higher mortality risk, compared to those without AF[47]. In addition to mortality risk, our study also revealed the association of pre-existing AF and incident AF with poor clinical outcomes following liver transplantation. New-onset AF following liver transplantation is also associated with post-transplant acute kidney injury, cerebrovascular events, and increased risk of graft failure among liver transplant recipients. There are several mechanisms that put the liver transplant patients with AF at higher risk of postoperative morbidity and mortality compared

Group by Type of study	Study name	Statistics for each study					Event rate and 95%CI	Relative weight
		Event rate	Lower limit	Upper limit	Z-Value	*P*-Value		
Case-Control	Bargehr *et al*	0.016	0.002	0.104	-4.094	0.000		7.32
Case-Control	VanWagner *et al* (1)	0.087	0.057	0.129	-10.307	0.000		41.00
Case-Control	VanWagner *et al* (3)	0.127	0.108	0.149	-20.542	0.000		51.69
Case-Control		0.094	0.055	0.156	-7.724	0.000		
Cohort	Fouad *et al*	0.025	0.011	0.060	-8.053	0.000		12.15
Cohort	Josefsson *et al*	0.194	0.143	0.257	-7.690	0.000		12.79
Cohort	Nicolau-raducu *et al*	0.031	0.018	0.054	-11.756	0.000		12.59
Cohort	Piazza *et al*	0.014	0.004	0.054	-5.976	0.000		11.15
Cohort	VanWagner *et al* (2)	0.006	0.005	0.007	-72.247	0.000		12.91
Cohort	VanWagner *et al* (4)	0.097	0.077	0.122	-17.105	0.000		12.86
Cohort	Wange *et al*	0.333	0.228	0.458	-2.594	0.009		12.65
Cohort	Xia *et al*	0.074	0.061	0.089	-24.629	0.000		12.89
Cohort		0.053	0.016	0.163	-4.518	0.000		
Overall		0.085	0.052	0.136	-8.903	0.000		

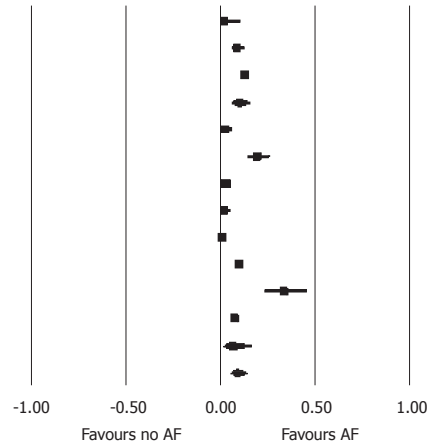

Figure 3 Forest plots of the included studies assessing incidence of atrial fibrillation following liver transplantation. AF: Atrial fibrillation.

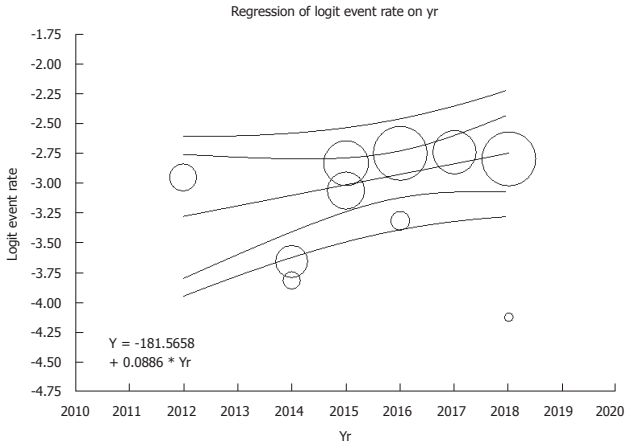

Figure 4 Meta-regression analysis showed no significant correlations between year of study and prevalence of pre-existing atrial fibrillation (*P* = 0.08).

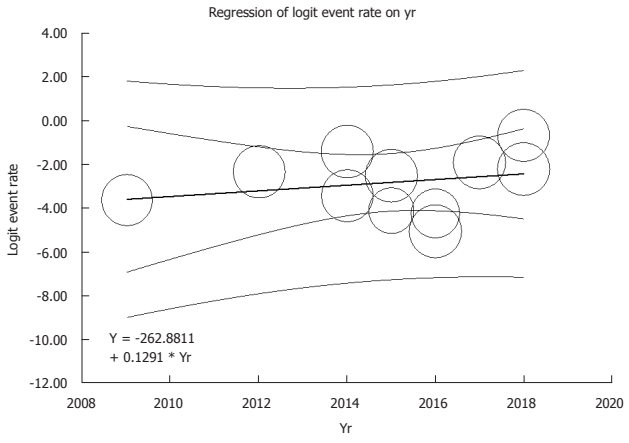

Figure 5 Meta-regression analysis showed no significant correlations between year of study and incidence of post-operative atrial fibrillation after liver transplantation (*P* = 0.54).

to those without AF[24,48]. Patients with AF reflect that they are frail and have already been at higher risk profiles accompanying with other cardiovascular risks (left ventricular hypertrophy, heart failure, stroke, *etc*.) at the time even before liver transplantation, so that they will inevitably develop higher complication rates at postoperative period[21,49,50]. Furthermore, AF itself plays a critical role as marker of underlying heart diseases that make patients vulnerable to perioperative hemodynamic challenges[51,52].

There are also several mechanisms explained why liver transplantation promotes the occurrence of AF during postoperative period (Figure 6). Firstly, conventional postoperative hemodynamic challenge could provoke AF through hemodynamic instability or inotropic administration[50]. Also, some preexisting liver diseases, such as nonalcoholic fatty liver disease (NAFLD), share a common risk factor, that is diabetes and obesity, with the AF patients[53]. In addition, NAFLD could also occur as de novo after liver transplantation and subsequently enhances the postoperative complications, contributed by systematic inflammatory mechanism[54-56]. Furthermore, immunosuppressive therapy increases the risk to develop insulin resistance which eventually leads to metabolic syndrome[57]. Various kind of cirrhosis-specific heart diseases, such as a well-known entity called congestive hepatopathy, prior to transplantation play a substantial arrhythmogenesis role as a substrate for pathogenesis of AF[50,58]. Various underlying medical problems including AF would, in the future, be used to identify high-risk patient population that needs to be optimized the treatment to achieve higher outcome after liver transplantation.

Leading cause of long term mortality in patients with liver transplantation is cardiovascular complications which, other than AF, include heart failure and myocardial infarction. These complications are predominantly driven by the development of metabolic syndrome after liver transplantation. However, this topic of interest is beyond the scope of our study and

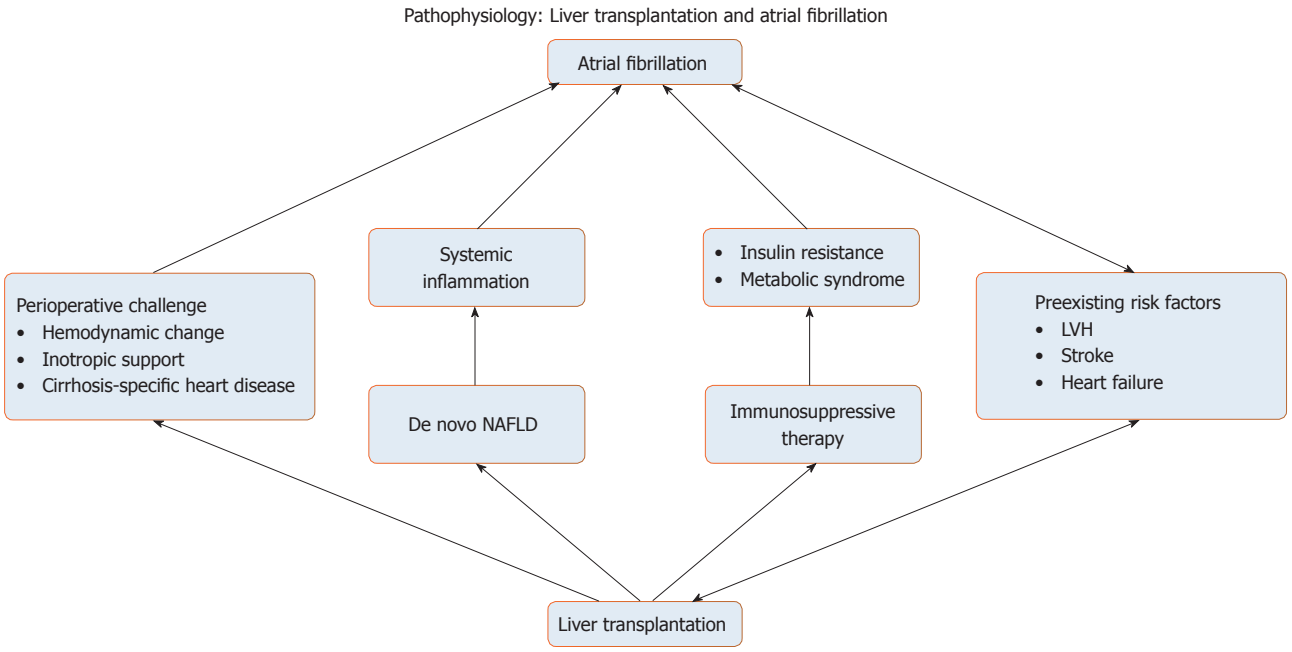

Figure 6 Potential mechanisms of atrial fibrillation in liver transplantation. NAFLD: Nonalcoholic fatty liver disease; LVH: Left ventricular hypertrophy.

could be explained elsewhere[50]. More or less, these cardiovascular complications were also considered as potential risk modification strategy that should not be overlooked. Our study has noteworthy limitations. Firstly, an inconsistent in definition, for an example how to define the timing of AF as an early or late onset, among the different studies preclude to draw the generalized conclusion. Such this limitation, data use needs tailoring to the individual patient. Secondly, duration of follow up during the postoperative period by some study prospectively monitored a cardiovascular event for just 30 d post-transplantation, which this time frame does not long enough to reveal the long-term morbidity and mortality outcome. However, with the potential of higher morbidity and mortality in liver transplant patients with AF by our meta-analysis, future studies, preferable with population-based or national database studies, are required to discover whether focused AF cares for liver transplanted patients can improve patient outcomes after liver transplantation. Finally, since our study is a meta-analysis of observational studies, it could entirely prove association, but could not demonstrate a cause-effect (causal) relationship, between liver transplantation and AF.

In conclusion, our study demonstrated the actual prevalence of preexisting AF in patient underwent liver transplantation, incidence of AF post-liver transplantation. Our study also highlighted the association of AF with higher morbidity and mortality among liver transplant recipients. Further well-designed studies are needed to explore the impact of AF in liver transplant patients, which we strongly believe that AF management, specified to liver transplant patients, would be an important strategy to augment standard of care in this particular population.

ARTICLE HIGHLIGHTS

Research background

Among liver transplant patients with atrial fibrillation (AF), there are lacks of data about incidence, prevalence and prognosis of AF in this specific group of patients. In spite of improvement of liver transplant care to the point of achieving almost 90% of 1-year survival rate, outcomes of liver transplantation related to AF remain unclear.

Research motivation

With excellent results of liver transplantation in term of survival, current indications of the transplantation have been extending into higher risk candidates due to higher amount of donors and more advanced treatment, which include preoperative preparation, surgical technique, immunosuppressive therapy and post-transplantation care. The high-risk liver transplant candidates tend to experience the adverse effects throughout perioperative period and worse outcomes, compared to those with less comorbidity. AF is one of the most common cardiac rhythm abnormalities and its prevalence increases with older age and higher comorbidities. Therefore, a number of patients with AF who received liver transplantation would definitely increase.

Research objectives

To examine outcomes of liver transplant recipients with AF, we performed this meta-analysis: (1) to assess prevalence of pre-existing AF and/or incidence of AF following liver transplantation, and the trends of patient's outcomes overtime; and (2) to evaluate impact of pre-existing AF and post-operative AF on patient outcomes following liver transplantation. Innovations and breakthroughs.

Research methods

We conducted a systematic literature search of EMBASE, Ovid MEDLINE, and the Cochrane Database (from database inception to March 2018): (1) to estimate prevalence of pre-existing AF and/or incidence of AF following liver transplantation; and (2) to evaluate impact of pre-existing AF and post-operative AF on patient outcomes following liver transplantation. Estimated prevalence, incidence and estimated risks from each study were incorporated by the random-effect, generic inverse-variance approach of DerSimonian and Laird.

Research results

There were significant associations of AF with worse clinical outcomes following liver transplantation including 2.3-fold higher risk of death and 5.1-fold higher risk of postoperative cardiovascular complications, and poor clinical outcomes such as stroke, acute kidney injury and graft failure. We also showed the incidence of postoperative AF, namely 8.5%, consistently across different type of studies without the change overtime by meta-regression.

Research conclusions

The overall estimated prevalence of pre-existing AF and incidence of AF following liver transplantation are 5.4% and 8.5%, respectively. Incidence of AF following liver transplant does not seem to decrease overtime. Pre-existing AF and new-onset AF are potentially associated with poor clinical outcomes post liver transplantation.

Research perspectives

This systematic review confirmed higher risks of death and postoperative complications in liver transplant patients with AF. Our findings indicate that AF may be an independent predictor for worse clinical outcomes following liver transplantation.

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CASE REPORT

Proton beam therapy in apneic oxygenation treatment of an unresectable hepatocellular carcinoma: A case report and review of literature

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Abstract

Presented here is the clinical course of a 63-year-old patient with a central, large and unresectable hepatocellular carcinoma (HCC) with liver metastases and tumor invasion of the portal and hepatic veins. After the tumor had been diagnosed, the patient was immediately treated with proton beam therapy (PBT), at a total dose of 60 Gy (relative biological effectiveness) in 20 fractions administered within 4 wk. To manage the respiratory movements, at the Rinecker Proton Therapy Center, apneic oxygenation was given daily, under general anesthesia. The patient tolerated both the PBT and general anesthesia very well, and did now show any signs of acute or late toxicity. The treatment was followed by constant reductions in the tumor marker alpha-fetoprotein and the cholestatic parameters gamma-glutamyltransferase and alkaline phosphatase. The patient commenced an adjuvant treatment with sorafenib, given at 6-wk intervals, after the PBT. Follow-up with regular magnetic resonance imaging has continued for 40 mo so far, demonstrating remarkable shrinkage of the HCC (maximal diameter dropping from approximately 13 cm to 2 cm). To date, the patient remains free of tumor recurrence. PBT served as a safe and effective treatment method for an unresectable HCC with vascular invasion.

Key words: Particle therapy; Proton beam therapy; Apneic oxygenation; Unresectable; Vascular invasion; Hepatocellular carcinoma; Intrahepatic metastasis

Core tip: Hepatocellular carcinoma (HCC) is one of the most common cancers in Asia. Patients with unresectable tumor disease require more options for in-principle curative therapies. We report here a patient with a large unresectable HCC due to vascular invasion and satellite metastases, who showed remarkable tumor shrinkage after completing proton beam therapy 4 years ago and who is still free of tumor recurrence to date.

Lin YL. Proton beam therapy in apneic oxygenation treatment of an unresectable hepatocellular carcinoma: A case report and review of literature. *World J Hepatol* 2018; 10(10): 772-779 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i10/772.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i10.772

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumor diseases worldwide, with particularly high incidence in the Asia-Pacific region, and is often associated with liver cirrhosis owing to alcohol abuse and chronic viral hepatitis[1]. Surgical resection and liver transplantation are the first-line curative treatments for large HCC, while small HCC (preferably < 2 cm in diameter) in an accessible location, away from critical structures, can be cured by local ablative techniques. For patients with large unresectable tumors, such as those with advanced tumor extension and vascular invasion, other locoregional treatment modalities are usually considered as palliative options; these include arterial catheter-based treatments, ablative techniques and radiation therapy.

In past years, many convincing results were published on the effectiveness and tolerability of charged-particle therapy with dose-escalating proton and heavy ion beam as a curative-intent treatment of unresectable HCC[2-4]. Based on the physical property of Bragg peak, proton beams deposit the dose maximum at a predefined depth in the tumor. Behind the maximal deposit, the dose drops rapidly, having no exit dose. This is a significant advantage in comparison to photon radiotherapy and enables the dose escalation to improve the local control. Because the surrounding uninvolved liver parenchyma can be spared from the unnecessary radiation dose, even larger tumor volume can be treated with proton beam therapy (PBT) without risk of fatal radiation-induced liver disease (RILD)[5,6].

CASE REPORT

In November 2013, a routine abdominal sonography of a 63-year-old German male revealed a huge tumor in the liver. Subsequent magnetic resonance imaging (MRI) and computed tomography (CT) scan of the abdomen revealed the main tumor mass to be of about 13.4 cm × 7.1 cm in size, encompassing segments Ⅰ, Ⅳ, Ⅴ and Ⅷ, as well as satellite metastases in segments Ⅱ and Ⅲ. The tumor involved all three hepatic veins and the portal vein, compressing the inferior vena cava (Figure 1). Liver biopsy, taken in January 2014, confirmed a diagnosis of HCC, Edmondson-Steiner-grade Ⅱ, with partially cirrhotic parenchymal modification. The additional esophagogastroduodenoscopy and colonoscopy demonstrated chronic gastritis with helicobacter pylori infection and sigmoid diverticulosis, but no evidence of other gastrointestinal malignancy. The patient reported not feeling any discomfort but having lost 12 kg of weight, which he had attributed to a dietary prescription to address his decompensated diabetes mellitus. Blood test (hepatic function panel) showed remarkably increased levels of gamma-glutamyltransferase (GGT) and alkaline phosphatase (AP), and moderately elevated levels of total bilirubin, glutamic-oxalocetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and cholesterol.

Because the initial consulting hospital classified this case of HCC as Child-Pugh score A, BCLC stage B, palliative treatments with transarterial chemoembolization (TACE) and sorafenib were recommended in the tumor board review. However, extended vascular invasion and tumor size > 10 cm are contraindications for TACE. The patient was informed about best supportive care and approximate survival time of 6 mo. He contacted our institute, the Rinecker Proton Therapy Center (RPTC), and was treated with PBT from February to March 2014. A total dose of 60.00 Gy (relative biological effectiveness, RBE) administered in 20 fractions within 4 wk was applied to both the main liver tumor and the satellite metastases, with a safety margin of 3 mm.

For precise targeting by PBT, special techniques to control the respiratory movements are required. At RPTC, we use apneic oxygenation (AO) with total intravenous anesthesia and oral intubation, which prolongs the safe apnea time during the irradiation (Figure 2). After preoxygenation, the artificial ventilation is stopped during the apneic phase. The patient stays connected, with delivery of 1 L/min oxygen and constant airway pressure. Due to the disproportion between the rate of oxygen removal from the alveoli compared to the rate of carbon dioxide delivery to them, the barometric pressure in the alveoli sinks, pushing the oxygen to move from the upper airway to the alveoli. That means, the consumed oxygen is replaced but the carbon dioxide is not removed, and the arterial partial pressure of carbon dioxide ($PaCO_2$) rises in the time following, in a range of 2-4 mmHg/min[7,8]. According to our standard operating procedure, the oxygen saturation during AO should not fall below 97%, and $PaCO_2$ is not allowed to exceed 61 mmHg.

The patient tolerated the PBT under general anesthesia daily, without any considerable toxicity

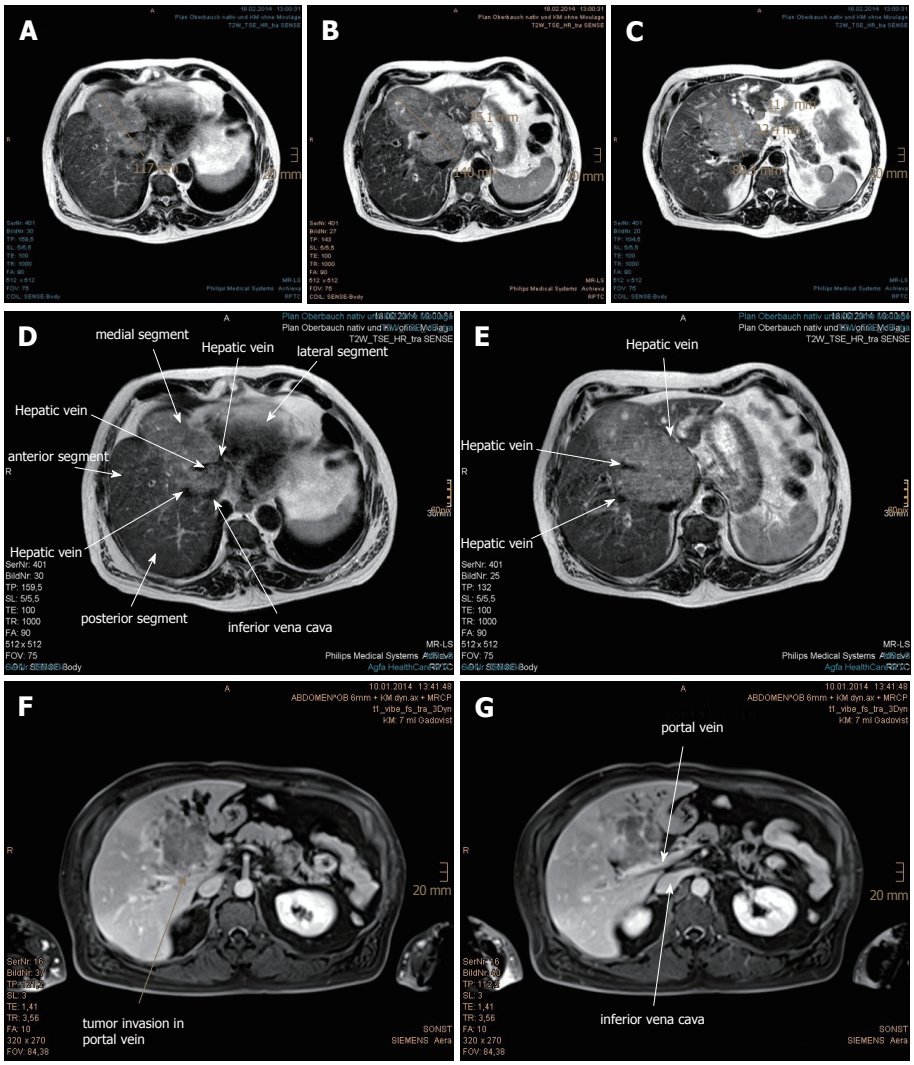

Figure 1 Large hepatocellular carcinoma with vascular invasion and satellite metastases in the magnetic resonance imaging scan prior to proton beam therapy. A: The upper portion of the main tumor abutting the heart in axial plane is shown; B: Extended hepatocellular carcinoma (HCC) with invasion of portal and hepatic veins in the axial plane is shown; C: The lower portion of the main tumor abutting the inferior vena cava is shown, with dilated segmental bile ducts and 2 liver metastases in the left hepatic lobe; D-G: The HCC involved all three hepatic veins as well as the portal vein. The inferior vena cava was also compressed.

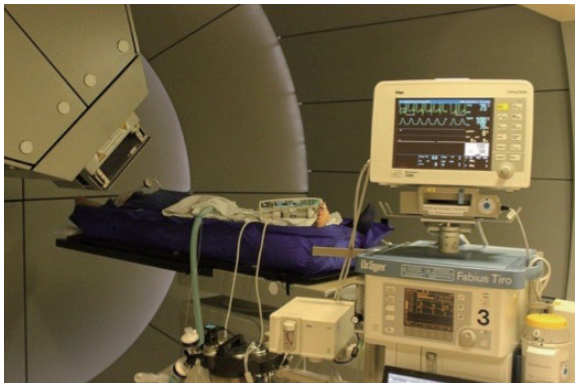

Figure 2 Proton beam therapy in use with apenic oxygenation under general anesthesia.

(Common Terminology Criteria for Adverse Events, grade 0). At the first follow-up (after 6 wk of treatment), the patient showed remarkable reduction in the tumor marker alpha-fetoprotein (AFP) (from 109 μg/L to 34 μg/L; normal range: < 15 ng/mL) and decrease in GGT (from 64 μkat/L to 25 μkat/L; normal range: < 1.00 μkat/L). From this time forward, the patient also commenced with targeted therapy, *i.e.*, tyrosine kinase inhibitor (sorafenib) and continued in his normal occupational activities (working as a driver). At 5 mo after the PBT, routine blood test showed leukocytosis (12.7 Gpt/L; normal range: 4.0-10.9 Gpt/L) with elevated C-reactive protein (101 mg/L; normal range: < 5.0 mg/L). Respiratory or urinary infection was excluded. The patient denied experience of fever but complained of night sweats, and was treated with antibiotics by his oncologist. Because of diarrhea, the sorafenib was stopped for a few months and restarted with the dosage halved (to 400 mg daily). At the end of 2014, both the leukocyte count and C-reactive protein level finally decreased and the GGT continued to fall (to 3.3 μkat/L). At 20 mo after the PBT, the AFP

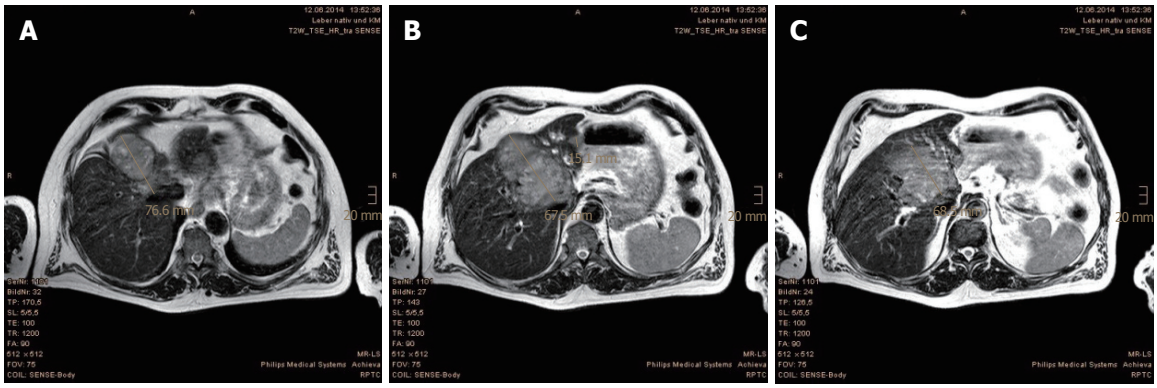

Figure 3 Tumor regression evidenced in the first follow-up magnetic resonance imaging scan at 3 mo after proton beam therapy. A-C: Size reductions of the main tumor and the liver metastases are shown, measured in three levels of axial plane.

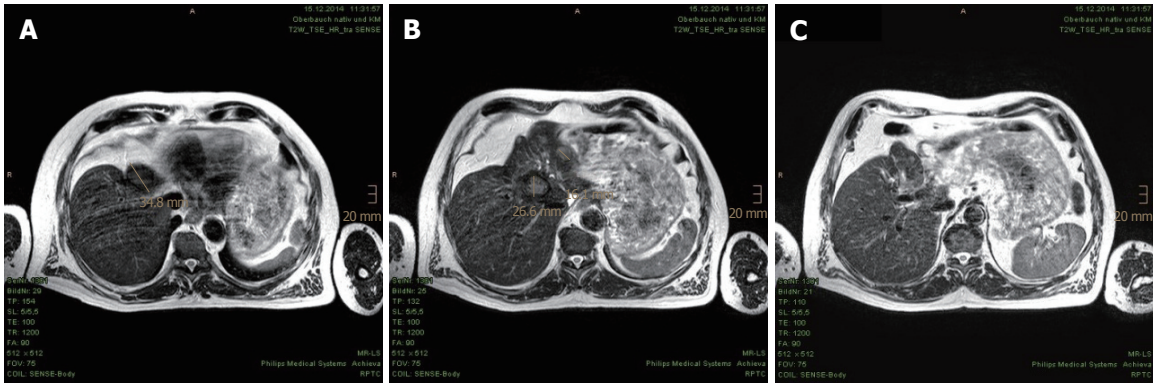

Figure 4 Continuous tumor shrinkage evidenced in the magnetic resonance imaging scan at 9 mo after proton beam therapy. A-C: The hepatocellular carcinoma and liver metastases presented as residual nodules without contrast enhancement.

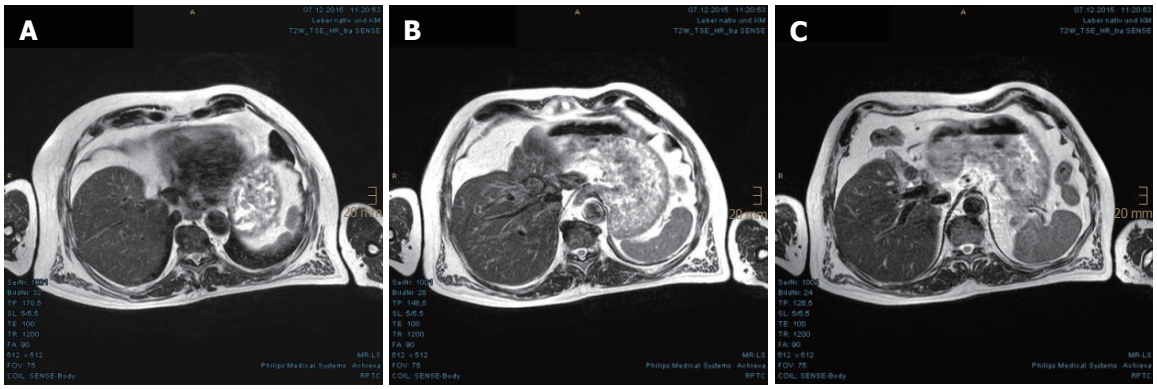

Figure 5 Continuous tumor shrinkage evidenced in the magnetic resonance imaging scan at 21 mo after proton beam therapy. A-C: Further size reductions of the residual tumor nodules in the axial plane are shown.

was within normal range (2.8 μg/L), remaining stable through the last measurement in June 2017 (2.5 μg/L). The thoracic CT scan at 19 mo after the PBT also did not reveal any suspicious metastasis.

In the first MRI control, taken 3 mo after the PBT, significant size reductions of the main tumor in the hepatic hilum and the satellite metastases in the left hepatic lobe were observed. The segmental cholestasis had also regressed, consistent with the falling GGT and AP (Figure 3). In the subsequent check-ups, continuous tumor shrinkage with indentation of the liver contour was observed, as if the patient had undergone a liver resection (Figure 4 and Figure 5). The latest MRI scan, performed in August 2017, revealed a residual nodule of approximately 2 cm × 2 cm without pathological enhancement in the hilar region (Figure 6). No signs of ascites, or new distant or lymph node metastases have been found in the 40-mo period following the PBT. To date, the patient is still receiving semi-dosed sorafenib and participating in

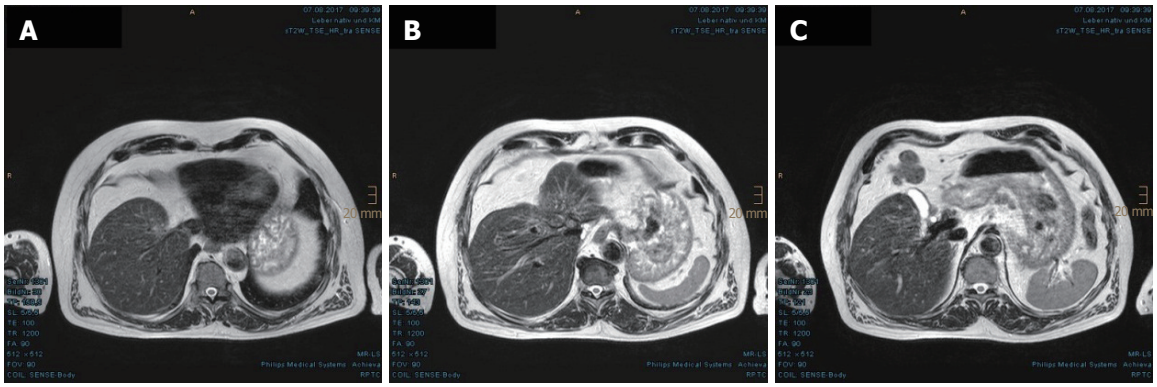

Figure 6 Residual nodules in the hepatic hilum without enhancement evidenced in the latest magnetic resonance imaging scan, at 40 mo after proton beam therapy. A: Complete regression of the upper portion of the hepatocellular carcinoma is shown; B: Pronounced indentation of the right-sided liver contour is shown, as if the patient had undergone a liver resection; C: The portal vein was able to be delimited again.

regular follow-up visits with his oncologist as well as in an annual MRI scan performed at the RPTC.

DISCUSSION

Patients with unresectable HCC are usually not deemed to be curable because surgical resection and liver transplantation are considered as the only curative options. According to guidelines, TACE and sorafenib are the standard of palliative care for unresectable HCC. Other local treatment methods, such as radiofrequency ablation, cryoablation and radioembolization, are often combined with the TACE and sorafenib regimen to improve the total response[9]. Yet, the therapeutic efficacy of combined treatments, such as that of radiofrequency ablation after TACE, is limited by the tumor size and morphology[10].

Katsanos *et al*[11] analyzed 55 randomized controlled trials on mono- or combined treatments with TACE for unresectable HCC and concluded that TACE in combination with external radiotherapy or local liver ablation may distinctly improve tumor response and the survival rates of patients. Choi *et al*[12] reviewed the different radiotherapy techniques and summarized the results of radiotherapy, partially combined with liver transplantation, TACE and concomitant chemotherapy, for HCC in each tumor stage. Radiotherapy can amend the total therapeutic assortments and outcomes of HCC by improving local control, enabling downstaging and treating unresectable HCC with vascular invasion or multiple intrahepatic metastases. The latest National Comprehensive Cancer Network (NCCN) guidelines[13] consider external-beam radiation therapy as a category 2B option for patients with unresectable HCC or those with contraindication for operation due to comorbidity. Besides, stereotactic body radiation therapy can be recommended as alternative to ablation and embolization techniques, particularly after their failure or in case of their contraindication. Because prospective randomized controlled trials evaluating the outcome of various techniques of external-beam radiation therapy *vs* ablation and arterially directed therapies are still pending or ongoing, clear guidelines of treatment recommendations for large unresectable HCC, specifically with vascular invasion and intrahepatic metastasis, are still missing. Mostly, only when further interventional treatments are no longer possible, will radiation therapy be considered.

The aim of modern radiotherapy techniques is to achieve delivery of an effective high-dose in the tumor, while sparing the surrounding structures as much as possible. If the liver function is restricted by coexistent liver disease, such as cirrhosis and/or viral hepatitis, the more essential the role of sparing the surrounding noncancerous liver tissues becomes. Crane *et al*[14] pointed out the challenges of radiotherapy for large HCC due to proximity of stomach and intestines, underlying liver disease, radiosensitivity of liver parenchyma and respiratory and interfractional motions. Because partial resection of the liver (with only 25% remnant) has been demonstrated as well tolerated by noncirrhotic patients[15], it allows radiation therapy of partial liver with higher doses if there is enough functional liver parenchyma left.

In order to prevent RILD, our institute uses the following dose constraints of liver: at least 700 cc of uninvolved liver parenchyma should receive less than 15 Gy (RBE), and the mean dose of liver should be less than 13 Gy (RBE). This is the Quantitative Analyses of Normal Tissue Effects in the Clinic (commonly known as QUANTEC) recommendation for stereotactic body radiation therapy of liver tumor with three fractions[16]. In the case of our patient, presented herein, the total liver volume prior to PBT was 2789 cc (including 638 cc gross tumor volume), and the mean dose of uninvolved liver parenchyma (*i.e.*, total liver volume with subtraction of clinical target volume) was 12.77 Gy (RBE). The volume of uninvolved liver that received less than 15 Gy (RBE) was approximately 1370 cc (Figure 7), while the remnant liver volume after tumor regression was 1470 cc, as measured by MRI scan at 40 mo after the PBT.

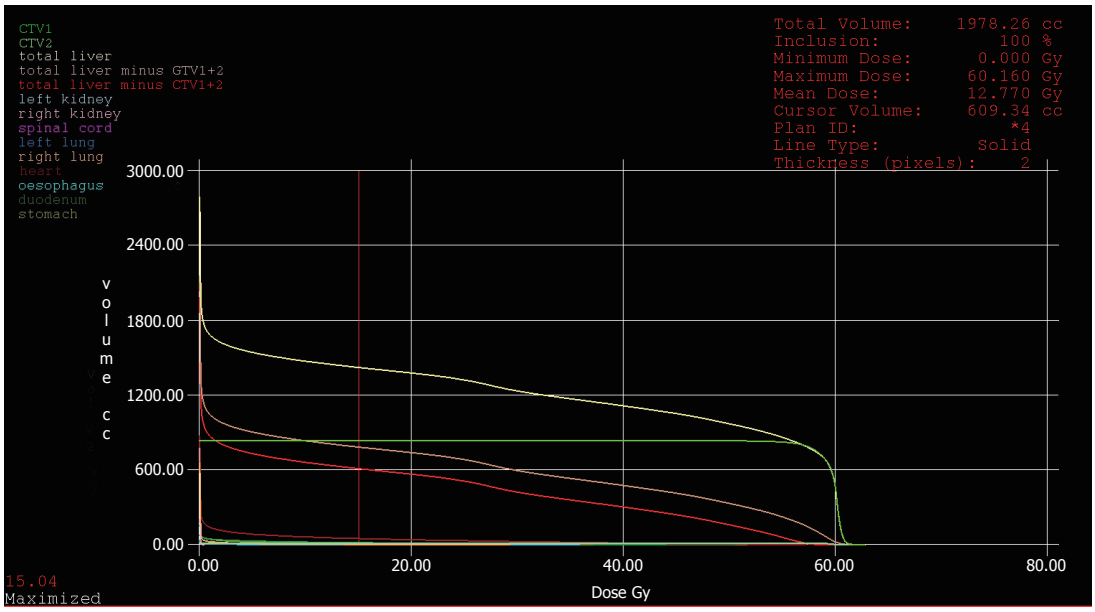

Figure 7 Dose-volume histogram of the clinical target volume and organs at risk.

Unlike photon radiotherapy, PBT requires less irradiation fields to encompass the target. Therefore, the dose burden of uninvolved liver parenchyma and other surrounding organs at risk, such as heart, stomach, intestines, spinal cord and kidney, as well as the risk of radiation-induced secondary malignancies is significantly reduced by the dosimetric advantage of proton beams[17,18]. Leung *et al*[19] compared the cost-utility of stereotactic radiation therapy and PBT, assuming PBT is a cost-effective therapy for inoperable advanced HCC due to the improved quality-adjusted life years. The spot scanning technique used at RPTC also facilitates more conformity to the extent of the target, in comparison to common scattering PBT[20]. In our case, we only used two irradiation fields, from 0 and 300 degrees, to embrace the main tumor in the central hepatic region and the satellite metastases in the left lobe (Figure 8). Irradiation of each field lasted 2.5-3.5 min.

The main challenge of radiotherapy for movable organs, in particular lungs and liver, is to manage the intra- and interfractional motion of the tumor. Measures like implantation of fiducial markers, respiratory gating or tracking, breath-hold method, abdominal compression and 4-dimensional CT simulation are applied in different institutions[21,22]. A unique feature of our center is the use of AO to control respiratory motion and to reduce the safety margin. To assess whether the patient can withstand apneic oxygenation, besides general preanesthesia evaluation, additional tests, such as body plethysmography, echocardiography and arterial blood gas test, can be required in case of relevant cardiopulmonary comorbidity. Since the inauguration of RPTC in 2009, we have treated over 500 patients with the apneic method. Up to the end of 2017, the total number of general anesthesia sessions was more than 6000, while the total time, including introduction and discharge, was 55 min on average. The longest period for AO was over 9 min, and the average apneic duration was approximately 3 min. Statistically speaking, the most treated tumor entities for the use of AO were thoracic malignancies and liver metastases.

Although the patient presented in our case report had several adverse prognostic factors, his case demonstrates that large unresectable HCC with vascular invasion and intrahepatic metastases can be treated excellently with PBT. RPTC has long-standing experience in PBT of movable tumors with AO, which securely solves the problem of organ motion, permits the reduction of safety margin and consequently the side effects, like RILD, and requires the least compliance of patients concerning breath control and abandonment of fiducial markers.

ARTICLE HIGHLIGHTS

Case characteristics

In a routine abdominal sonography of a 63-year-old asymptomatic male, a large central tumor in the liver was detected.

Clinical diagnosis

According to the abdominal sonography, the magnetic resonance imaging and the pathologically increased tumor marker alpha-fetoprotein and liver function panel, a hepatic malignancy was urgently suspected.

Differential diagnosis

Liver cirrhosis, cholangiocellular carcinoma, liver angiosarcoma, liver metastases of non-hepatic origin.

Laboratory diagnosis

The laboratory tests showed significant increase of the tumor marker alpha-fetoprotein and the cholestatic parameters gamma-glutamyltransferase and

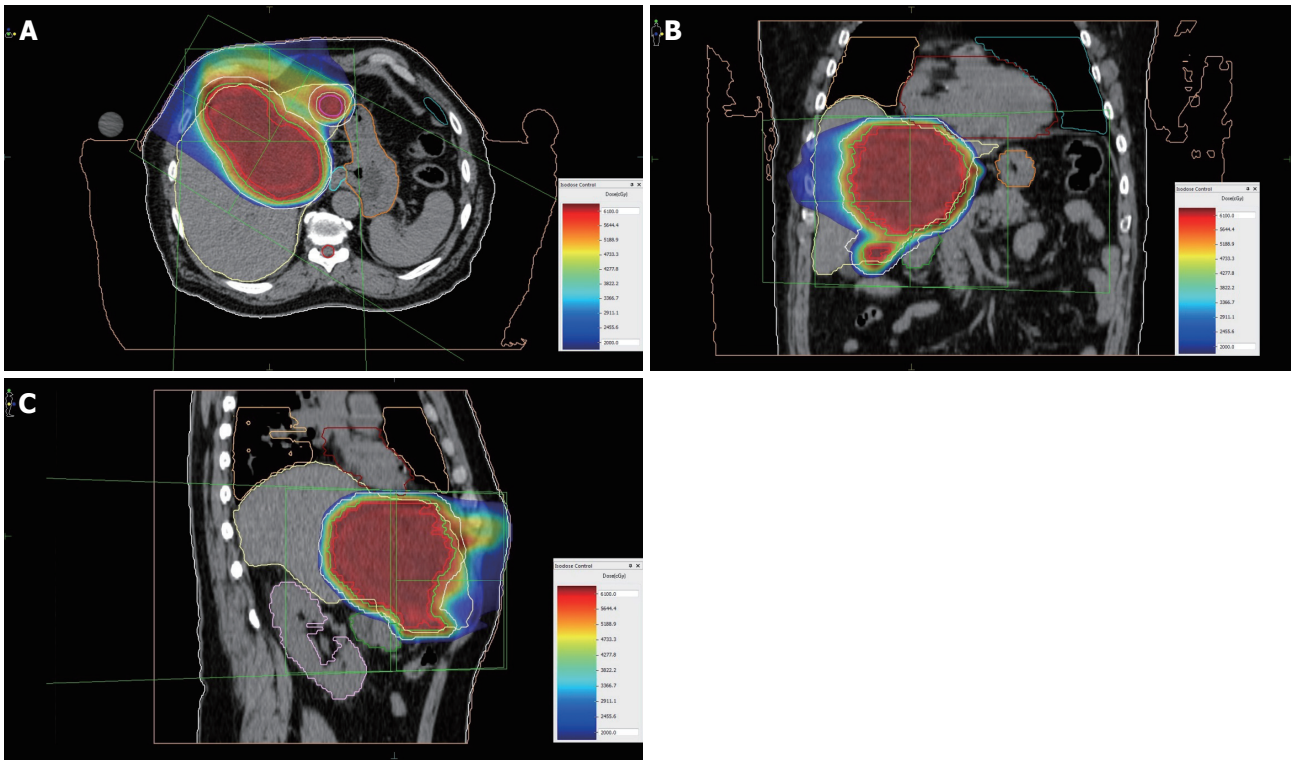

Figure 8 Treatment plan of proton beam therapy with isodose distributions. A: Axial plane; B: Coronal plane; C: Sagittal plane. Red line: Gross tumor volume; Green line: Clinical target volume.

alkaline phosphatase.

Imaging diagnosis

Magnetic resonance imaging and computed tomography of the abdomen revealed the main tumor mass to be of about 13.4 cm × 7.1 cm in size, encompassing segments Ⅰ, Ⅳ, Ⅴ and Ⅷ, as well as satellite metastases in segments Ⅱ and Ⅲ. The tumor involved all three hepatic veins and the portal vein, compressing the inferior vena cava.

Pathological diagnosis

A liver biopsy confirmed a diagnosis of hepatocellular carcinoma (HCC), Edmondson-Steiner-grade Ⅱ, with partially cirrhotic parenchymal modification.

Treatment

The patient was treated with proton beam therapy (PBT) in apneic oxygenation at the Rinecker Proton Therapy Center, at a total dose of 60 Gy (relative biological effectiveness) in 20 fractions.

Term explanation

We use the apneic oxygenation to manage respiratory motion of the tumor and to reduce the safety margin, which enables the sparing of uninvolved liver parenchyma and other surrounding organs at risk as well as a dose escalation.

Experiences and lessons

The patient is free of tumor recurrence in a period of 4 years after the treatment. PBT is a safe and effective therapy for large unresectable HCC with vascular invasion and intrahepatic metastases.

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CASE REPORT

Neuroendocrine tumor incidentally detected during living donor hepatectomy: A case report and review of literature

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Author contributions: Akbulut S, Isik B, and Cicek E designed the report; Akbulut S collected the patient's clinical data; Samdanci E provided histopathological information; Akbulut S and Yilmaz S analyzed the data and wrote the paper.

Informed consent statement: The patient involved in this study gave his informed written consent prior to study enrollment, authorizing the use and disclosure of his protected health information.

Conflict-of-interest statement: The author declares no potential conflict of interest.

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Abstract

To our best knowledge, no case of a tumor that was incidentally detected during living donor hepatectomy (LDH) has been reported in the English language medical literature. We present two cases in which grade I neuroendocrine tumors (NET) were incidentally detected during our twelve-year LDH experience. First Case: A 26-year-old male underwent LDH for his brother suffering from HBV-related chronic liver disease (CLD). After right lobe LDH, intestinal length was measured as part of a study concerning the relationship between small intestinal lengths and surgical procedure. At this stage, a mass lesion with a size of 10 mm × 10 mm was detected on the antimesenteric surface, approximately 90 cm proximal to the ileocecal valve. A wedge resection with primary intestinal anastomosis was performed. Second Case: A 29-year-old male underwent right lobe LDH for his father with hepatitis B virus (HBV)-related CLD. An abdominal exploration immediately prior to the closure of the incision revealed that the appendix vermiformis was edematous and had firmness with a size of 8-10 mm at its tip. An appendectomy was performed. The pathological examinations of the specimens of both patients revealed

grade 1 NET. In conclusion, even if patients undergoing LDH are healthy individuals, whole abdominal cavity should be gently palpated and all findings recorded after completing laparotomy. Suspected masses or lesions should be confirmed by frozen section examination. Such an approach would avert potential medicolegal issues.

Key words: Living donor hepatectomy; Incidental tumor; Neuroendocrine tumor; Chronic liver disease; Hepatitis B virus

Core tip: To our best knowledge, no case of a tumor that was incidentally detected during living donor hepatectomy (LDH) has been reported in the English language medical literature. Herein, we present two cases in which grade I neuroendocrine tumors were incidentally detected during our twelve-year LDH experience. Even if patients undergoing LDH are healthy individuals, the whole abdominal cavity should be gently palpated and all findings be recorded after completing laparotomy. Suspected masses or lesions should be confirmed by frozen section examination. Such an approach would avert potential medicolegal issues.

Akbulut S, Isik B, Cicek E, Samdanci E, Yilmaz S. Neuroendocrine tumor incidentally detected during living donor hepatectomy: A case report and review of literature. *World J Hepatol* 2018; 10(10): 780-784 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i10/780.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i10.780

INTRODUCTION

While deceased donors are an important part of the liver donor pool in western countries, living donors constitute an important portion of the donor pool in many Asian countries including Turkey[1]. The most important problem with living donor liver transplantation is the mortality and morbidity risk faced by completely healthy donor candidates due to a major surgery like liver resection. In order to minimize those risks, all living liver donor (LLD) candidates undergo an examination according to an algorithm consisting of biochemical blood tests and advanced radiological instruments[2]. Incidental hemangioma, focal nodular hyperplasia, cystic lesions, median arcuate ligament, and ventricular septal defect have been rarely reported to be detected during examinations of LLD candidates[3-5]. On the other hand, no publication other than our study has ever reported unusual findings such as cancer detected incidentally in the liver or other intraabdominal organs during either preoperative investigations or in a living donor hepatectomy (LDH) procedure[6]. In this study we report two neuroendocrine tumor (NET) cases detected incidentally during our 12-year LDH experience.

CASE REPORT

Case 1

A 26-year-old healthy man applied to our transplant center for being a LLD to his 37-year-old brother with chronic liver disease (HBV). The donor candidate was taken into the operating room for LDH in May 2017. A laparotomy was performed through an incision starting from xiphoid to umblicus and extending laterally on the right side. As no macroscopic finding was detected in the liver, a right lobe LDH was performed. As part of a study conducted in our department investigating the relationship of mesenteric and antimesenteric lengths of small intestine with the surgical procedure, intestinal lengths of this patient were also measured. At this time, a mass lesion measuring approximately 10 mm was detected on the antimesenteric face of the intestine, approximately 90 cm proximal to the ileocecal valve (Figure 1). The mass was resected together with the mesentery of the adjacent intestinal segment. A wedge resection with primary intestinal anastomosis was performed. Intestinal mucosa and submucosa were closed with polyglactin 910 suture material while the seromuscular layer was closed with polypropylene. The patient experienced no complications during his postoperative follow-up and was discharged. A histopathological examination revealed a grade I neuroendocrine tumor (carcinoid tumor) with a size of 7 mm × 5 mm, which had intact surgical margins (Figure 2). An immunohistochemical analysis showed that it was NSE (+), Chromogranin (+), Synaptophysin (+), and Ki67 proliferation index (1%-2%) positive (Figures 3 and 4). The patient was put under follow-up by the medical oncology department, and a thoracoabdominal computerized tomography taken in the first controls revealed no additional lesions.

Case 2

A 29-year-old healthy man applied to our transplant center for being a LLD candidate for his 51-year-old father who had chronic liver disease (HBV + HCC). His preoperative biochemical tests and radiological studies were normal. The patient was taken into the operating room for right donor hepatectomy in May 2008. A laparotomy was performed through an incision starting from xiphoid to umblicus and extending laterally on the right side. As no macroscopic finding was detected in the liver, right lobe LDH was performed. At the time when the surgeon suspended the anterior abdominal wall in order to place a drain into the surgical field, it was noticed that the appendix vermiformis was edematous and had prominent superficial arterioles. In addition, a firmness measuring 8-10 cm in size was palpated at the tip of the appendix. Therefore, an appendectomy was performed. As no complications developed at the postoperative follow-up, the patient was discharged to be seen at

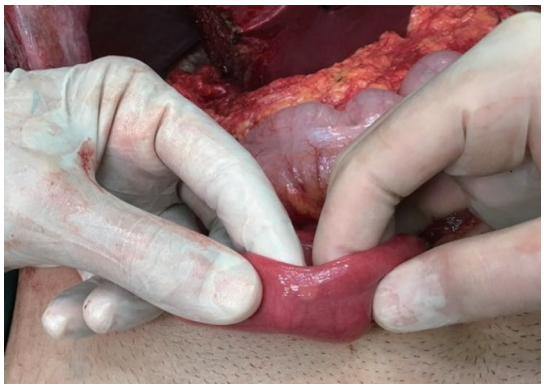

Figure 1 Intraoperative view of the tumor located in the antimesenteric border of the small intestine.

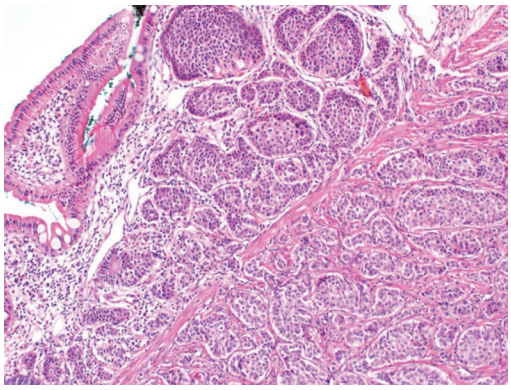

Figure 2 Tumor cells are seen in the submucosa with insulary pattern (HE × 100).

control visits. The pathology report of the appendix vermiformis showed a grade Ⅰ neuroendocrine tumor (NET) with a diameter of 10 cm at the tip of the appendix vermiformis, which showed full-thickness invasion but did not spare the mesoappendix (Figure 2). In addition, the lesion was NSE (+), Chromogranin (+), Synaptophysin (+), and Ki67 (1%) positive upon immunohistochemical analysis (Figures 3 and 4). As the patient was living in another city, he is currently under follow-up of another center there. He has recently informed us that no problems have occurred in his final control visit.

DISCUSSION

The World Health Organization has classified NETs into two main categories: well differentiated (low grade, intermediate grade, high grade neuroendocrine tumor) and poorly differentiated (high grade neuroendocrine carcinoma). The basic criteria for this classification are the Ki67 proliferation index (< 3%, 3%-20%, > 20%) and number of mitosis (< 2 mitosis, 2-20 mitosis, > 20 mitosis).

NETs most commonly involve the small intestine. NETs are most commonly located in the first 60 cm segment before the ileocecal valve. Unlike appendix NETs, small intestinal NETs have the potential to make both nodal and distant metastasis independent of their size. Therefore, the most appropriate surgical approach is to perform a partial small intestinal resection to involve lymph nodes and mesentery, independent of the tumor size. As small intestinal NETs have the potential of being multiple, all intestinal loops should be carefully inspected during the operation[7-9].

Appendix vermiformis is the second most commonly involved organ by NETs in the gastrointestinal system[10]. The majority of appendix NETs are incidentally detected in patients taken into operating room for a presumed diagnosis of acute appendicitis. NETs are diagnosed at a rate of 0.3%-2.27% in patients diagnosed with acute appendicitis while they are detected in 1.8%-2.3% of patients undergoing incidental appendectomy[7-9]. The prognosis and metastasis potential of appendix NETs depend on their size. Ninety-five percent of appendix NETs are smaller than 2 cm at the time of diagnosis and have a low metastasis potential (4%). In tumors with a diameter ⩾ 2 cm or patients with mesoappendix invasion, right hemicolectomy is the most appropriate approach. In tumors with a diameter ranging between 1.0 cm and 1.9 cm, adjunct hemicolectomy is the most appropriate approach when one or more of the following parameters exists: mesoappendix invasion, vascular invasion, high proliferation index (grade 2), suspicious/positive surgical margins, and mixed histology (goblet cell carcinoid, adenocarcinoid). Simple appendectomy is sufficient for patients without mesoappendix invasion despite a diameter of 1.0-1.9 cm and appendix NETs with a diameter < 1 cm. No adjunctive surgical procedure was undertaken since the patient presented here had a grade Ⅰ NET.

The follow-up of patients with NET depends on tumor size and grade. One or a combination of several diagnostic modalities of biochemical (5-hydroxyindoleacetic acid, serum chromogranin), endoscopic (pan endoscopy, colonoscopy), radiological (computed tomography, magnetic resonance imaging, Indium-111 pentetreotide (OctreoScan), and functional PET imaging with 68-Ga DOTA-TATE can be used for diagnosis. Patients with an appendix NET having a diameter of ⩾ 2 cm should be evaluated clinically and with biochemical/radiological studies whenever indicated at three and twelve months postoperation. Controls should be carried out after one year and advanced radiological tests should be performed as needed. In cases with a tumor size of less than 2 cm routine controls are not needed. The follow-up protocol of small intestinal NETs and appendix NETs should be in the form of close follow-up.

This case study brings into question whether it is necessary to palpate the whole abdominal cavity and especially gastrointestinal organs in healthy individuals undergoing laparotomy for LDH. Many studies so far have recommended not to touch the gastrointestinal tract unless indicated because even powder on surgical gloves may trigger the development of postoperative adhesions. In addition, handling, palpating, or removing the bowel

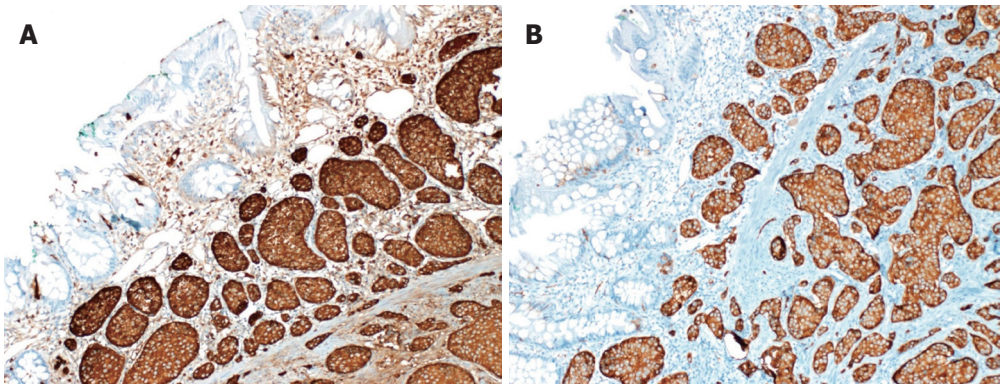

Figure 3 Positive immunostaining of tumor cells with chromogranin or synaptophysin antibody (HE × 100). A: Chromogranin; B: Synaptophysin.

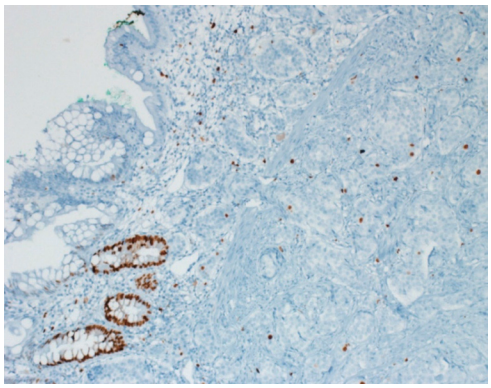

Figure 4 The low proliferation index in the tumor with Ki67 antibody (HE × 100).

loops may delay the return of intestinal motility during the postoperative period. Another question is what to do when a mass lesion is encountered in the gastrointestinal system. We suggest two methods: (1) when a tumoral lesion is detected in the gastrointestinal system before the LDH procedure, the lesion's gross macroscopic features should be evaluated and the entire mass or part of it should be resected and sent for frozen examination. When the result of the latter is a benign pathology, the hepatectomy procedure should be resumed. Whenever the result of the frozen examination is a malignant condition, hepatectomy procedure should be aborted and a definitive surgery should be performed against the mass; and (2) when a tumoral lesion is encountered incidentally at the exploration after the completion of the LDH procedure, a frozen examination of the tumoral lesion should be performed. In these patients, too, the result of the frozen examination should be obtained and surgical treatment should be carried out on the basis of oncological principles. However, an important caveat is what would be the fate of the liver graft harvested from these patients. In our opinion, ultrasonography should be performed for the living liver graft on the back table, and it should be transplanted when no hepatic lesion is identified. We assume that preoperative hepatic MDCT and dynamic MRI examinations are already negative for hepatic lesions in such patients. Recipients of such a liver should still be closely followed.

Unfortunately, we did not perform frozen examination in both patients presented here. The lesion of the patient with the appendix NET was both very small and located at the tip of the appendix. Hence, the frozen report would not affect our surgical strategy. As for the intestinal NET case, the lesion was of completely benign appearance, as can be seen in Figure 1. Despite this, we resected the segment with the lesion on the basis of the oncological principles. The recipients of both donors were put under close follow-up, and neither developed any lesion suspected to be a tumor to the date of the writing of this article.

In patients undergoing LDH operation, the abdominal cavity may be explored very gently using powderless gloves. This approach both creates a chance for early detection of some unexpected lesions and avoids potential medicolegal problems.

Whenever a suspicious lesion is detected before starting the LDH procedure, a frozen examination should be done from that lesion. When the frozen report indicates a malignant lesion, the LDH procedure should be aborted and the lesion should be resected on the basis of oncological principles. However, the question whether donors with NETs having a diameter smaller than 2 cm that are located to the tip of the appendix should be used is open to discussion.

ARTICLE HIGHLIGHTS

Case characteristics

We aimed to present two cases in which grade Ⅰ neuroendocrine tumors were incidentally detected during our twelve-year living donor hepatectomy experience.

Clinical diagnosis

Intraoperative appearance of the lesions of both patients was compatible with benign disease.

Differential diagnosis

Differential diagnosis of the first case included calcified nodule, benign intestinal tumor, and early stage malignant tumor. Differential diagnosis of the second

case includes acute appendicitis, mucocele, and carcinoid tumor

Laboratory diagnosis
No abnormal findings were detected in preoperative biochemical blood tests in both living liver donor candidates.

Imaging diagnosis
No abnormal findings were detected in preoperative radiological examinations in both living liver donor candidates.

Pathological diagnosis
The immunohistochemical examinations of the specimens of both patients were reported as grade Ⅰ neuroendocrine tumor.

Treatment
While a wedge resection with primary intestinal anastomosis was performed in the first case, simple appendectomy was performed in the second case.

Related reports
To our knowledge, no publication other than our study has ever reported unusual findings, such as cancer, detected incidentally during a living donor hepatectomy procedure.

Experiences and lessons
Even if patients undergoing living donor hepatectomy are healthy individuals, the whole abdominal cavity should be gently palpated and all findings recorded after completing laparotomy. Suspected masses or lesions should be confirmed by frozen section examination.

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MINIREVIEWS

Decision modelling for economic evaluation of liver transplantation

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Author contributions: Qu Z, Krauth C and Schrem H conceptualized the overview. Qu Z, Gwiasda J and Liersch S drafted the manuscript. Amelung VE, Kaltenborn A, Harries L, Beneke J and Schrem H critically reviewed the manuscript and contributed important intellectual contents.

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Abstract

As the gap between a shortage of organs and the immense demand for liver grafts persists, every available donor liver needs to be optimized for utility, urgency and equity. To overcome this challenge, decision modelling might allow us to gather evidence from previous studies as well as compare the costs and consequences of alternative options. For public health policy and clinical intervention assessment, it is a potentially powerful tool. The most commonly used types of decision analytical models include decision trees, the Markov model, microsimulation, discrete event simulation and the system dynamic model. Analytic models could support decision makers in the field of liver transplantation when facing specific problems by synthesizing evidence, comprising all relevant options, generalizing results

to other contexts, extending the time horizon and exploring the uncertainty. For modeling studies of economic evaluation for transplantation, understanding the current nature of the disease is crucial, as well as the selection of appropriate modelling techniques. The quality and availability of data is another key element for the selection and development of decision analytical models. In addition, good practice guidelines should be complied, which is important for standardization and comparability between economic outputs.

Key words: Cost benefit analysis; Decision tree; Liver transplantation; Decision analysis; Decision support models; Resource allocation; Cost effectiveness

Core tip: This overview focuses on providing an understanding of decision modelling approaches and their application to liver transplantation, outlining the major characteristics of decision analytic models as well as the individual strengths and weaknesses of several main techniques for modelling. We believe decision modelling may be able to provide tools by bringing all evidence from other studies together and comparing the costs and consequences of alternative options to reach a decision. It is a particularly powerful tool for public health policy and clinical intervention assessment.

Qu Z, Krauth C, Amelung VE, Kaltenborn A, Gwiasda J, Harries L, Beneke J, Schrem H, Liersch S. Decision modelling for economic evaluation of liver transplantation. *World J Hepatol* 2018; 10(11): 837-848 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i11/837.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i11.837

INTRODUCTION

The improvement of immunosuppression, innovation of splitting technique and growing clinical experience in liver transplantation have increased the utilization of available donor organs and survival rates[1,2]. Nevertheless, the crisis of organ shortage is subsisting. In 2013, 5921 livers were donated for transplantation in the US, while 12407 patients were waiting for an appropriate donor[3]. In 2016, 1567 patients received liver grafts, while 1704 remained on the waiting list in eight European countries[4]. The situation in Germany has been under particularly increasing pressure due to publicly discussed transplant scandals. Furthermore, the gap between donated organs and the necessity of transplants has been widening due to regulatory issues highlighting the relevance of public trust[5]. In 2011, 1191 liver transplantations were performed in Germany, but 1792 patients were listed for liver transplantation[6]. Although living donation and split-liver transplantation have been established to relieve the shortage of organs, the immense demand for liver grafts constantly increases[7-10]. Managing this widening gap remains a major challenge both ethically as well as economically. Decision modelling, based on real clinical and economic data, might provide the tools to overcome these challenges.

Due to this scarcity, every available donor liver should be allocated in a manner that maximizes its utility, urgency and equity. Required resources, funding and coverage by health care insurance for transplant systems need reliable information based on validated economic models to support political and practical decisions. The recent liver allocation system has been urgently confined in the past two decades, and prioritizes candidates by the Child-Turcotte-Pugh score or Model for End-Stage Liver Disease (MELD) score and its adaptions[11]. However, MELD scores and similar systems lack the predictive power for short- and long-term outcome of liver transplantation[12], in addition to the consideration of utility and transplant benefit. Furthermore, care management interventions and extensive treatment of liver transplant recipients are commonly required and consume considerable financial healthcare resources[13]. Therefore, selection and evaluation in this lifesaving procedure is an important topic in health economics[14].

Economic evaluation involves different aspects of transplantation. Evaluations of donor organ quality, recipient characteristics, as well as strategies for organ allocation demand an economically-based decision evaluation. Considerations of alternative therapies other than transplantation, as well as adequate immunosuppression therapy regimes after transplantation, require intensive evaluation. In addition, comorbidities and complications play an important role in the estimation of the cost-effectiveness of transplantation[13].

Decision analytical models combine information from various sources to assess the implications of different decisions, and could therefore generalize evidence from other contexts when local data and studies are unavailable. This sets them apart from statistical models[15]. Furthermore, when randomized clinical trials (RCT) cannot be performed due to practical or ethical issues, the power of decision analytical models lies in their ability to generate results without primary data[16]. This review focuses on providing an understanding of decision modelling approaches and their application to liver transplantation.

WHAT IS DECISION MODELLING?

Decision analytic modelling uses mathematical relationships to define a series of consequences that derive from a set of options[17]. Although it shares a common theoretical foundation with statistic models and has a

close association with Bayesian statistics[18], the key feature of decision analytic modelling accounts for the variability and uncertainty in all possible decisions. Moreover, it combines evidence from other studies like clinical-, cost- and health-related quality of life (QOL) data as utility values and compares the cost and consequences of alternative options. This generates a framework to reflect on the key differences of possible end points from all the alternative options in terms of cost and effect. It is thus a powerful tool for public health policy and clinical intervention assessment.

Even though the methods of decision analysis have been applied to medicine for over 40 years, their rather modest impact on real-world decision-making[19] has only recently been on the rise. To illuminate decision analytic models, we introduce the most commonly used types of models: Decision trees, the Markov model, microsimulation, discrete event simulation and the system dynamic model, illustrating how decision analytical models perform in the context of liver transplantation.

Decision trees

A decision tree model is recognized as the simplest structural decision analytical model and represents both the clinical decision procedure as well as consequential results in aggregate levels[15,20]. All clinical outcomes of patients in a decision tree model are visualized as a series of decision nodes and follow pathways with probabilities for each respective branch.

An example is given by Kantola *et al*[21] in Figure 1. This study was designed to determine cost-utility of molecular adsorbent recirculating system treatment in acute liver failure. The square node at the start of the tree represents the decision between alternative treatment strategies. The circular chance node shows the possible alternative events for a patient. Pathways (the "branches") following each node represent a series of alternative events, which are mutually exclusive. Probabilities show the likelihood of certain events, multiplying along the nodes and branches to estimate the overall probability of reaching the distinct outcome. Probabilities for all events assessed sum up to a total of one.

Following these branches and nodes, a total cost can be derived for the distinct combination of therapy options and compared to the potential benefit, such as in this case Quality Adjusted Life Years. Nevertheless, interpretation needs to account for clinical reason and include a careful discussion when assessing the most beneficial choice for combining therapies.

The simplicity and transparency of decision trees are their main advantages and may illustrate which possible set of options may be most promising. However, decision tree models can be very complex when used to model complicated long-term prognoses[18]. In other words, when they are used to model a chronic disease, decision trees can have numerous lengthy pathways representing recurring events, which is very time-consuming to analyze and interpret.

Markov models

Markov models are commonly used to provide a framework that represents sequences of events as a large number of complexity modelling options over time. Certain events lead to different health states (patients with different probabilities of transitioning from one state to another) given a defined period of time (cycle length). They commonly include large numbers of complexity modelling options. The number of states and the association among them are pre-defined in accordance with the decision problem, as well as the transition probabilities and cycle length[17,22].

Sarasin and his colleagues[23] showed an example of using Markov models to compare the gain of life expectancy and the cost-effectiveness of living and deceased donor liver transplantation in Figure 2. Each patient starts at the state of "cirrhosis, hepatocellular carcinoma (HCC), with no contraindications to cadaveric liver transplantation". With this initial state of health, they can then make a transition into several other states with different probabilities for each transition state for each defined, discrete time interval or cycle. They also might stay in their current state. The chances of transferring between different states are a set of defined transition probabilities derived from the appropriate transaction of longitudinal research data. Lengths of these cycles (one month in this example) depend on the disease or interventions of interest. To end the transition process in this Markov model, an absorbing state "death" was set that the patients obviously cannot leave once reached. Then, the Markov process modeled an integrity profile of both donor and recipient life expectancy over a lifetime-long horizon. The application of this Markov model handled the complexity of patients with early HCC and revealed that living donor liver transplantation is cost-effective compared to deceased donor liver transplantation under certain conditions[23].

A major advantage of Markov models is that they account for time dependency and can model changing probabilities over time. Therefore, Markov models are eligible to analyze chronic and complex conditions and clinical matters[24], such as the transplant field, relatively quickly and easily[13]. The important limitation of Markov models is the "Markov property", also called "Markov assumption", which assumes that transition probabilities only depend on the current health status but not on past history. Moreover, the Markov assumption might over-simplify the nature of disease, as it handles patient cohorts homogenously[18]. For higher resolution in this regard, an alternative approach known as patient-leveled simulation can be applied.

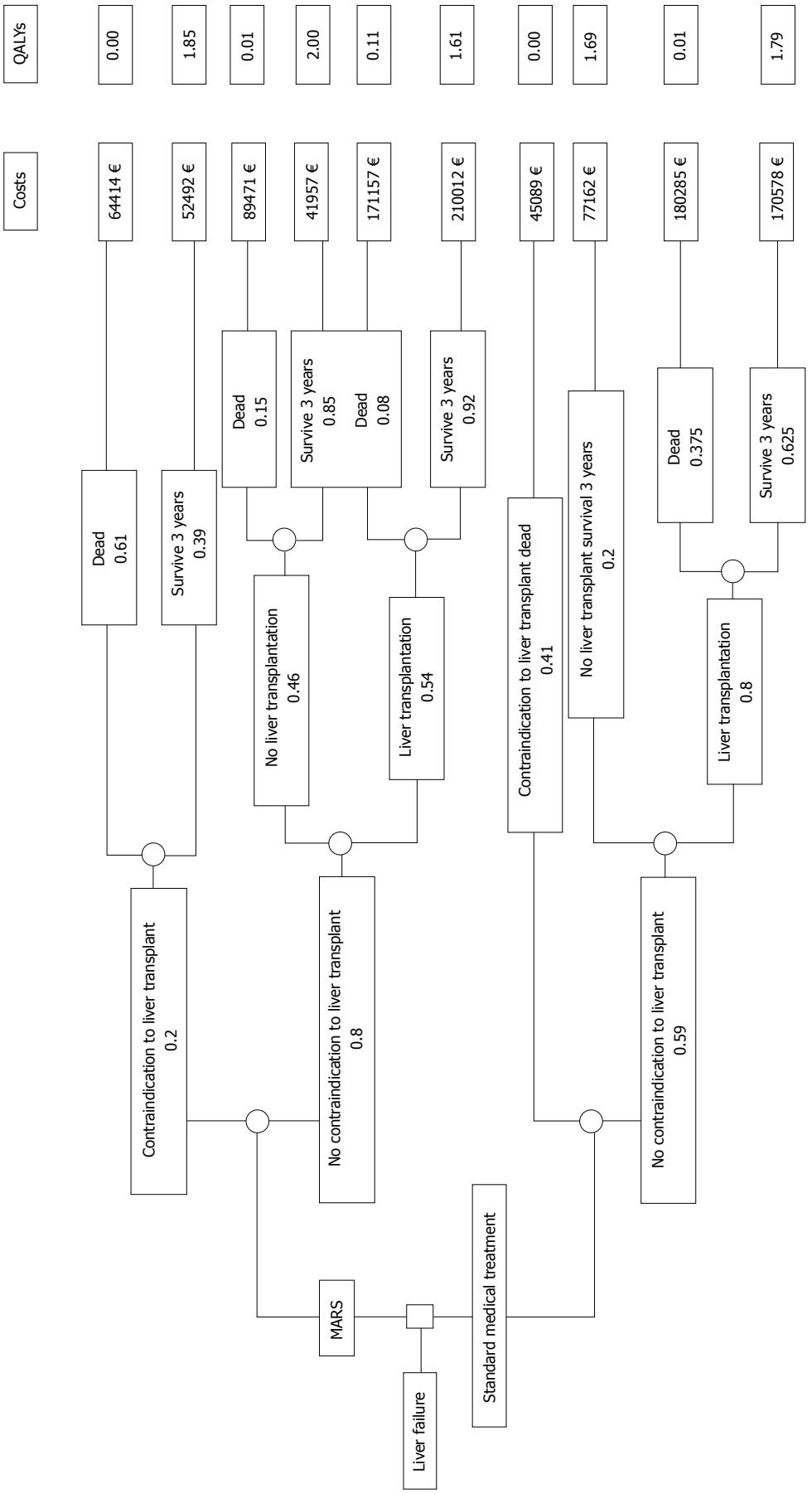

Figure 1 Decision tree for determining the short-term cost-utility of treatment in acute liver failure. Choice between strategies (decision node) and occurrence of chance events (chance node). Kantola *et al*[21]. MARS: Molecular adsorbent recirculating system.

Patient level simulation (microsimulation)

The microsimulation model is featured by "individual sampling", which means to simulate one individual at a time, rather than the whole cohort.

Perkins *et al*[25] developed a Markov-based microsimulation model to compare results under the present liver allocation policy in the United States (Figure 3). This model simulates how each patient proceeds through the model with the chance of multiple parallel events. One individual case is randomly selected from all patients.

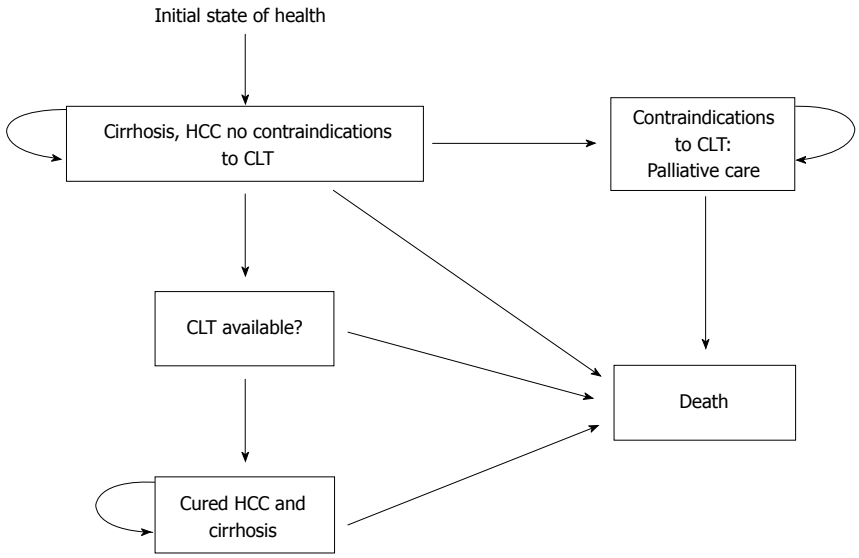

Figure 2 States of health in the decision model. Each square represents a state of health. Straight arrows represent the changes that may occur during each month. Curved arrows mean that the patient may remain in the same state of health. Sarasin *et al*[23]. HCC: Hepatocellular carcinoma.

The initial state is "alive". Patients can enter other states based on fixed transition probabilities, which simulate events in one cycle (three months in this study) until reaching the state "death". This study modeled the changes of the allocation policy, which demonstrated the survival benefit for the patients who have a MELD score $\leqslant$ 14 from transplantation, among the highly diverse patient population in the waiting list. The results appear more reliable than models based on aggregated data and could also be validated.

The advantage of microsimulation models is flexibility, in regards to different patterns of disease processes and intervention, because these models keep track of each individual's history[19]. Moreover, it can be useful when accumulating the history of each patient to determine the different transitions, costs and health benefits. However, there are also disadvantages in using microsimulation: First, outcome effective determinants in patients' history demand more detailed data, which challenge simply structured database research. Secondly, the simulation and computation of patient level simulation are time-consuming. Consequentially, the uncertainty assessment is not flexible when compared with other types of decision analytic models.

Discrete event simulations

Discrete event simulations (DES) can represent the competition for resources and investigate the changes in stochastic systems[26,27], and are mainly used to evaluate health care systems. The capacity and utility of allocation systems have previously been assessed before and after policy changes[28-31]. Another example reported by Shechter *et al*[26] is a biologically-based discrete-event simulation model, which represents the biological progression of end stage liver disease (ESLD) and examines the impact of changing allocation policies on this issue. The model was comprised of five modules: The patient generator, organ generator, pretransplant natural history, matching algorithm, and post-transplant survival (Figure 4). DES allows different modules to run independently, as this study shows, where pre-transplant history and allocation policy stand in parallel and individual patient attributes may influence the pathway, costs and outcomes. Unlike patient-level simulation models, DES is appropriate to model situations where constraints on resources could affect treatment options[15,32].

DES has several methodological advantages compared to other commonly used models, because it simulates the time until the next event for a given patient, which reduces the amount of time required for model construction and interim computations[18]. The output is not limited to survival only, but also allows estimations of event counts and sub-group analyses[33]. Moreover, statistical processing tools for relevant input parameters can be deployed. In contrast, structural complexity is the most prominent disadvantage of DES, which makes it difficult to apply to clinical research[34]. The complicated structure also makes computations more extensive, in regards to time and resources compared to Markov models when dealing with the same decision problem[35].

System dynamic models

System dynamic models allow modeling interactions within a population and with their environment over time; hence, they are especially suited for studies related to infectious diseases. The theoretic background of a system dynamic model is that complex behaviors of systems are a result of ongoing accumulations of

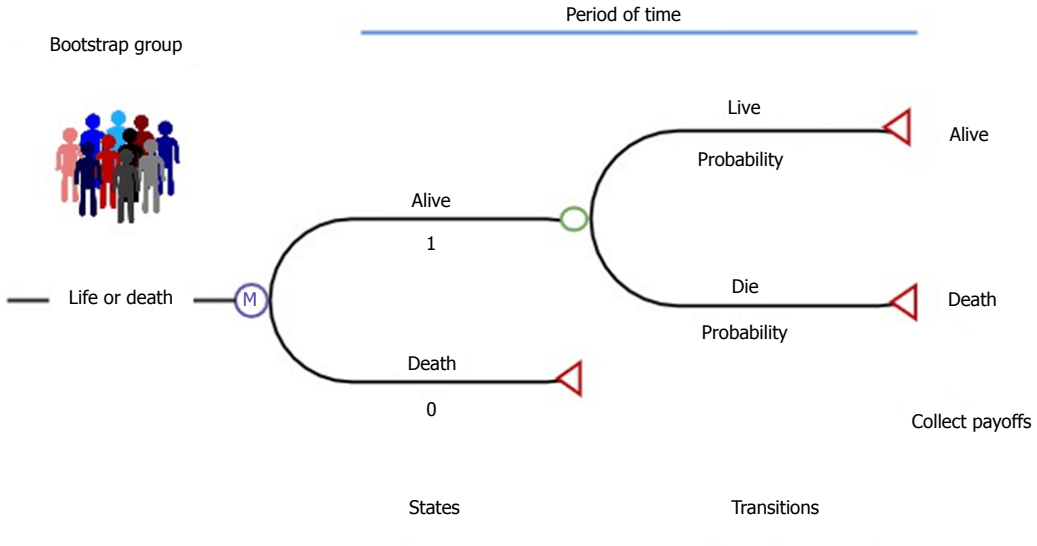

Figure 3 Simple example of a Markov microsimulation model. Perkins *et al*[25].

people, resources as well as biological and physiological states[36]. The probabilities of events can change through feedback of such accumulations. To the best of our knowledge, there is no application example of systematic dynamic models in the context of liver transplantation. However, examples of kidney[37] and corneal[38] transplantation showed that the predicted number of transplantations are consistent with observed results, which indicates the potential usefulness of system dynamic models for this field.

The advantage of system dynamic models is the information on interactions between individuals, which may quantify the impact of intervention on outcomes more accurately. Disadvantages are similar to DES, where information and interaction on individual level exponentially increase the complexity of model structure, computational burden, as well as lower transparency more than Markov models[19].

HOW DECISION ANALYTIC MODELLING CAN BE USED IN THE FIELD OF LIVER TRANSPLANTATION

Decision analytic models, in contrast to statistical models, incorporate decision-making into analysis[19]. Established in economic evaluations within other fields, decision analytic models in liver transplantation aim to inform decision-makers in two main areas: Decision analysis and measurement[18]. There are several aspects in which decision analytical modelling could help decision-makers in this field.

Combining different sources of evidence

In concordance with the principle of evidence-based medicine, decision-making on the basis of economic evaluation also requires the use of all accessible evidence related to the intervention effectiveness[39]. A decision analytical model offers a logic framework for the integration of data from very different sources, such as clinical trials, observational studies, insurance claim databases, case registries, public health statistics, and preference surveys[40]. In addition, more parameters related to resource utilization and utilities like unit cost, health-related QOL and preferences of patients are important evidence in economic evaluation[18]. This series of non-clinical indicators complement clinical data within the framework of decision analytics and support a much more complete picture of expectations from various parties involved. In particular, the specific data for patients with ESLD should also be organized into informative resources. Cillo *et al*[41] recommend a prospective assessment, which will substantially help decision analysis and support the decision-making process[42].

Comprising relevant options

In most instances, a single study cannot compare all the relevant alternative options for treatment paths for diseases such as ESLD. Decision-makers might therefore be challenged by a lacking comparison of all potentially effective interventions. New techniques like network meta-analysis extend the concept of indirect comparison by including multiple pairwise comparison information from clinical trials to constitute a network of evidence[43,44]. However, system dynamic models are more eligible to combine different types and sources of evidence, like clinical trials and patient questionnaires, and therefore adds fundamental information to the shared decision-making process.

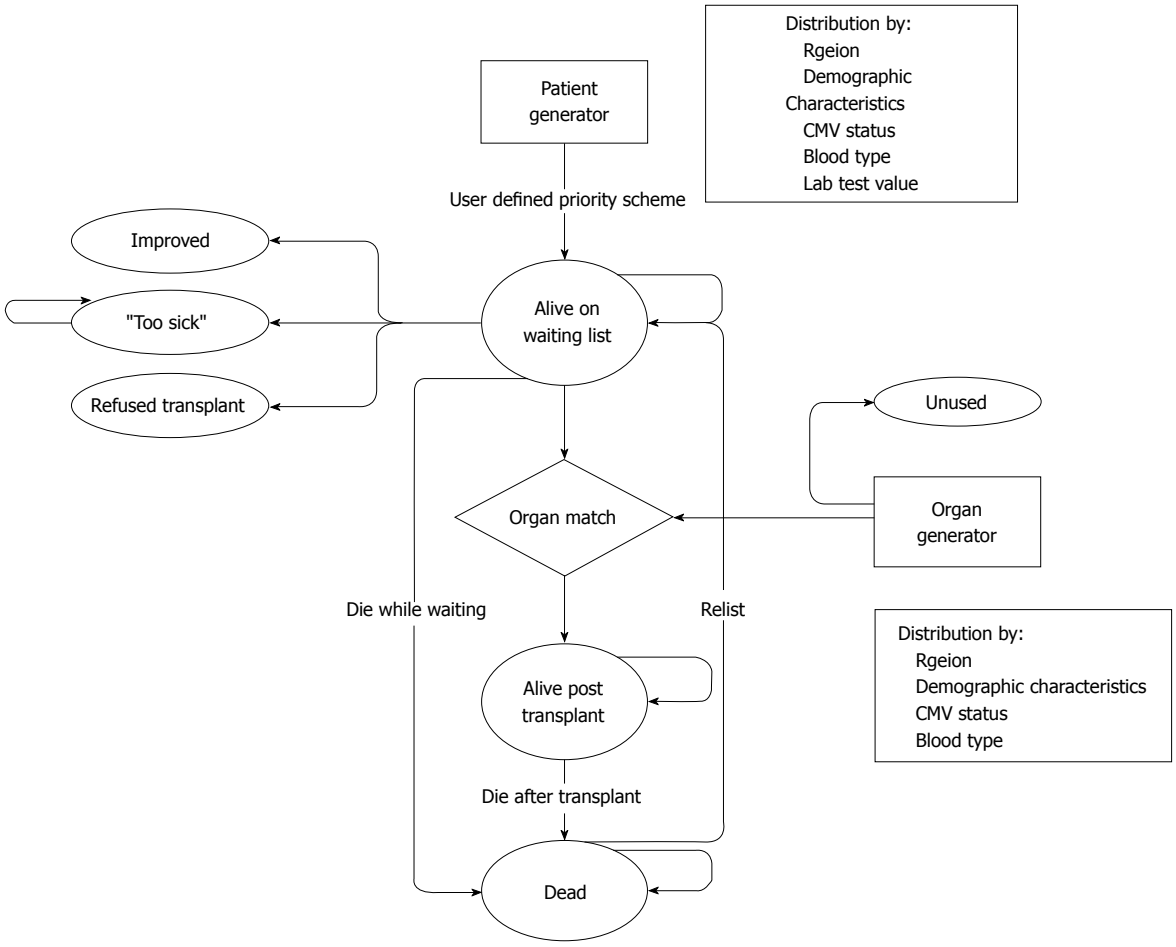

Figure 4 Model structure for patients entering the liver transplantation program. Shechter *et al*[26].

Applying results to other context/subgroups

The differences between patient subgroups can, for example, derive from either baseline characteristics like age, gender, comorbidity severity or variations in the healthcare context. The application of findings from one context can be difficult to transfer to other situations. Cucchetti *et al*[45] performed a study to measure the risk of age for salvaging transplantation in patients resected for HCC. A Markov model was developed to quantify the effect of patient's age above Milan criteria. Next, the risk of resection at two or three years below the age limit could be evaluated. The clinical evidence may not be able to show the difference between subgroups in heterogeneous patients over a long horizon[46]. However, in this research, the reduction of life expectancy of hepatic resection in different patient groups was clearly shown using a decision model.

Extending the time horizon

Many of the interventions for liver transplant patients require long time periods, and the weight of personal value added by these therapy options takes a long time for patients to assess. Therefore, models that evaluate the benefits of interventions for patients should cover sufficient time horizons. Long-term consequences, as well as costs of alternative options and interventions, are substantially affected by time. Even lifetime horizons are often needed for many models and are almost always required for models in which options have different time-varying survival rates[40]. Decision models offer the framework to include the effect and cost over time by adding respective results, and can evaluate the effects of main interventions beyond primary data sources and their continuous treatment effects[40].

Exploring the uncertainty

A key interest in liver transplantation is weighing the probabilities of risk and success between different options, especially in organ resource allocation and the decision of appropriate time-point selection for certain interventions. Not only are patients affected, but so are other potential organ recipients. Transplantation itself is not a definitively curing option, but leads to a life-long immunosuppressive treatment. Population variation, parametric imprecision as well as modelling selections and other aspects challenge predictive modelling, with uncertainty in different layers[47]. Clinical and economic data accessibility and validity also contribute to this

uncertainty[16].

In the face of these challenges, decision modelling methods are not only for reflecting this uncertainty but also to assess their influence so that the decision-makers can make choices with the relevant possibilities known. Analyses estimating the uncertainty due to parameters of interest is the most common approach to perform in modeling, which could be represented *via* deterministic sensitivity analysis (DSA) or probabilistic sensitivity analysis (PSA)[48]. In DSA, parameters in modelling are specified as multiple point estimates, and are varied manually to test the sensitivity of modeling results. In PSA, model inputs are specified as a distribution and varied to predefined probability distributions accordingly.

Along with the probabilistic analysis mentioned above, expected value of perfect information analysis is argued to be the most appropriate presentational technique for representing decision uncertainty. Jay and colleagues[49] showed the cost-effectiveness of organ donation after cardiac death versus after brain death. This novel sensitivity analysis represents both the probability of whether a decision is appropriate and its consequence, which is important for comparing the incremental net benefits under different accessibilities with the information of probabilities.

KEY POINT IN DECISION ANALYTICAL MODEL DEVELOPMENT

Understanding the nature of disease history

Model construction should combine efforts from multiple parties, including clinical and economic experts as well as decision-makers from the context of interest, and make best utilization of all available evidence. Neither the modeler nor the clinician alone can complete the task that conceptualizes an accuracy-simplicity balanced model. The accuracy of the model depends on whether the structure accounts for all important events or transitions and probabilities[25]. A thorough understanding of the disease is crucial for defining the possible health states in the model as well as capturing the occurrence of clinical events beyond follow-up[50].

Model characteristics and techniques

The key consideration of decision analytical model selection is the acceptance of the modelling technique, model "error", model appropriateness, dimensionality, and ease and speed of model development[32]. Decision trees are typically used when the process is not complicated, the recurrence of disease is not important and the time frame is short. Markov models are more feasible when simple chronic interventions are conducted. When the interaction is important, discrete event time and system dynamics could construct a more comprehensive and interactive system, but the development time and cost may significantly increase[51].

Figure 5 shows a flow chart for selecting the appropriate decision models based on the mentioned summaries and guidelines recommended by Barton *et al*[15] and Cooper *et al*[32].

Data quality and availability

The quality and availability of data is another key element for the selection and development of decision analytical models. Without sufficient and high quality data, the development of models will be difficult and result in low validity. As discussed above, synthesizing evidence is one of the most important fields that decision analytical modelling could help with during economic evaluation. In general, the information needed as input parameters for economic evaluation is derived from different kinds of data sources[52], including RCT, observational studies, secondary data analysis (*e.g.,* Meta-analysis) and expert opinions[50]. For topics of interest in liver transplantation, ethical considerations may additionally constrain the option of performing RCT. Therefore, data from published literature needs to be consolidated and considered in this context. In particular, reviews and reports from both the European Liver Transplant Registry, Organ Procurement and Transplantation Network as well as the Scientific Registry of Transplant Recipients database are valuable sources.

However, the consolidated data may cause incorrect estimations of parameters within the models, especially when multiple inputs are derived from single publications. In this situation, individual data from electronic medical databases or re-analysis of available published individual level data will be more appropriate. When several studies provide results on the same parameters of interest, researchers usually need to combine different results using meta-analytical methods[53] or adopt the results reported in meta-analyses. When potential biases in the original research or meta-analyses are handled appropriately, the inclusion of these results might increase uncertainty, which will be noted when constructing the model. Although expert opinion is the least preferable data source due to its subjectivity, it may still play an important role in the evaluation of cost and resource use when other sources of evidence are absent[54].

Good practice guidelines for modelling

The development of decision analytical models is a sophisticated task requiring the modelers to have sufficient experience, as well as the ability to evaluate, present and interpret the model's output. The complexity of the clinical pathway for complex interventions such as liver transplantation and the differences of health care environments between transplant centers and countries (*e.g.,* organ availability, allocation strategy, financial assistance for transplantation, post-

Table 1 Summary of types of decision models in liver transplantation

Model type	Model description	Type of scenario most suited for
Decision tree	Clinical outcomes are modelled as a series of decision nodes and follow pathways with probabilities for each respective branch.	Disease without relapse or recurrence.
Markov model	Represents sequences of events that lead to different health states with different probabilities of transitioning from one state to another over a defined period of time.	Chronic conditions involving recurrent events over time.
Microsimulation	Simulates one individual patient proceeding through the model with the chance of multiple parallel events.	Individual level information is important.
Discrete event simulation	Represents the competition for resources and investigates the changes in stochastic systems.	Interactions of resource allocation between individuals are of importance.
System dynamic model	Modeling interactions within a population and with their environment over time.	Spread of infectious diseases.

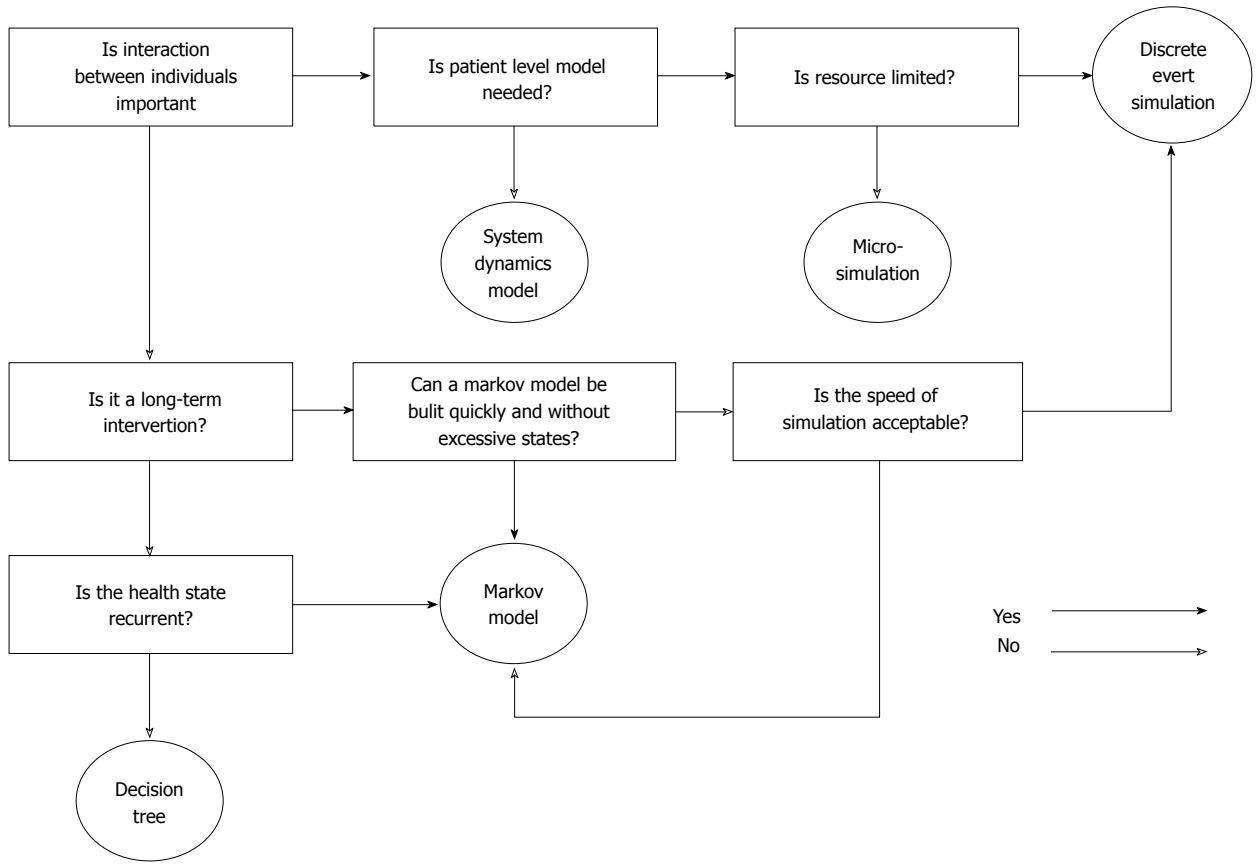

Figure 5 Scheme of selecting the appropriate model type.

transplant management and the consequential influence on QOL) challenge even the most experienced modeler to develop a model of economic outcomes of interest[51]. The best practice guidelines have therefore been significantly improving the process of model development, which is important for standardizing and comparing economic outputs.

The International Society for Pharmaco-economics and Outcomes Research Task Force group published a series of guidelines on good practice standards for modeling research, which set the standards for modeling practice[48,55-60]. However, these very detailed guidelines may not be well-understood in practice when performing a modeling study for the first time. To bridge this gap, Rautenberg *et al*[61] developed a beginner's guide to support modelers alongside the development of decision analytical cost-effectiveness models. This guide is especially helpful for researchers who are interested in utilizing this economic evaluation instrument, which is an easy-to-use practical guideline recommended for elementary modelers to initiate studies in this field.

CONCLUSION

This review demonstrates the major characteristics of decision analytic models (Table 1) as well as the

individual strengths and weaknesses of several main techniques for modelling. Decision trees are fit for disease interventions without relapse or recurrence. Markov models are suitable for interventions for chronic conditions involving recurring events over time. When individual level information is important, microsimulation models should be considered. If interactions between individuals are of importance, discrete event time models are suitable for simulation of the interaction of resource allocation. Dynamic models are fit to simulate the spread of infectious diseases.

Besides this, choosing the best depends on advanced understanding of the disease and its related interventions. Inter-professional cooperation is likely needed to combine methodological and clinical knowledge into a purposeful model. Furthermore, data availability and quality must be taken into account, which is as important as the definition and measurement of critical model components. The availability, weight and information of details for interventions, alternatives, target populations, health outcomes and time horizons have to be considered when conceptualizing the model. This is in regards to the modelling technique, model appropriateness and both the ease and speed of model development.

This framework of methods guides the analysis and interpretation of various data sources that further conclusions and a more advanced understanding of various elements and aspects of liver transplantation.

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EDITORIAL

Exosomal microRNAs as a potential therapeutic strategy in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the second cause of cancer-related death worldwide. The incidence of HCC is constantly increasing in correlation with the rise in diabetes and obesity, arguing for an urgent need for new developments in the treatment of this lethal cancer. Exosomes are small double-membrane vesicles loaded with distinct cargos, particularly small non-coding RNAs called microRNAs, representative of each donor cell and secreted to affect the features of neighboring cells or recipient cells located further away, like in the case of metastasis. A better understanding of the role of exosomes with a microRNA signature in cancer pathogenesis gave rise to the concept of their use as a non-invasive diagnostic biomarker and in the treatment of cancer, including HCC. In this communication, we review recent works that demonstrate that hepatic stellate cells establish an epigenetic communication with liver cancer cells, which affects their pro-malignant features. If naturally secreted patient-derived exosomes show major limitations concerning their clinical use, bio-engineered exosome mimetics that incorporate controlled components and exhibit no protumoral properties could be promising carriers for the treatment of liver cancers, which is the organ preferentially targeted by systemic injection of exosomes.

Key words: MicroRNAs; Hepatocellular carcinoma; Targeted therapy; Exosomes

Core tip: Despite the intensive research efforts to identify the molecular events responsible for the emergence of liver cancer, hepatocellular carcinoma (HCC) remains a major health problem worldwide. Thus, the identification of new therapeutic opportunities to

counteract the challenging issues linked to HCC heterogeneity and resistance to conventional treatments is a short-term necessity. Over the last few decades, microRNAs have appeared as interesting therapeutic strategies with their pleiotropic inhibitory action, but the use of a delivery system is a requirement for miRNA mimic administration. Exosomes, which are small vesicles naturally produced by immune cells and aberrantly by cancer cells, have recently emerged as a promising vehicle.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the second cause of cancer-related death worldwide for which therapeutic options are very limited. Indeed, because of its heterogeneity, the development of effective therapies against this cancer remains a challenging issue. HCC is considered to be a paradigm of inflammation-associated cancer, since 80% of HCC emerges following vast liver remodeling. Briefly, HCC primarily affects men with cirrhosis due to hepatitis B and C viruses, alcohol abuse, genotoxic exposure and metabolic disorders, and the incidence is increasing due to diabetes and obesity[1]. Efforts in the molecular and genetic profiling of HCC revealed that among the mutational landscape of HCC, the Wnt/β-catenin, p53 and Ras pathways are the most frequently mutated. Other prevalent mutations occur in epigenetic modifiers such as chromatin remodelers and imprinted clusters[2].

Despite these molecular findings, the molecular pathogenesis of HCC is still not fully understood, and novel strategies are urgently needed to cure this lethal disease with high incidence. Over the last decades, other crucial epigenetic regulators, the small non-coding RNAs named microRNAs (miRNAs), have been largely found to be disturbed during hepatocarcinogenesis[3]. Promisingly, due to their large spectrum of action on proliferation, inflammation and metabolism, miRNAs have emerged as robust therapeutic opportunities in various cancers. Regarding liver diseases, miRNA-based therapies have been successfully tested[4,5] - this type of molecule is preferentially delivered to the liver[6]. Despite promising results obtained with miRNA-based therapies, a number of challenges remain to improve the efficiency of this type of treatments. Their limitations are similar to those which have delayed the use of therapies based on small interfering RNAs: Improvement of stability, free or encapsulated administration, problems of specificity, tissue distribution, response persistence and secondary effects.

Recently, new therapeutic candidates for anticancer drug delivery have been proposed that are based on a biological system of transporting active cargos called exosomes. Exosomes are double membrane cell-derived microvesicles defined by a diameter of 30 to 100 nm that contain a great diversity of nucleic acids, protein and lipids - 3408 mRNAs, 2838 miRNAs and 9769 proteins according to the Exocarta database based on 286 studies[7]. Most cells, and particularly immune cells, are physiologically secreting exosomes that originate from multivesicular bodies. Multivesicular body fusion with the plasma membrane is orchestrated by Rab, soluble N-ethylmaleimide-sensitive-factor attachment protein (commonly known as SNAP) and SNAP receptor (commonly known as SNARE) proteins[8]. In response to different activating signals like antigenic, cytokinic or mitogenic stimuli, immune cells are able to increasingly release these small vesicles[9]. Recent studies revealed that disequilibrium in exosome formation and/or delivery contributes to pathological processes leading to immunological disorders and cancers. Indeed, during tumorigenesis, tumor cells aberrantly secrete exosomes to communicate with stromal cells and to modify secondary sites favoring metastasis[10]. In consequence, the detection of exosomal miRNAs in body fluids has appeared as a potent non-invasive diagnosis tool for cancer, including HCC[11], but also as a new therapeutic opportunity.

Exosome-based therapies have emerged over the past decade as an attractive strategy for tissue repair, immune vaccine and against cancer, firstly because of their biocompatibility. Second, their small size facilitates their crossing through biological barriers, notably the blood-brain barrier, and limits their renal clearance. These microvesicles might prove to be suitable for liver disease, especially for liver cancer treatment, since exosomes accumulate in the liver after systemic injection. In particular, exosomes preferentially target the resident macrophages, the Kupffer cells. Interestingly, the uptake of extracellular vesicles by Kupffer cells increases in the case of liver damage[12]. Over the past two years, the Selaru laboratory published two compelling manuscripts studying exosomes carrying miRNAs as a way to dialog between stromal cells, in particular stellate cells, and cancer cells in cholangiocarcinoma (CCA)[13] and in HCC[14]. Both studies revealed the clinical potential of miRNAs loaded in stellate cell-derived exosomes for *in vivo* delivery in mice and, on a longer-term perspective, CCA or HCC treatment.

STUDY ANALYSIS

The work from Wang *et al*[14] focused on miR-335-5p, a microRNA already described as a tumor suppressor in HCC[15] and that is known to gradually decrease in activated hepatic stellate cells (HSCs) during hepatic fibrosis[16]. This miRNA is also reduced in the serum of

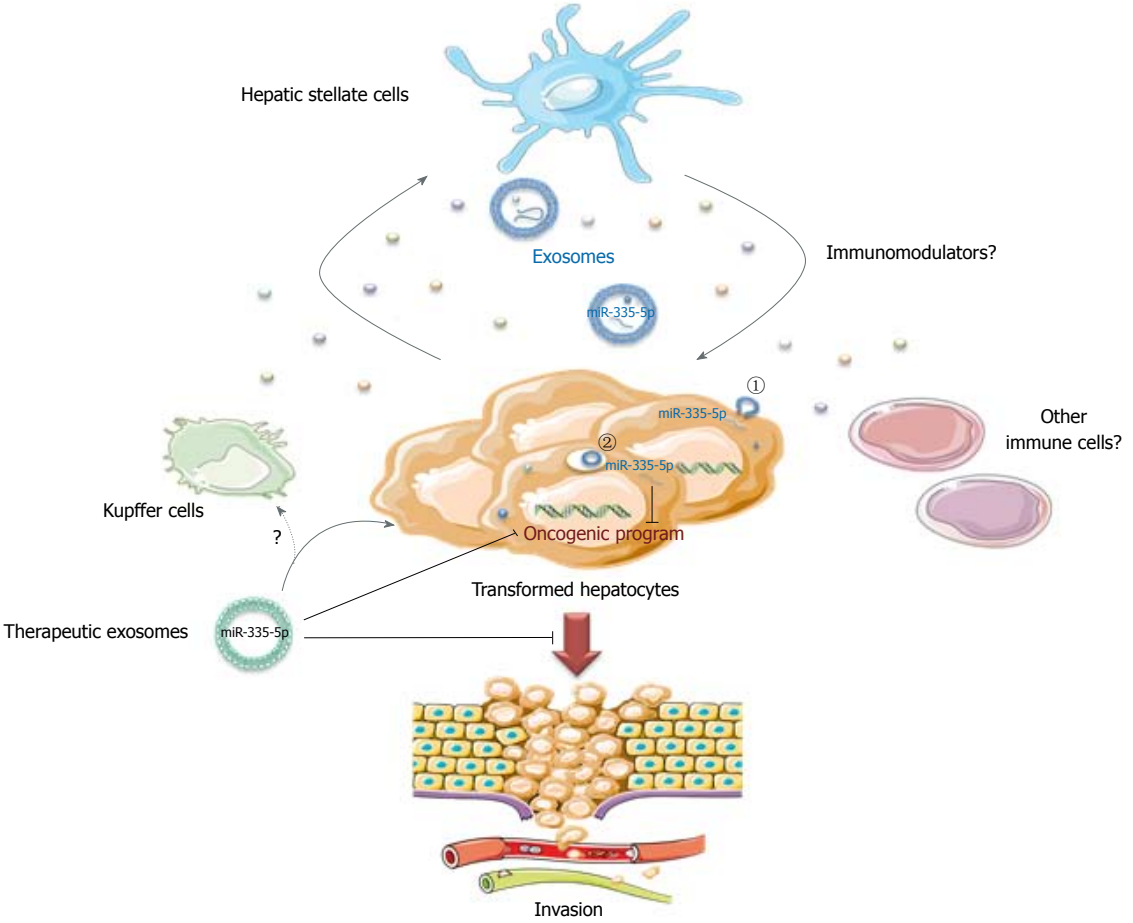

Figure 1 An exosomal miR-335-5p-based therapy for hepatocellular carcinoma. In the case of hepatocellular carcinoma, miR-335-5p is lost in cancer cells, favoring cell proliferation and invasion. The hepatic stellate cells could counteract these pro-malignant features by secreting exosomes containing nucleic acids and miRNAs, including miR-335-5p, which are captured by HCC cells by a direct fusion with recipient cell membrane (1) or by endocytosis (2). Mimicking this biological process, therapeutic exosomes, either isolated from patients or bioengineered exosome mimetics, loaded with miR-335-5p might slow cell proliferation, promote apoptosis and limit cell invasion. It remains to be determined whether other immune cells could participate in this material transfer and which immunomodulators could regulate this exchange.

HCC patients in association with progressive features and is predictive of chemo-embolization response[17]. For their study, the authors used different HCC cell lines either mutated for p53 (HuH-7) or β-catenin (HepG2), or infected with HBV (MHCC97) with low or high metastatic features. They confirmed a global tumor suppressive role of miR-335-5p associated with miR-335-5p loss in all cell lines compared to the LX-2 HSC line[14]. A miR-335-5p mimic inhibited HCC cell proliferation and invasion upon transfection, but also, crucially, when transfected in LX-2 cells seeded in co-culture with HCC cells. This suggests that a miRNA dialog between HCC and stromal cells might be established to favor tumor progression and invasion (Figure 1).

To confirm the material transfer *via* the exosomal route between HSC and HCC cell lines, the authors used an elegant system with two fluorophores coupled to a stop signal based on the Cre-lox strategy (loxP-dsRED-loxP-stop-eGFP) transfected in HCC cells. Using this tool, they demonstrated that LX-2 cells transferred Cre recombinase to neighboring HCC cells, as evidenced by green labeling. An efficient transfer was also observed when exosomes were purified from LX-2 cells and later added to HCC cell culture. *In vivo*, intra-tumoral injection of LX-2 Cre-positive exosomes to a subcutaneous HCC xenograft also led to GFP signal in the tumors. This means that the construct was efficiently recombined in HCC *via* exosome capture. Finally, with a view to treatment, Wang *et al*[14] showed that LX-2 isolated exosomes, extemporaneously enriched with miR-335-5p, reduce HCC growth after intra-tumoral injection every 2 d for 4 wk in MHCC97H cell xenografts.

The intra-tumoral administration was preferred to concentrate exosomes into the tumor mass and to mimic the historically intra-arterial administration of chemotherapeutic agents. Exosome treatment led to overexpression of miR-335-5p by 30-fold in the tumors and modification of its target landscape, resulting in an attenuation of proliferation and an increase in apoptosis. This study confirmed the previous observations

performed by the Selaru laboratory showing that LX2 cells secrete miR-195-enriched exosomes, which can communicate with CCA cells and decrease *in vivo* tumor growth in a CCA rat model after intravenous injections every 2 d[13].

PERSPECTIVES

In conclusion, these proof of concept studies performed in two different models of liver cancer support the existence of an epigenetic dialog between cancer cells and stromal cells driven by exosomes to favor tumor progression. In particular, HSCs play an important part in this dialog, but other non-parenchymal cells like Kupffer cells could also probably participate. They also highlight that HSC-derived exosome manipulation succeeds in restoring a more physiological expression of miRNAs found deregulated in cancer cells *in vivo*. The restoration of miRNA expression modifies gene expression, and subsequently limits cell proliferation and favors apoptosis. These results, and others generated in various cancer models, support extracellular vesicles as an attractive modality for personalized treatment for liver cancer, especially since this type of particle primarily targeted the liver after systemic injection.

Several studies have used exosomes as a targeted delivery system for chemotherapeutic agents, leading to cancer cell death and promoting a domino effect through the release of secondary cytotoxic vesicles[18]. Additionally, exosomes produced by mesenchymal stem cells have been largely studied in liver disease and found to be modulators of the immune response, favored by their engulfment by resident macrophages. They are also key modulators of oxidative stress and fibrotic processes[19]. Despite all these encouraging features, a number of limitations for their clinical feasibility are currently a brake for using these delivery systems. Indeed, the production of patient-derived exosomes for clinical application appears expensive, time-consuming and complex (preparation method, loading, characterization, *etc*). Since these biological carriers present pro-tumoral characteristics, the pro-malignant factors have to be preliminarily identified and removed before re-injection. The reproducibility also remains a major barrier, since a previous study suggested that three independent preparations of exosomes from mesenchymal stem cells only shared 20% of their proteome[20].

A promising alternative for extracellular vesicle-based therapeutics is the synthesis of bioengineered exosome mimetics, which could allow the production of exosome preparations suitable for clinical use (sterile, characterized, reproducible)[21]. In conclusion, even if about a hundred clinical trials are currently testing the benefit of exosomes as therapeutic agents, a gold standard method for their isolation and loading has to be approved. A better characterization of their specificity, functionality and safety is required, for which liver cancer, characterized by its pro-inflammatory microenvironment and its refractoriness to conventional treatments, undoubtedly constitutes a model of choice.

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EDITORIAL

Treating nonalcoholic steatohepatitis with antidiabetic drugs: Will GLP-1 agonists end the struggle?

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is highly associated with insulin resistance (IR), type 2 diabetes mellitus and metabolic syndrome, being characterized as the hepatic component of metabolic syndrome. Despite its high prevalence, no pharmacological treatment has been established, as of yet. A growing body of evidence, however, shows that reducing IR can result in improvement of the biochemical and histological features of nonalcoholic steatohepatitis (NASH)-the aggressive form of NAFLD that can lead to cirrhosis and hepatocellular carcinoma. Unfortunately, the several trials that have assessed the effect of various antidiabetic agents to date have failed to establish an effective and safe treatment regimen for patients with NAFLD. Glucagon-like peptide-1 (commonly known as GLP-1) agonists are a novel class of antidiabetic drugs that improve insulin sensitivity and promote weight loss. They also appear to have a direct effect on the lipid metabolism of hepatocytes, reducing hepatic steatosis. Several trials have demonstrated that GLP-1 agonists can reduce aminotransferase levels and improve liver histology in patients with NAFLD, suggesting that these agents could serve as an alternative treatment option for these patients. This manuscript discusses the role and potential mechanisms of GLP-1 agonists in the treatment of NASH.

Key words: Nonalcoholic steatohepatitis; Cirrhosis; Glucagon-like peptide-1 receptor agonists; Nonalcoholic fatty liver disease

Core tip: There is an urgent need for an effective treatment of nonalcoholic fatty liver disease (NAFLD). Growing evidence indicates that reducing insulin resistance can result in improvement of the biochemical

and histological features of patients with nonalcoholic steatohepatitis (NASH). However, no antidiabetic agent to date has been proven as both safe and effective for the treatment of patients with NASH. Recent studies have demonstrated that glucagon-like peptide-1 agonists, a novel class of antidiabetic drugs, may be effective in slowing the progression of NAFLD, highlighting their potential role in the treatment of this complex disease.

Kalogirou M, Sinakos E. Treating nonalcoholic steatohepatitis with antidiabetic drugs: Will GLP-1 agonists end the struggle? *World J Hepatol* 2018; 10(11): 790-794 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i11/790.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i11.790

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of clinical and histopathological conditions, ranging from simple steatosis [*i.e.,* nonalcoholic fatty liver (NAFL)] to liver injury [*i.e.,* nonalcoholic steatohepatitis (NASH), the aggressive form of NAFLD that can lead to cirrhosis and hepatocellular carcinoma][1,2]. NAFLD is highly associated with metabolic syndrome and type 2 diabetes mellitus (T2DM)[3]. In fact, the prevalence of NAFLD in T2DM has been estimated to be around 60%[4].

The pathophysiology of NAFLD is not yet fully elucidated; however, it is widely believed that insulin resistance (IR) may play a critical role in the pathogenesis of the disease. Several studies have shown that patients with NAFL and NASH are characterized by IR and hyperinsulinemia, irrespective of glucose tolerance or body mass index[5,6]. The multi-hit hypothesis, initially described by Day and James[7], claims that IR is the key factor in the pathogenesis of steatosis. IR causes dysregulation of peripheral lipolysis and increases *de novo* lipogenesis, leading to elevated levels of circulating fatty acids and lipid accumulation within hepatocytes-the "first hit" that predisposes to liver injury, inflammation and fibrosis[8]. Disrupted insulin signaling is also involved in inflammatory cascade activation, lipid peroxidation and liver injury-"the second hit" leading to NASH[9].

Currently, NAFLD is reported to be the most common chronic liver disease worldwide[10]. However, despite huge efforts, there is still no established pharmacotherapy. Lifestyle modifications remain the sole therapeutic approach[11]. Given that IR is considered as the main pathogenetic factor for the development of NAFLD, drugs targeting IR have been investigated the most as potential treatment options for NAFLD, but the studies have yielded conflicting results.

Metformin, the most widely used insulin-sensitizing agent, improves insulin sensitivity by mechanisms that are not yet fully understood[12]. A meta-analysis assessing the effect of metformin in NAFLD revealed that, while it can improve the biochemical and metabolic features of NAFLD, it does not improve the patients' histological response[13]. Metformin is not currently recommended for the treatment of NAFLD by either the American Association for the Study of Liver Diseases or the European Association for the Study of Liver (commonly referred to by their acronyms, AASLD and EASL, respectively)[14,15].

Thiazolidinediones, another class of insulin-sensitizers, act by redistributing fat from ectopic tissues to the adipose tissue, and by increasing levels of adiponectin-an adipokine that has insulin - sensitizing properties[16,17]. Several studies have evaluated the efficacy of thiazolidinediones in patients with NAFLD. The "Pioglitazone *vs* vitamin E *vs* placebo for the treatment of nondiabetic patients with nonalcoholic steatohepatitis" trial (published as the PIVENS trial) was the largest one performed, involving 247 nondiabetic patients with biopsy-proven NASH[18]. The patients were randomized to receive either pioglitazone (30 mg/d) or vitamin E (800 IU/d) or placebo. The pioglitazone treatment was associated with a significant reduction in steatosis and lobular inflammation compared to placebo; however, it did not improve fibrosis. A randomized, placebo-controlled trial performed in patients with NASH and prediabetes or T2DM showed that pioglitazone achieved the primary endpoint of an $\geqslant$ 2-point decrease in NAFLD activity score without worsening fibrosis, and was associated with improvement in steatosis, inflammation and ballooning necrosis[19]. The AASLD and EASL have suggested the use of pioglitazone in patients with biopsy - proven NASH[14,15], although concerns about the side effects and long-term safety of this drug have limited its widespread use. Pioglitazone has been associated with weight gain that is persistent (even after discontinuation of the treatment), fluid retention, deterioration of heart failure, bone fractures, and increased risk of bladder cancer[20-23].

ROLE OF GLUCAGON-LIKE PEPTIDE-1 AGONISTS IN NAFLD

Glucagon-like peptide-1 (GLP-1) agonists represent a novel class of antidiabetic drugs. They mimic the action of endogenous GLP-1, a gastrointestinal hormone of the incretin class of proteins that is secreted from Langerhans cells in response to nutrient ingestion[24]. This hormone has several metabolic effects, including the stimulation of glucose-dependent insulin secretion, inhibition of glucagon release, induction of pancreatic β-cell proliferation, and delay of gastric emptying[25]. While native GLP-1 is rapidly degraded by the enzyme dipeptidy1 peptidase-4 (otherwise known as DPP-4), GLP-1 agonists have increased resistance to DPP-4, thus prolonging the half-life time[26]. These agents have been shown to have beneficial effects on IR and weight control[25]. Several studies have demonstrated the

presence of GLP-1 receptor in hepatocytes, implying that GLP-1 agonists may also exert a direct effect on the liver. Gupta *et al*[27] found that the GLP-1 receptor plays a key role in the decrease of hepatic steatosis *in vitro*, by modulating elements of the insulin signaling pathway. GLP-1 agonists have demonstrated protection of hepatocytes from fatty acid-related death by prohibition of a dysfunctional endoplasmic reticulum stress response. They also appear to reduce fatty acid accumulation by activation of both macroautophagy and chaperone-mediated autophagy[28]. Evidence suggests that GLP-1 secretion is impaired in patients with NAFLD and NASH, highlighting the role of GLP-1 agonists as potential candidates for NAFLD treatment[29].

Liraglutide

Multiple trials have evaluated the efficacy of GLP-1-based therapies in NAFLD. Among the GLP-1 agonists, liraglutide is the most widely studied drug. In the "Liraglutide Efficacy and Action in NASH" study (published as the LEAN study), a double-blind randomized control trial, Armstrong *et al*[30] assessed the effect of 48 wk of treatment with liraglutide in patients with biopsy-proven NASH. Fifty-two patients with (n = 17) or without (n = 35) T2DM were randomly allocated to receive either liraglutide (1.8 mg/d) or placebo. The primary endpoint of the study was the resolution of definite steatohepatitis without worsening fibrosis. Secondary histological endpoints included change in the overall NAFLD activity score (steatosis, ballooning, lobular inflammation) and its individual components. Overall, 9/23 patients in the liraglutide group showed resolution of NASH with no worsening fibrosis compared to 2/22 patients in the placebo group (P = 0.019), successfully meeting the primary endpoint. Regarding the secondary outcomes, fewer patients in the liraglutide group showed progression in fibrosis compared to the placebo group (2/23 *vs* 8/22, P = 0.04). However, results concerning lobular inflammation and overall NAFLD activity score were not statistically significant when compared between the two groups. The authors used histological primary endpoints, being able to evaluate the direct effect of liraglutide on the liver. The study was performed on patients with biopsy-proven NASH, avoiding the inclusion of those without definite NASH. Their findings suggested that liraglutide led to the histological resolution of NASH, with the small sample size being, however, a major limitation.

Ohki *et al*[31] performed a retrospective cohort study evaluating the efficacy of liraglutide compared to sitagliptin and pioglitazone in patients with NAFLD. They reported a significant reduction in serum aminotransferase levels for all groups, while the aspartate aminotransferase (AST)-to-platelet counts ratio index was significantly reduced only for the liraglutide and pioglitazone groups. Body weight significantly decreased in the liraglutide group, while it increased in the pioglitazone group and did not retain a statistically significant difference for the sitagliptin group. Administration of liraglutide was identified as an independent factor for body weight reduction in multivariate analysis.

In a recent open-label trial by Feng *et al*[32], 87 patients with NAFLD were randomized to receive liraglutide, metformin or gliclazide for 24 wk. All three groups showed reduced intrahepatic fat, but the liraglutide group had the greatest reduction. In addition, the researchers found a statistically significant decrease in serum AST and alanine aminotransferase levels only in the liraglutide and metformin group, reporting slightly better results for the liraglutide group. However, a study by Khoo *et al*[33] demonstrated that liraglutide was as effective as structured lifestyle modification for reduction of liver fat fraction and serum aminotransferase levels.

Exenatide

Two trials examined the use of exenatide in patients with NAFLD and T2DM[34,35]. In the first, Shao and colleagues[34] studied 60 patients with NAFLD and T2DM[34]. The patients were randomized to receive exenatide plus insulin glargine U-100 (exenatide group) or insulin glargine U-100 plus insulin aspart (intensive insulin group) for 12 wk. The levels of alanine aminotransferase, AST, and gamma-glutamyl transferase were significantly lower in the exenatide group than in the intensive insulin group. The exenatide plus insulin glargine treatment was also found to be superior to the intensive insulin therapy concerning the reversal rate of fatty liver (93.3% *vs* 66.7%, respectively). The second study, conducted by Fan *et al*[35], compared the efficacy of exenatide *versus* metformin in patients with NAFLD and T2DM. The results revealed that exenatide was more effective than metformin in reducing body weight and improving liver enzymes. Nevertheless, the efficacy of exenatide has not been evaluated in randomized trials with histological outcomes in patients with NASH, as of yet. Lastly, a recent meta-analysis of six studies assessing the efficacy of GLP-1 agonists (liraglutide and exenatide) in NAFLD, revealed that these agents improve liver histology and reduce serum aminotransferase levels, indicating that they might be effective in patients with biopsy-proven NASH[36].

Semaglutide

Semaglutide is a novel long-acting GLP-1 analogue, and has been recently approved for T2DM[37]. It has 94% sequence homology to human GLP-1 and a half-life of 165 h, supporting a once weekly scheme of administration[38]. Semaglutide has shown beneficial effects on glucose control and weight loss compared to placebo and other antidiabetic drugs in patients with T2DM in the "SUSTAIN" trial program[37,38]. It is currently under investigation for its potential as a treatment option for patients with NASH. A 72-wk, randomized, double-blind trial of 372 patients comparing the effi-

cacy and safety of three dose levels of subcutaneous semaglutide once daily *vs* placebo in NASH patients is ongoing (NCT02970942). This trial is expected to be completed during 2019 and will provide additional information on the effectiveness of GLP-1 agonists in patients with NAFLD.

CONCLUSION

GLP-1 agonists are not currently recommended by the AASLD and EASL for the treatment of NAFLD. In their latest guidelines, it was pointed out that it is still premature to consider these agents as a specific treatment for patients with NASH without diabetes, due to inadequate evidence[14,15]. Future research is, therefore, needed to confirm their efficacy in these patients.

In conclusion, current evidence suggests that GLP-1 agonists may be an attractive therapeutic option for patients with NAFLD. However, larger studies of longer duration with histological endpoints are still required to establish their exact role in the management of NAFLD.

Perspective for future study

GLP-1 agonists have been shown to be effective in improving liver histology and reducing aminotransferase levels in patients with NASH. So, the question arises as to whether these agents could serve as a treatment option for such patients. While data are promising, they are still limited. Large-scale randomized, placebo-controlled trials with complete histological outcomes are warranted to elucidate the efficacy of GLP-1 agonists in treating NASH. Another major limitation of the currently available studies is the lack of long-term outcomes. Studies of longer duration are required to properly evaluate the histological improvement in NASH. What is more, it would be interesting if future trials would include both diabetic and nondiabetic patients, in order to clarify the effect of GLP-1 agonists in NASH, regardless of changes in glycemic control. It will also be significant to assess whether GLP-1 agonists affect NAFLD in a dose-dependent manner, in order to search for preferred doses.

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EDITORIAL

Novel insights in the prevention of perinatal transmission of hepatitis B

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Abstract

Perinatal transmission of hepatitis B virus (HBV) infection is major contributor to the growing burden of chronic hepatitis B worldwide. Administration of HBV immunoglobulin and HBV vaccination as soon after pregnancy as possible are the mainstay of prevention of perinatal transmission of HBV infection. In women with high viral loads, antiviral prophylaxis also appears to be useful. Lamivudine, telbivudine and tenofovir have been shown to be both safe and effective in this setting but tenofovir is the first-line option due to its low potential for resistance and more favorable safety profile.

Key words: Tenofovir; Perinatal transmission; Hepatitis B; Lamivudine; Telbivudine

Core tip: Administration of hepatitis B virus (HBV) immunoglobulin and HBV vaccination as soon after pregnancy as possible are the mainstay of prevention of perinatal transmission of HBV infection. In women with high viral loads, antiviral prophylaxis with tenofovir also appears to be useful.

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INTRODUCTION

Perinatal transmission of hepatitis B virus (HBV) is a major healthcare problem, particularly in low-income countries with high prevalence of chronic hepatitis B (CHB)[1]. In regions where CHB is endemic, HBeAg(+) mothers transmit HBV in 70%-90% of their children if prophylaxis is not administered[1]. In addition, high CHB prevalence, poor compliance with medical care and barriers to health care among low-income population groups, especially in immigrants and Roma population, are associated with increased perinatal HBV transmission even in developed European countries[2,3]. Women with high viral loads are at particularly increased risk to transmit hepatitis B to their offspring[4-7]. In many CHB endemic areas, perinatal transmission of hepatitis B is the major cause of transmission of hepatitis B[8,9]. Moreover, progression from HBV infection to CHB is substantially more frequent in the offspring of HBeAg(+) women than in patients who are exposed to HBV during adulthood[10,11]. Indeed, approximately 90% of the former will progress to CHB[10,11].

ROLE OF HBV IMMUNOGLOBULIN AND HBV VACCINATION

Administration of HBV immunoglobulin and HBV vaccination prevents most cases of perinatal HBV transmission[1]. Nevertheless, children born from women with high viral load are still at considerable risk for acquiring HBV despite the administration of HBV immunoglobulin and HBV vaccination [8%-18% when HBV deoxyribonucleic acid (DNA) levels are > 10^7-10^8 copies/mL][4,12-14]. On the other hand, a recent study reported that prompt administration of HBV immunoglobulin (*i.e.* within 4 h after birth) and/or an increase in the number of HBV vaccination doses (at birth and at 1, 2, 4 and 6 mo) resulted in very low rates of perinatal HBV transmission (2%) in HBeAg-positive women with HBV DNA levels > 200000 IU/mL[15].

ROLE OF NUCLEOSIDE ANALOGUES

Several studies also showed that nucleoside analogues combined with administration of HBV immunoglobulin and HBV vaccination are more effective in the prevention of perinatal HBV transmission than administration of HBV immunoglobulin and HBV vaccination alone[16]. In a meta-analysis of 5 small randomized controlled trials (RCTs, *n* = 444 pregnant women), treatment with lamivudine combined with administration of HBV immunoglobulin and HBV vaccination reduced infant HBsAg seropositivity by 11.7% and infant HBV DNA positivity by 21.2% compared with administration of HBV immunoglobulin and HBV vaccination[17]. In a meta-analysis of 4 small RCTs (*n* = 293 pregnant women), telbivudine also reduced infant HBsAg seropositivity by 15.8% and infant HBV DNA positivity by 16.2% compared to the control group[17]. Three early small nonrandomized studies (*n* = 307 pregnant women) showed that tenofovir also reduces the risk for perinatal HBV transmission[18-20]. In a more recent RCT in HBeAg-positive mothers with viral load > 200000 IU/mL (*n* = 200), HBV transmission was observed in 5% of cases who received tenofovir in addition to HBV immunoglobulin/HBV vaccination compared with 18% in mothers treated with HBV immunoglobulin/HBV vaccination alone[14]. In contrast, in a larger RCT (*n* = 331), tenofovir combined with HBV immunoglobulin/HBV vaccination did not reduce the risk of HBV transmission compared with HBV immunoglobulin/HBV vaccination alone[15]. However, rates of HBV transmission in the latter group were very low (2%) and it is possible that the study was not powered to show superiority of tenofovir[15].

Very few studies compared the efficacy of different nucleoside analogues in the prevention of perinatal HBV transmission. In two non-randomized studies (*n* = 690 pregnant women), lamivudine was equally effective with telbivudine[21,22] and in another non-randomized study (*n* = 120 pregnant women), lamivudine was similarly effective with tenofovir[18]. Lamivudine, telbivudine and tenofovir also appear to be safe during pregnancy and do not increase the risk of congenital malformation, prematurity or maternal complications[17,23]. However, it should be emphasized that tenofovir and telbivudine are both Food and Drug Administration (FDA) pregnancy category B drugs (*i.e.,* no risk in animal studies, unknown in humans) whereas lamivudine is FDA pregnancy category C drug (*i.e.,* teratogenic in animal studies, unknown in humans)[24]. It has also been shown that in the United States, a country with very low prevalence of CHB, combining a nucleoside analogue with HBV immunoglobulin/HBV vaccination is more cost-effective than HBV immunoglobulin/HBV vaccination alone[25]. Nevertheless, it should be emphasized that none of these agents are licensed for use during pregnancy.

Current guidelines recommend screening of all pregnant women for CHB during the first trimester of pregnancy[24,26,27]. In all pregnant women with HBV DNA levels > 200000 IU/mL and/or > 6-7 $\log_{10}$ IU/mL or HBsAg levels > 4 $\log_{10}$ IU/mL, antiviral prophylaxis with tenofovir should start at week 24-32 of gestation and continue for up to 4-12 wk after delivery[24,26,27]. Tenofovir is preferred over lamivudine and telbivudine because of lower resistance rates and because it is a FDA pregnancy category B drug[24,26,27].

ROLE OF CAESAREAN SECTION

The role of caesarean section in the prevention of perinatal transmission of HBV infection is unclear. In a recent meta-analysis of 10 studies (*n* = 5091 new-

borns), caesarean section reduced the incidence HBV transmission by 38% compared with vaginal delivery (95%CI: 0.40-0.98; P = 0.04)[28]. However, the benefit of caesarean section was smaller in studies where hepatitis B immunoglobulin was administered to all women[28]. Moreover, caesarean section did not reduce the risk of vertical HBV transmission in HBeAg(+) women[28]. Accordingly, current guidelines do not recommend caesarean section for the prevention of perinatal transmission of HBV infection due to insufficient data[26].

BREASTFEEDING IN HBsAg(+) WOMEN

Regarding breastfeeding, current guidelines state that it is not contraindicated in HBsAg(+) women who are not receiving nucleoside analogues, since breast milk contains the lowest concentrations of HBV among body fluids and breast feeding does not increase the risk of HBV transmission in women who receive HBV immunoglobulin and HBV vaccination[24,26,27,29]. Moreover, breastfeeding is also not prohibited in women who are receiving prophylaxis with tenofovir, since this agent is excreted in very small amounts in breast milk[24,26,27,30,31].

CONCLUSION

Perinatal transmission of HBV infection is major contributor to the growing burden of CHB worldwide. Administration of HBV immunoglobulin and HBV vaccination as soon after pregnancy as possible are the mainstay of prevention of perinatal transmission of HBV infection. In women with high viral loads, antiviral prophylaxis with tenofovir also appears to be useful. Strategies to improve the awareness of this major healthcare problem are also needed to curb the rising incidence of CHB infection.

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REVIEW

Role of traditional Chinese medicine in the management of patients with hepatocellular carcinoma

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Abstract

Traditional Chinese Medicines (TCMs) have been employed for centuries in the treatment of patients with hepatocellular carcinoma (HCC). Previous reviews of this topic have focused on certain aspects of TCM treatment rather than an overall assessment of their value and mechanisms of action. Both the Chinese and English medical literatures were reviewed to identify where TCM might be of value in the treatment of HCC and the justification for such treatment. TCM treatment corrects the "internal disequilibriums" thought to be responsible for the development, growth, and spread of the tumor. It has also been used to manage symptoms associated with HCC and the adverse effects of chemo- and radiation-therapies. Recent research has documented the precise effects of TCM on tumor biology. There are also increasing efforts to identify which of the many components of TCM herbal remedies are primarily responsible for these beneficial effects. This review outlines the benefits of TCM treatment of HCC and the laboratory data describing their anti-tumor properties.

Key words: Hepatoma; Herbal medicine; Liver disease; Liver; Cancer

Core tip: Traditional Chinese Medicines (TCMs) are commonly employed by patients with hepatocellular carcinoma (HCC). This review identifies which herbal concoctions are most frequently recommended by TCM authorities. TCMs serve to correct internal imbalances that contribute to HCC. TCMs favorably alter HCC cell biology.

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INTRODUCTION

Traditional Chinese Medicine (TCM) is a comprehensive medical system that utilizes herbal remedies, acupuncture, dietary therapy, exercise, and massage to prevent, treat, and rehabilitate disease states by restoring the internal environments of an individual to a state of equilibrium. It is based on traditional medical theories and the practice experiences of Chinese TCM physicians. The traditional medical theories describe two components of illness: "holism" (the concept of viewing the situation as a whole) and "syndrome differentiation" (the consequences of disrupted holism). Thus, rather than focusing on the tumor *per se*, TCM focuses on correcting the internal disequilibriums responsible for tumor development and progression.

Given the phylogeny of the oncogenic hepatitis B virus (HBV), it can be assumed that hepatocellular carcinoma (HCC) has been prevalent in the Chinese population for centuries[1]. Hence, Chinese TCM physicians have had extensive experience in identifying, developing, and refining treatments for this potentially lethal tumor. This longstanding experience and commitment to treating HCC is an important feature of TCM. Specifically, unlike "Western Medicine" where effective treatments are identified by the results of prospective, randomized, placebo-controlled trials, in TCM, the value of a particular herbal concoction is gauged by the number of recommendations it has received by TCM authorities over the course of centuries.

MOST COMMONLY EMPLOYED TCMS FOR HCC

TCM physicians have identified various Chinese herbal medicines that represent every category of the Chinese materia medica recognized by the International Organization for Standardization (ISO)[2]. The majority of these agents are deficiency-supplementing herbs, heat-clearing herbs, and blood-quickening stasis-transforming herbs (Table 1).

The ten most commonly employed individual herbs are provided in Table 2. They are: Poria (Fuling), Rhizoma Atractylodis Macrocephalae (Baizhu), Radix Astragali Mongolici (Huangqi), Herba Hedyotidis (Baihuasheshecao), Radix Glycyrrhizae (Gancao), Radix Bupleuri Chinensis (Chaihu), Radix Codonopsis (Dangshen), Radix Paeoniae Alba (Baishao), Radix Angelicae Sinensis (Danggui) and Carapax Trionycis (Biejia).

Often, combinations of herbs are advocated such as qi-boosting spleen-supplementing herbs being combined with heat-clearing toxin-resolving herbs, blood-quickening stasis-transforming herbs and/or liver-soothing qi-rectifying herbs (qi is the vital life force that is thought to animate the body internally)[3]. The ten most commonly advocated combinations of herbs are provided in Table 3.

TCMS FOR THE TREATMENT OF HCC SYMPTOMS

Anorexia, fatigue, weakness, and right upper quadrant discomfort are the most common symptoms of HCC while ascites and jaundice are the most common signs[4]. TCMs are often used in the treatment of these and the other features listed in Table 4. In a recent cluster analysis performed by Liu *et al*[4], Endothelium Coreneum Gigeriae Galli (Jineijin) and Fructus Hordei Germinatus (Maiya) were the most commonly-used herbal medicines for treating anorexia; Radix Astragali Mongolici (Huangqi) for fatigue and weakness; Rhizoma Corydalis Yanhusuo (Yanhusuo) and Fructus Toosendan (Chuanlianzi) for right upper quadrant discomfort; Pericarpium Arecae (Dafupi), Polyporus (Zhuling) and Poria (Fuling) for ascites; and Herba Artemisiae Capillaris (Yinchen) for jaundice[5]. Other herbal medicines used to treat less common symptoms and signs of HCC are also provided in Table 4.

TCM FOR IMPROVED QUALITY OF LIFE AND SURVIVAL IN HCC PATIENTS

The use of TCM to correct disequilibriums in a patient's internal environment has been associated with improved quality of life for HCC patients. For example, the Jianpi Jiedu Decoction has been reported to improve quality of life by attenuating symptoms in 30 patients with advanced HCC[6]. Similar results have been obtained with other combinations[7-10].

Other studies have described improved survival. Specifically, compared to untreated controls, treatment with a Ruanganlidan Decoction and Rhizoma Curcumae Longae increased median disease-free survival by approximately 12 mo in 78 HCC patients[11]. In another study, Qudu Huayu Xiaoji Formula not only improved the quality of life in 77 HCC patients after hepatic arterial chemoembolization, but also prolonged survival by 5-9 mo when compared to 76 patients treated with chemoembolization alone[12].

TCM AND ADVERSE REACTIONS TO CHEMOTHERAPEUTIC AGENTS

Side effects of chemotherapy are major concerns for cancer patients and often interfere with treatment.

Table 1 Types of herbal medicines and frequency of use in the treatment of patients with hepatocellular carcinoma

Category	Relative frequency	Category	Relative frequency
Herbs that supplement deficiency: Baizhu, Huangqi, Dangshen, Danggui, Shanyao, Gancao, Baishao, Biejia	27.70%	Herbs that drain downwards: Dahuang, Yuanhua	1.37%
Herbs that clear the heat: Baihuasheshecao, Banzhilian, Shengdihuang, Zhizi, Huangqin, Qinghao	19.26%	Herbs that astringe: Wuweizi, Shanzhuyu	1.01%
Herbs that invigorate blood and dissolve stasis: Ezhu, Danshen, Yujin, Tubiechong	13.67%	Herbs that counteract toxins, kill parasites and relieve itching: Fengfang	0.68%
Herbs that promote urination and percolate dampness: Fuling, Yiyiren, Yinchen, Cheqianzi, Yumixu	12.04%	Herbs that warm the interior: Wuyao	0.54%
Herbs that rectify qi: Zhiqiao, Chenpi	8.39%	Herbs that expel wind and damp: Sangjisheng, Qinjiao	0.46%
Herbs that release the exterior: Chaihu, Guizhi	4.14%	Herbs that calm the mind: Suanzaoren, Longgu	0.42%
Herbs that promote digestion: Jineijin	3.18%	Herbs that calm the liver and extinguish wind: Muli, Wugong	0.25%
Herbs that relieve cough, dissolve phlegm and calm panting: Banxia, Tinglizi, Walengzi	2.94%	Herbs that open the orifices: Shexiang	0.11%
Herbs that stanch bleeding: Sanqi, Xianhecao, Baimaogen	1.91%	Herbs that expel parasites: Binglang	0.08%
Herbs that transform dampness: Houpo	1.86%	Herbs that induce vomit: Changshan	0.02%

Table 2 The most frequently prescribed herbal medicines used in the treatment of patients with hepatocellular carcinoma

Herb name	Relative frequency	Herb name	Relative frequency
Poria (Fuling)	5.20%	Radix Angelicae Sinensis (Danggui)	2.35%
Rhizoma Atractylodis Macrocephalae (Baizhu)	5.20%	Carapax Trionycis (Biejia)	2.22%
Radix Astragali Mongolici (Huangqi)	4.07%	Radix Bupleuri Chinensis (Chaihu)	3.66%
Herba Hedyotidis (Baihuasheshecao)	3.75%	Radix Codonopsis (Dangshen)	3.26%
Radix Glycyrrhizae (Gancao)	3.71%	Radix Paeoniae Alba (Baishao)	3.03%

Numerous TCM herbs have been identified that reduce the side effects and non-tumor toxicity of chemotherapeutics. For example, Ciji Hua'ai Baosheng Granule Formula (CHBGF) attenuates the decreases in white blood cell and platelet counts of H_{22} hepatoma transplanted tumor caused by chemotherapy[13]. Combining Rhizoma Zingberis Recens (Shengjiang) and Rhizoma Phragmitis (Lugen) reduces the vomiting caused by chemotherapy in H_{22} hepatoma carcinoma-bearing mice[14], and Danggui Beimu Kushen attenuates cisplatin toxicity (in the same animal model). Other TCMs such as Panaxan, Fufang Ejiao Jiang, Lianqi Capsule, and the aqueous extract of Fructus Akebiae (Bayuezha) have also been reported to reduce side effects and improve the efficacy of chemotherapy for HCC in H_{22} hepatoma bearing mice[15-18]. Compared to chemotherapy alone, Tremella Polysaccharide, extracted from Polyporus (Zhuling), improved quality of life and physical activity and attenuated fatigue, nausea, vomiting, constipation, diarrhea, and white blood cell counts during chemotherapy in 50 patients[19]. Jianpi Jiedu Formula minimized hepatic dysfunction following transarterial chemoembolization (TACE) treatment in 16 patients[20]. Similarly, the Zipi Decoction was associated with improved hepatic function following TACE when compared to TACE alone[21]. Jian Pi Li Qi Decoction in 52 patients and Jiedu Granules combined with Cinobufacini in 60 patients alleviated signs and symptoms of the postembolization syndrome following TACE[22]. Finally, it should be noted that on occasion, TCM can adversely affect patient outcomes when TCM and chemotherapy drugs interact[23].

TCM AND HCC TUMOR BIOLOGY

Recent developments in molecular and cell biology have provided important insights into the pathogenesis and course of HCC. They have also provided investigators with an opportunity to identify the mechanisms whereby TCM impacts HCC. To date, such research has focused on HCC proliferative activity, apoptosis, metastasis, angiogenesis, immune reactivity, and multidrug resistance.

The effects of TCM on the proliferative activity and growth of malignant hepatocytes and tumors

A large number of herbs have been reported to inhibit malignant hepatocyte proliferation and tumor growth. In many instances, the precise mechanisms and signaling pathways have also been identified. For example, Akebia trifoliate (Thunb.) and Koidz (Sanyemutong) seed extract inhibited the proliferation of various human HCC cell lines *via* induction of endoplasmic reticulum stress *in vitro*[24] whereas the ethyl acetate extraction from a Chinese herbal formula, Jiedu Xiaozheng Yin inhibited proliferative activity by suppression of the

Table 3 Descending frequency of herbal medicine combinations used in the treatment of patients with hepatocellular carcinoma

Precedence	Herbal medicine combinations
1	Rhizoma Atractylodis Macrocephalae (Baizhu) and Poria (Fuling)
2	Radix Astragali Mongolici (Huangqi) and Rhizoma Atractylodis Macrocephalae Baizhu)
3	Radix Astragali Mongolici (Huangqi) and Radix Codonopsis (Dangshen)
4	Radix Astragali Mongolici (Huangqi) and Radix Angelicae Sinensis (Danggui)
5	Radix Astragali Mongolici (Huangqi) and Poria (Fuling)
6	Rhizoma Atractylodis Macrocephalae (Baizhu) and Radix Curcumae Wenyujin (Yujin)
7	Rhizoma Atractylodis Macrocephalae (Baizhu) and Radix Bupleuri Chinensis (Chaihu)
8	Rhizoma Atractylodis Macrocephalae (Baizhu) and Radix Glycyrrhizae (Gancao)
9	Rhizoma Atractylodis Macrocephalae (Baizhu) and Pericarpium Citri Reticulatae (Chenpi)
10	Rhizoma Atractylodis Macrocephalae (Baizhu) and Radix Codonopsis (Dangshen)

Table 4 Herbal medicines and the frequency of their use in treating symptoms and signs associated with hepatocellular carcinoma

Symptoms and signs	Herb and frequency of use (*n*)
Anorexia	Endothelium Coreneum Gigeriae Galli (Jineijin) (18), Fructus Hordei Germinatus (Maiya) (12), Fructus Amomi (Sharen) (9), stir-baking Fructus Hordei Germinatus et Massa Fer-mentata Medicinalis (Jiaosanxian) (7), Fructus Setariae Germinatus (Guya) (6), Massa Medicata Fermentata (Shenqu) (5) and Fructus Crataegi Pinnatifidae (Shanzha) (5)
Fatigue	Radix Astragali Mongolici (Huangqi) (23) and Radix Codonopsis (Dangshen) (14)
Discomfort	Rhizoma Corydalis Yanhusuo (Yanhusuo) (15), Fructus Toosendan (Chuanlianzi) (13), Radix Curcumae Wenyujin (Yujin) (10), Olibanum (Ruxiang) (9), Myrrha (Moyao) (7), Fructus Citri Sarcodactylis (Foshou) (7), Radix Aucklandiae (Muxiang) (5) and Rhizoma Cyperi (Xiangfu) (5)
Ascites	Pericarpium Arecae (Dafupi) (30), Polyporus (Zhuling) (22), Poria (Fuling) (18), Rhizoma Alismatis (Zexie) (13), Semen Plantaginis (Cheqianzi) (8) and Cortex Magnoliae Officinalis (Houpo) (5)
Jaundice	Herba Artemisiae Capillaris (Yinchen) (37), Rhizoma Polygoni Cuspidati (Huzhang) (13), Radix et Rhizoma Rhei Palmati (Dahuang) (11), Herba Hyperici Japonici (Tianjihuang) (8), Fructus Gradeniae (Zhizi) (8), Herba Lysimachiae (Jinqiancao) (7), Radix Paeoniae Rubra (Chishao) (6) and Radix Scutellariae Baicalensis (Huangqin) (6)
Abdominal distention	Fructus Aurantii Submaturus (Zhiqiao) (11), Cortex Magnoliae Officinalis (Houpo) (8), Semen Raphani Sativi (Laifuzi) (7), Pericarpium Citri Reticulatae Viride (Qingpi) (6), Radix Aucklandiae (Muxiang) (6), Fructus Amomi (Sharen) (5) and Fructus Aurantii Immaturus (Zhishi) (5)
Nausea and vomiting	Caulis Bambusae in Taeniam (Zhuru) (27), Rhizoma Pinelliae (Banxia) (19), Flos Inulae (Xuanfuhua) (17), Fructus Amomi (Sharen) (10), Ochra Haematitum (Daizheshi) (7) and Pericarpium Citri Reticulatae (Jupi) (6)
Fever	Gypsum Fibrosum (Shigao) (9), Cortex Moutan Radicis (Mudanpi) (8), Radix Bupleuri Chinensis (Chaihu) (8), Herba Artemisiae Annuae (Qinghao) (6), Rhizoma Anemarrhenae (Zhimu) (6) and Fructus Gradeniae (Zhizi) (6)
Diarrhea	Poria (Fuling) (7), Rhizoma Alismatis (Zexie) (7), Semen Euryales (Qianshi) (6) and Fructus Schisandrae Chinensis (Wuweizi) (5)
Constipation	Radix et Rhizoma Rhei Palmati (Dahuang) (12), Fructus et Semen Trichosanthis Kirilowii (Gualou) (6), Semen Pruni Japonicae (Yuliren) (5) and Fructus Cannabis (Huomaren) (5)

polycomb gene product Bmi1 and Wnt/β-catenin signaling and inducing G0/G1 phase arrest *in vitro* and *in vivo*[25,26]. Coptischinensis (Huanglian) restrained HepG2 cell proliferation through activation of the the NAG-1 gene enzyme *in vitro*[27].

Other TCM herbs have been reported to inhibit malignant hepatocyte proliferative activity and tumor growth through mechanisms that have yet to be identified. Of these, Bufalin, a component of Venenum Bufonis (Chansu), inhibited both proliferation and invasion of HCC cells *in vitro*[28], and Chaiqiyigan granula enhanced Taxol-induced growth inhibition of HCC xenografts in nude mice[29]. Other herbal medicine extracts that have been reported to possess tumor growth inhibiting properties *via* yet to be defined mechanisms include Jianpi Huayu Formula, which inhibited BEL7402 cell proliferation *in vitro*[30], Compound Recipe Kushen SMMC, which inhibited 7721 cell proliferation *in vitro*[31], and Fuzheng Yiliu Granule, which inhibited PLC tumor growth in H_{22} hepatoma-bearing ICR mice and the HepG2 cell line[32].

The effects of TCM on apoptosis and autophagy of malignant hepatocytes

Dysregulation of apoptosis and autophagy are important components of tumor development, often resulting from activation of oncogenes and/or mutations in tumor suppressor genes. Thus, much effort has been expended on identifying TCM herbs that induce malignant hepatocyte apoptosis. Kangai Fuzheng Prescription was found to promote apoptosis and inhibit the growth of human hepatoma SMMZ-7721 cells by downregulating *p53* gene expression *in vitro*[33]. TCM matrine, a component of Radix Sophorae Flavescentis (Kushen), induced apoptosis and cell arrest by altering Bcl-2, Bax, and miR122a expression in human HepG2 cells and murine HCC cells[34,35]. Quercetin, an extract

from multiple herbal medicines, promoted apoptosis in the same HepG2 cells by increasing the transcription of the apoptosis-related *fas* gene[36]. Ligustrumlucidum Aitfruit (Nüzhenzi) extract could induce apoptosis and cell senescence through upregulation of p21 in human HCC cell lines[37]. Finally, modified Yi Guan Jian, a Chinese herbal formula, induced apoptosis in Bel-7402 cells[38] and Rhizoma Panacis Majoria (Zhuzishen) in H22 hepatoma cells[39].

In addition to inducing apoptosis, Baicalein, from Radix Scutellariae Baicalensis (Huangqin), enhanced autophagy *via* increasing endoplasmic reticulum stress in HCC cells[40]. Similarly, Arenobufagin (Chansu), a natural bufadienolide from toad venom, induced apoptosis and autophagy in human HCC cells but through inhibition of the PI3K/Akt/mTOR pathway in human HCC cells[41].

The effects of TCM on malignant hepatocyte metastases

Controlling HCC metastases is an important strategy for preventing tumor recurrence. Various TCM herbs have been reported to possess this property. Specifically, Sini-San inhibited HBx-induced migration and invasiveness of HCC cells by inhibiting multiple signal transduction pathways including ERK/phosphatidylinositol 3-kinase/Akt upstream of NF-κB and AP-1 in human HCC cells[42] while Biejiajian Pill suppressed the invasiveness of HepG2 cells by inhibiting the Wnt/β-catenin pathway in HCC cells[43]. Jinlong Capsule decreased the adhesive ability of highly metastatic MHCC97H cells *in vitro* and thereby significantly inhibited their movement and invasion[44].

In animal studies, Ginsenoside Rg3 from Ginseng (Renshen) inhibited the growth and metastasis of the highly metastatic human LCI-D20 cells in nude mice. This affect was ascribed to regulating the expression of nm23 and CD44 proteins[45]. By inhibiting SMMC-7721 cell invasion, Radix Salviae Miltiorrhizae (Danshen) decreased intrahepatic and distant metastasis of these cells in nude mice[46]. Another TCM that inhibits malignant hepatocyte metastases is Berberine, which inhibited the growth and development of spontaneously developed lung metastases in an orthotopic model of HCC (MHCC-97L) in mice by suppressing Id-1 expression[47].

The effects of TCM on HCC angiogenesis

HCC survival, growth, and metastases are dependent on new blood vessel growth or angiogenesis (Figure 1). TCM herbs that inhibit HCC angiogenesis include the alkaloids of Rubusalceifolius Poir (Cuyexuangouzi) and Livistonachinensis seeds (Pukuizi), which interfere with Notch signaling in a mouse model of HCC[48,49]. Resveratrol [typically extracted from Rhizoma Polygoni Cuspidati (Huzhang) or Fructus Mori (Sangshen)] decreases microvessel density of transplanted hepatic tumors in nude mice and inhibits tumor growth[50]. By significantly reducing vascular endothelial growth factor expression, Celastrusorbiculatus Thunb (Nansheteng) inhibited Hep-G2 induced tumor growth in orthotopic nude mice[51]. Finally, Qinggan Huayu Formula has been reported to inhibit tumor development and growth by reducing vascular endothelial growth factor and transforming growth factor-β1 protein expression and neovascularization in HCC rats[52].

The effects of TCM on the immunologic response to HCC

In the absence or setting of a suboptimal immune response, tumor cell growth, metastasis, and rates of recurrence are enhanced. Thus, the status of natural killer cells, T lymphocyte subpopulations such as CD_3^+, CD_4^+ and CD_8^+, and pro- as well as anti-inflammatory cytokines are important, and the ability of TCM to enhance the immune response to HCC would be of therapeutic value. Ganodermalucidum polysaccharides (GLPS) is an extract from Ganoderma Lucidum (Lingzhi) that significantly increases the ratio of T effector to regulatory T cells and suppresses tumor growth in HCC-bearing mice[53]. Moreover, GLPS eliminates regulatory T cells suppression of T effector proliferation resulting in increased pro-inflammatory IL-2 secretion. GLPS has also been reported to inhibit T cell Notch1 and FoxP3 expression by increasing miR-125b expression in hepatoma-bearing mice[53]. Another TCM with immuno-modulant properties is Radix Astragali Mongolici (Huangqi), a polysaccharide, which inhibits the growth of mouse HCC HepA by promoting pro-inflammatory TNF-α and IFN-γ production[54]. Combining Jiedu Xiaozheng Yin and Fuzheng Yiliu Formula improved the immune function of mice with H_{22} HCC by increasing CD_3^+ and CD_3^+/CD_4^+[55]. Shaoyao Ruangan Recipe, Biejiajian Pill, Ginsenoside Rg3, Fructus Lycii (Gouqizi) polysaccharide, and Fructus Schisandrae Chinensis (Wuweizi) polysaccharides are other herbal medications that have been reported to inhibit HCC by enhancing the host's immune responsiveness in HCC-bearing mice[56-60].

The effects of TCM on the multidrug resistance of malignant hepatocytes

Increased expression of multidrug resistance (MDR) protein activity, the family of transporters responsible for exporting xenobiotics from within cells, is considered the principal explanation for the failure of chemotherapy in HCC treatment. Many TCM herbs have been reported to reverse MDR expression and/or activity. For example, Tetramethylpyrazine, a bioactive constituent isolated from the root of Ligusticum chuanxiong Hort (Chuanxiong) downregulated P-gp, MRP2, MRP3, and MRP5 expression in HCC BEL-7402/ADM cells[61]. Bufalin, extracted from Venenum Bufonis (Chansu) and Hedyoticdiffusa (Baihuasheshecao) injection, achieved the same effect in BEL-7402/5-FU cells[62-63], and Hirudo (Shuizhi) extract, Qizhu Decoction, Shehuang Xiaoliu

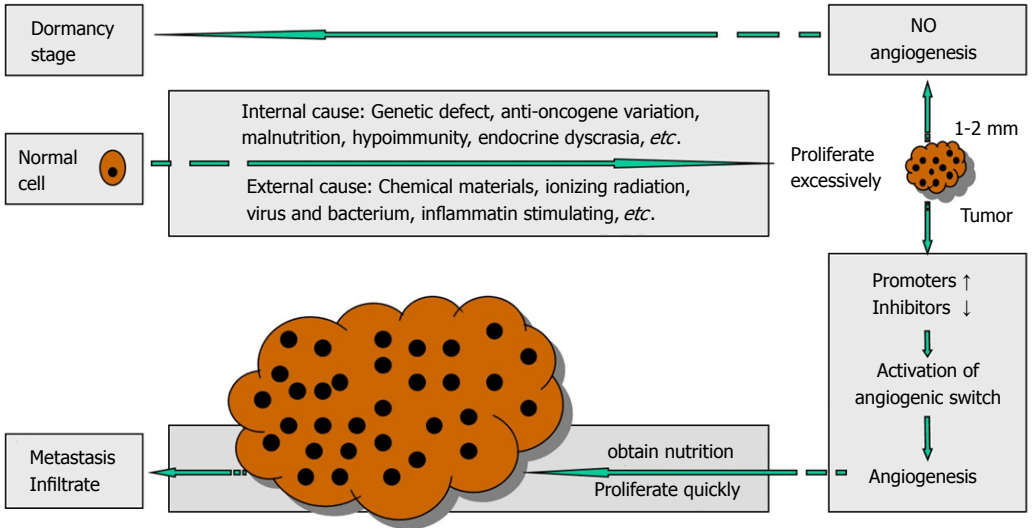

Figure 1 Solid tumor's occurrence and angiogenesis.

Decoction, Jianpi Huayu Formula and Quercetin all reversed MDR activity in HCC tissues[64-68].

CONCLUSION

Although much progress has been made in our utilization and understanding of TCMs for the treatment of HCC, additional experimentation and research is still required. Clearly, no single herbal medicine, active component, or compound recipe has been identified to be curative. Moreover, the mechanism(s) involved in achieving the benefits described are multiple and complex. Nonetheless, empiric and experimental data suggest that TCM is effective in limiting symptoms, reducing treatment associated side effects, inhibiting tumor growth, and altering key intracellular signaling pathways. While a combination of TCM and Western medicine may evolve as the optimal approach to treating HCC, certain challenges remain. Principal amongst these is the need for Western Medicine physicians to consider and where appropriate accept the concept of "holism" for cancer treatment. These physicians must also be willing to consider empiric findings, albeit of century's duration, as an additional measure of efficacy, particularly for compounds such as TCM herbs that due to their unique fragrance, do not always lend themselves to testing in placebo-controlled clinical trials.

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REVIEW

Genetic diversity of hepatitis viruses in West-African countries from 1996 to 2018

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Abstract

The severity of hepatic pathology and the response to treatment depend on the hepatitis virus genotype in the infected host. The objective of this review was to determine the distribution of hepatitis virus genotypes in West African countries. A systematic review of the literature in PubMed, Google Scholar and Science Direct was performed to identify 52 relevant articles reporting hepatitis A, B, C, D, E and G viruses genotypes.

Hepatitis B virus (HBV) genotype E with a prevalence of 90.6% (95%CI: 0.891-0.920) found in this review, is characterized by low genetic diversity. Hepatitis C virus (HCV) genotypes 1 and 2 represented 96.4% of HCV infections in West African countries, while hepatitis delta virus, hepatitis A virus, hepatitis G virus genotypes 1 and HEV genotype 3 were reported in some studies in Ghana and Nigeria. HBV genotype E is characterized by high prevalence, low genetic diversity and wide geographical distribution. Further studies on the clinical implications of HBV genotype E and HCV genotypes 1 and 2 are needed for the development of an effective treatment against this viral hepatitis in West African countries. Surveillance of the distribution of different genotypes is also needed to reduce recombination rates and prevent the emergence of more virulent viral strains.

Key words: Hepatitis virus; Mutations; Genotypes; Recombination; West African Economic and Monetary Union

Core tip: The determination of hepatitis viruses genotypes is very important for the management and treatment of infected patients. Indeed, mutation development, disease progression and antiretroviral response are all dependent on the genotype of the infecting virus. Genotype determination is therefore very important to identify patients who are at increased risk of disease progression and to optimize treatment. The objective of this review was to determine the prevalence and distribution of different hepatitis viruses genotypes in 10 West African countries.

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INTRODUCTION

Viral hepatitis is an inflammation of the liver that may progress spontaneously to healing or lead to cirrhosis or hepatocellular carcinoma (HCC). Viral hepatitis caused 1.34 million deaths in 2015, a figure comparable to deaths from tuberculosis (TB) and human immunodeficiency virus (HIV). However, mortality attributable to TB and HIV is decreasing, while that due to hepatitis is constantly increasing[1]. There are five types of hepatitis viruses designated by the letters A, B, C, D and E. The most common forms of the disease are hepatitis A, B and C. Another human lymphotropic virus belonging to the Flaviviridae family and closely related to hepatitis C virus (HCV) was identified as hepatitis G virus (HGV) or GB virus C (GBV-C). However, several studies have shown that GBV-C/HGV infection is not clearly associated with any disease and may play a role in modulating HIV disease[2,3]. In a study conducted in Burkina Faso, a prevalence of 7.4% of HGV was reported in blood donors[4]. Viral hepatitis usually occurs as a result of parenteral contact with infected body fluids: Blood transfusions or contaminated blood products.

Hepatitis B virus (HBV) is ubiquitous, but the prevalence of infection varies across different regions of the world. According to the World Health Organization, about two billion people have been in contact with HBV worldwide, with more than 240 million cases of chronic infections[5]. About 80 to 120 million cases of chronic HBV infection occur in sub-Saharan Africa[6,7]. According to the carriage of hepatitis B surface antigen (HBsAg), there are zones of low endemicity (< 2%) such as Western Europe or North America; areas of average prevalence (2%-7%) such as North Africa or Eastern Europe and finally high endemic areas (> 8%) such as West Africa or Southeast Asia[8]. Indeed, hepatitis B is highly endemic in West Africa, with the highest prevalence in the world (> 8%). In sub-Saharan Africa, about 47% of HCC have been attributed to HBV[9]. Despite the availability of a vaccine, HBV remains a major public health problem, with approximately 686 thousand deaths per year worldwide due to the consequences (cirrhosis and HCC) of this infection[5].

More than 71 million people worldwide are chronically infected with HCV and may develop liver cirrhosis and/or HCC[10]. There is currently no effective vaccine against HCV; and about 399000 people die each year from hepatitis C, mainly cirrhosis and HCC. In North and West Africa, the prevalence of HCV infection ranges from 0.5% to 1.0%[10]. Hepatitis delta virus (HDV) is a small defective ribonucleic acid (RNA) virus that depends on HBV for the assembly of new virions and proliferation of infection to hepatocytes. HDV infection can be therefore prevented through vaccination or any strategy to eliminate HBV infection. Approximately 15 to 20 million people worldwide are co-infected with these two viruses, with a high risk of severe liver disease[11]. In a study of pregnant women in Benin, the prevalence of HDV was 11.4% in 15.5% of HBsAg-positive individuals[12].

There are several genotypes of hepatitis viruses with different clinical implications and distinct geographic distributions. HBV is classified into 10 genotypes (A-J) and about 40 subgenotypes with a correlation between genotypes and their modes of transmission[13]. In fact, HBV genotype A is found in North America, Europe, South-East Africa and India; genotypes B and C in Asia and Oceania while genotype D is the most common in North America, North Africa, Europe, the Middle-East and Oceania. HBV genotype E is hyperendemic in West Africa; genotype F is found in

South America; and genotypes G and H are in Central and South America[13]. HCV has a high genetic diversity with a predominance of genotype 1 and 3 worldwide. The endemic strains of genotypes 1 and 2 are mainly found in West Africa, genotype 3 in Asia, genotypes 4 in Central Africa and the Middle East, while genotypes 5 and 6 are predominant in South Africa and South East Asia, respectively[14]. HCV genotype 2 is the most common genotype in West African countries, followed by genotype 1 and genotype 3[15]. HDV genotype Ⅰ is more common in Europe and North America, genotype Ⅱ is predominant in the Far East and Japan while genotype Ⅲ is predominant in the Amazonian area and northern South America[16].

The severity of hepatic pathology and the response to treatment depend on the virus genotype in the infected host. For example, HBV genotype A infection tends to chronicity, whereas genotype D has a high frequency of mutation influencing response to treatment. Liver cirrhosis and progression to HCC are strongly associated with HBV genotypes C and D compared to other genotypes[17,18]. Furthermore, superinfection of chronic HBV patients by HDV leads to increased liver damage and more rapid progression of cirrhosis in 90% of cases[16]. HDV genotype Ⅲ is thought to be associated with severe forms of liver disease, while a more moderate clinical evolution and a wide variety of clinical conditions are observed with genotypes Ⅱ and Ⅰ, respectively. The response to interferon treatments is more effective against HBV genotypes A and B compared to genotypes C, D and Ⅰ. HBV genotype E seems to have the worst response to treatment[18]. Rapid progression of hepatic disease and HCC has also been associated with HBV genotype A1[13]. HCV subtype 1b is associated with a high risk of developing HCC compared to other genotypes[19]. Early generations of vaccines protected against subtype 1b, while genotype 3, which accounts for 30.1% of HCV global infections, is less likely to respond to first and second generation direct-acting antivirals currently used for HCV treatment[14,20].

Determination of the viral genotype is an essential element of the pre-therapeutic assessment, because it is one of the predictors of the response to treatment and determines the choice of molecules used with the new anti-HCV treatments. It has also been shown that HBV genotypes differ according to disease course, mutation development and response to antiviral therapy[21]. Indeed, genotype determination is important to identify patients who are at increased risk of disease progression and to optimize treatment[22]. Here, we review various publications on the genotypes of hepatitis viruses in West African countries [West African Economic and Monetary Union (WAEMU) countries, Ghana and Nigeria] in order to map the genotypes distribution and discuss the infections associated with the different viruses identified.

HEPATITIS VIRUSES' INFECTION IN WEST AFRICA

HBV infection

HBV belongs to the *Hepadnaviridae* family and the *Orthohepadnavirus* genus[23]. It is a double-stranded circular DNA enveloped virus of small circumference (1.6 million Dalton) associated with a DNA-dependent DNA polymerase that acts as a reverse transcriptase during replication. HBV is highly contagious, 100 times more contagious than HIV and can remain stable at 25 ℃ for seven days in dried blood. Sexual, parenteral (through the blood), mother-to-child or even close intrafamily non-sexual contact over a long period of time are the different modes of infection. The most common modes of spread of hepatitis B in endemic areas are mother-to-child transmission and exposure to infected blood. The appearance of a chronic infection is very common for infants infected by their mother before the age of 5 years.

Markers, such as the HBsAg, the HBs antibody (anti-HBs), the core antigen (HBcAg), the HBe antigen (HBeAg) and the HBe antibody (anti-HBe) make it possible to monitor the evolution of this virus. Despite the small size of the genome and the constraints imposed by its organization, HBV is highly variable.

HBV strains are divided into several genotypes. These genotypes are defined by a divergence of at least 8.0% of the whole genome nucleotide sequence and at least 4.1% in the S gene[24]. The main genotypes were divided into subgenotypes based on the divergence between 4.1% and 8.0% of their complete nucleotide sequence[25]. In the last decade, phylogenetic studies of sequences of different viral genomes have tentatively classified HBV into 10 genotypes (A-J)[26]. Genotypes and subgenotypes have a distinct geographic distribution[27]. Genotype A is the only predominant genotype in East Africa, where the prevalence of other genotypes is less than 5%[24,28]. The subgenotype A1 is predominant in Africa, while subgenotypes A3-A6 are found in Central and West Africa[29].

Subgenotype D1 is highly prevalent in East Africa[30]; D7 has been isolated in Tunisia[31]; and D8 has been characterized in Niger[32]. West Africa is the main focus of genotype E. Vaccination is the safest way to prevent HBV infection[33]. Major advances in the treatment of chronic HBV have been made with the development of nucleoside reverse transcriptase inhibitors with anti-HBV activity, such as L-nucleosides (lamivudine 3TC) or alkylphosphates (tenofovir disoproxil fumarate)[34].

HCV infection

HCV belongs to the family Flaviviridae and the genus *hepacivirus*[35]. HCV mainly infects hepatocytes but may also be present in blood mononuclear cells and dendritic cells[36]. HCV is a small single-stranded RNA virus of positive polarity, enveloped 55-65 nm in diameter.

Parenteral route is the major mode of transmission of HCV. Transfusion and intravenous drug addiction are also routes of transmission. To this day, the main cause of HCV transmission in developed countries is drug abuse[37]. The HCV genome shows a high rate of mutations with considerable genetic heterogeneity of the virus in infected people worldwide. Phylogenetic approaches made it possible to classify HCV into 11 major genotypes (designated by the Arabic numerals from 1 to 11), with many subtypes (indicated by lower case letters a, b, c, *etc*.)[38].

Subgenotypes 1a, 1b, 2a, 2b and 3a are widely distributed worldwide[39], while 5a and 6a are common in South Africa and Southeast Asia[40,41]. Genotype 4 is predominant in Central Africa[42,43] and in North Africa[44]. In Africa, divergent HCV genotype 1 and 2 strains were found endemic in the West African subregion[45-47]. Recently, analysis of the epidemic history of HCV infections has traced modern HCV lines in West Africa to the 17th and 20th centuries[46]. The current standard treatment for chronic infection is the combination of pegylated interferon alpha (pegIFNα) and ribavirin (RBV)[48]. Currently, new therapeutic approaches using direct-acting antivirals have been developed for the treatment of chronic hepatitis C. These molecules inhibit certain stages of the viral cycle and prevent the production of viral particles by infected hepatocytes.

Others hepatitis virus infection (HDV and HGV)

HDV infection: HDV is an infectious agent that can only infect patients previously or simultaneously infected with the HBV[49]. It is a single-stranded RNA negative polarity virus, 1700 nucleotides in size, which encodes a single structural protein, the hepatitis delta antigen (HDAg), and requires HBV to replicate. HDV infection can only occur with simultaneous coinfection with HBV or superinfection[1]. HDV transmission is predominantly parenteral, and the sexual route is less effective than HBV. Mother-to-child transmission is rare. Hepatitis D is a liver disease that can take the acute form and chronic form. There can be no hepatitis D in the absence of HBV. Co-infection with HBV or HDV superinfection results in more severe disease than HBV mono-infection[1]. HDV infection is diagnosed by high titers of immunoglobulin G (IgG) and immunoglobulin M (IgM) anti-HDV and confirmed by serum detection of HDV RNA by polymerase chain reaction[1]. HDV isolates in the world are divided into at least eight phylogenetically distinct genotypes[50].

Genotype 1 is the predominant form of HDV with worldwide distribution, while genotypes 2 and 4 are present in Japan and Taiwan and are often associated with a milder form of disease[50]. Genotype 3 has been reported in the Amazonian region[51]. Genotypes 5-8 were detected in the sera of patients of African origin[52]. In addition, genotype 8 infection was also detected in the state of Maranhão in northeastern Brazil[53]. Some studies have shown that genotypes 3 and 4 can be associated with particularly severe clinical forms of hepatitis (fulminant hepatitis)[51]. HDV infection is rarely studied in West Africa despite the high prevalence of HBV. There is currently no effective antiviral therapy for hepatitis D. The prevention of hepatitis D involves vaccination against hepatitis B. pegIFNα is the only effective anti-HDV drug; nucleoside analogues active against HBV have little or no effect on HDV replication[1].

HGV infection: The HGV, called GBV-C or HGV, is a flavivirus, such as HCV, that causes spontaneously resolving acute hepatitis or fulminant hepatitis. It can cause chronic infections. HGV is a single-stranded positive-strand RNA virus[54]. Its transmission is mainly parenteral. Maternal-fetal and sexual transmissions are higher than those seen with HCV. IFN is effective in normalizing hypertransaminasemia in infected patients, but relapse appears to be common when treatment is discontinued.

METHODOLOGY

Research strategy and selection criteria

A systematic review of the literature was conducted to identify relevant articles reporting genotypes of hepatitis viruses in WAEMU countries including Ghana and Nigeria from 1996 to 2018. The research was conducted in French and/or English in three databases: PubMed, Google Scholar and Science Direct. The keywords used were "HBV and/or HBV and other viruses "+" the name of each of the 10 countries included in the study". A filter limiting the search for keywords in the title and/or abstract of articles was used [PubMed: (tiab); Google Scholar: Allintitle and Science Direct: TITLE-ABSTR-KEY]. Searches with similar terms such as "hepatitis virus", "hepatitis virus", "hepatitis virus genotypes" or "hepatitis virus genotype" were also conducted.

The studies were then selected on the basis of the following criteria: (1) data published in a peer-reviewed scientific journal; (2) only patients residing in one of the WAEMU countries, Ghana or Nigeria; and (3) patients from these countries infected with hepatitis viruses whose genotypes have been identified. All scientific publications (52) that reported data on genotypes of hepatitis viruses in populations from WAEMU countries, Ghana and Nigeria, between 1996 and 2018 and met the selection criteria were included in this systematic review (Figure 1). Eligible studies had to report the genotype of viral hepatitis in populations from included countries regardless of method used for viremia detection. Both risk groups or general population were eligible for inclusion.

HBV and HCV viremia detection were based on

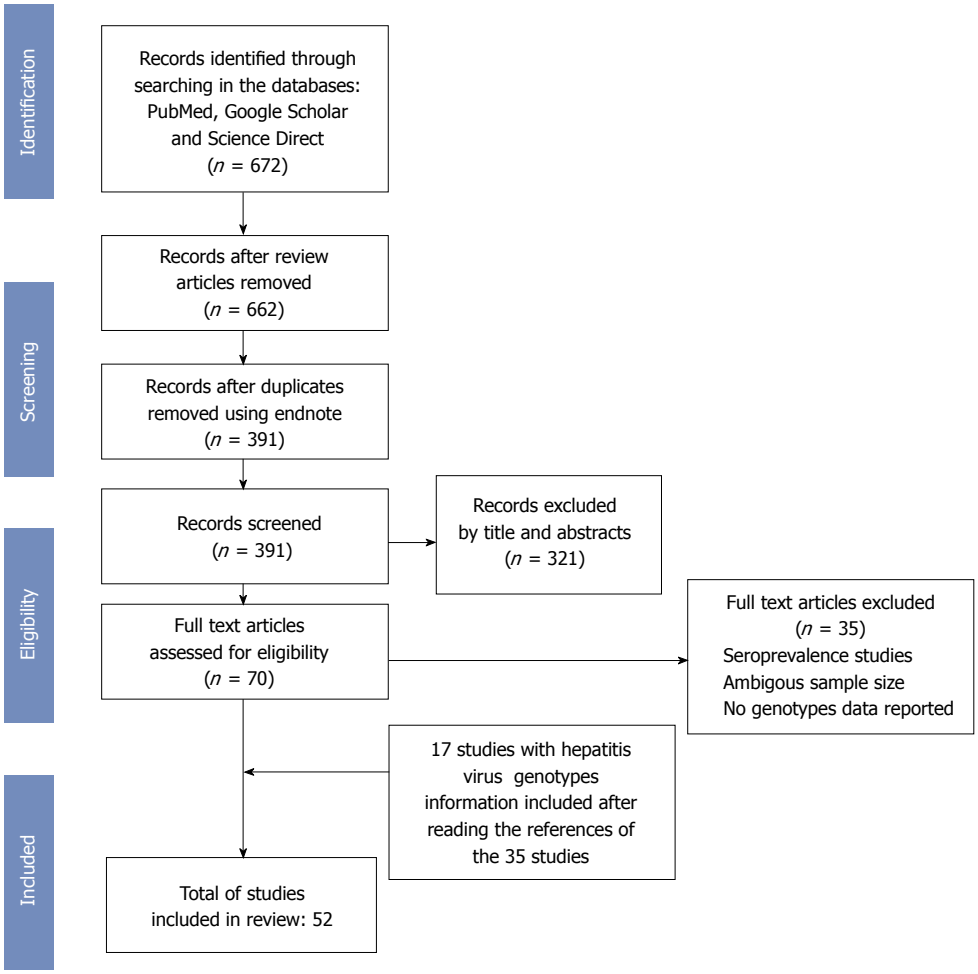

Figure 1 Flow diagram showing the method for the study selection. The database search for the search strategy described in the section was cleaned up to exclude review articles and duplicates. Titles and abstracts were included in the literature review. Seroprevalence articles, articles with ambiguous data that did not meet the inclusion criteria were then excluded during the full-text review. Fifty-two (52) relevant articles were finally included for this review.

DNA/RNA amplification. Genotypes detection was performed using polymerase chain reaction or direct sequencing. HCV genotype classification was considered because in many studies, HCV cases were classified at the genotype level but not at the subtype level. Journal articles, publisher correspondence, news, letters, book chapters and studies whose data were ambiguous or could not be extracted were systematically excluded. The search and selection of the relevant articles in the three databases were carried out by two independent reviewers. The inclusion of a study by both reviewers was a requirement. In case of disagreement on the eligibility of a study, the problem was solved through a discussion and/or consensus with a third reviewer.

Extractions of data and analysis

The genotyping data were extracted from the different studies carried out in Ghana, Nigeria and WAEMU countries. The data extracted from the various studies included in this review are: The first author, the year of the data publication, the study population, the type of study or data collection (prospective or retrospective), the country, the number of samples successfully genotyped and the results of identified genotypes.

In multi-center studies, only data from the countries included in this mini-review were considered. Prevalence was determined by making the ratio of the genotype considered to the total number of samples tested for that genotype. Confidence intervals were calculated using the R software. Phylogenetic analysis was performed with 53 HBV sequences using the neighbor-joining algorithm based on the Kimura two-parameter distance estimation method. Only bootstrap values of > 80% are shown (1.000 replicates). The maps were made using genotyping data from each country (source: Dr. Ouattara AK).

SEARCH RESULTS

Selection of studies

The initial search in the three databases, according to the search strategy described in the methodology, found 391 articles after elimination of reviews and duplicates. Examination of titles and abstracts led

to the elimination of 321 studies that did not meet the inclusion criteria of this review. Only 70 studies were considered eligible after full text review. This step allowed the exclusion of 35 articles presenting data reporting only seroprevalences or presenting ambiguous genotyping data. Finally, 52 studies, 35 of which were obtained after the full text examination and 17 after references examination of the 35 articles selected, were included in this systematic review.

Characteristics of included studies

Tables 1 and 2 present the characteristics of the different studies included in this systematic review. The majority of included studies used a prospective method of sample collection. A case-control study, two Cas reports, two multi-center studies and four cohort studies were included in the review, while the rest were cross-sectional or prospective studies. Twelve studies were performed in HIV-infected individuals compared to 11 in blood donors and seven in pregnant women, while populations and age groups were variable for the rest of the studies.

HBV genotypes

The frequency of the different genotypes was determined by dividing the number of samples presenting the genotype considered by the total number of successfully sequenced samples. In this systematic review, the largest number of successfully sequenced samples were recorded in Ghana (457/1620), followed by Nigeria (269/1620) and Côte d'Ivoire (251/1620). Genotyping studies of hepatitis viruses were rare in Guinea-Bissau and almost non-existent in Togo. The HBV genotype E was the predominantly isolated genotype in the various studies conducted in the WAEMU countries, Ghana and Nigeria (Table 1).

Indeed, out of a total of 1620 successfully sequenced HBV samples, E genotypes were individually isolated in 90.6% (1468/1620, 95%CI: 0.891-0.920) of HBV infection cases. In addition, its prevalence of recombination or coinfection with genotypes A and D was estimated at 0.86% (14/1620, 95%CI: 0.005-0.014) in our study area. HBV genotype E is characterized by low genetic diversity compared to other genotypes, including genotype A (Figure 2). The second HBV genotype reported in terms of frequency in the countries included in this review was genotype A with an individual prevalence of 7.8% (126/1620, 95%CI: 0.065-0.092), while a prevalence of 0.74% (12/1620, 95%CI: 0.004-0.013) of genotypes D was observed in the study area. A slight decrease in the overall frequency of HBV genotype E in West African countries was found between 2003 and 2010 (94.4%) compared to 2011-2018 (90.0%) with emergence of genotypes A and D. Some studies have focused on the genotyping of other hepatitis viruses with a predominance of HCV infections (528/570, Table 2). Figure 3 shows the geographical distribution of the different HBV genotypes in the countries included in this review.

Genotypes of other hepatitis viruses (HCV, HDV, HAV)

Of the 535 strains of HCV isolated and sequenced successfully, genotype 1 was found in the majority of cases of infections (56.4% or 298/528) against 40.0% (211/528) for genotype 2 while 3.6% (19/528) of the samples had genotypes 3 (9/19), 4 (7/19) and 5 (3/19) of HCV. HCV genotype 2 was most common in Benin, Burkina Faso, Ghana Guinea Bissau and Mali, while genotype 1 was predominant in Côte d'Ivoire, Senegal and Nigeria (Figure 4), with a high number of sequenced samples. Genotypes 1 of other hepatitis viruses such as HDV, a satellite virus still found in coinfection with HBV, HAV, HGV, and HEV genotype 3, have been reported in some studies in Ghana and Nigeria (Table 2).

DISCUSSION

Selection of studies

The aim of this review was to map the genotypes of the different hepatitis viruses identified in WAEMU countries, Ghana and Nigeria. The systematic review in the PubMed, Google Scholar and Sciences Direct databases included 52 studies reporting genotypes of hepatitis A, B, C, D, E and G. The availability of genetic data varied across country due to the prevalence or clinical relevance of the virus or the difficulty of sequencing in a context of limited resources. Indeed, most of the genotyping studies (29/52) focused on HBV because of its endemicity in sub-Saharan Africa and its clinical implications[1]. In West Africa, chronic carriage of HBV in the general unvaccinated population is estimated to be between 10% and 18%[55]. Several studies (18/52) have also provided HCV genotype data, which is the second virus of clinical interest in this West African sub-region after HBV, while very little genetic data is available on HDV, a satellite of HBV. The genetic data on HAV come only from Nigeria where it is endemic[56], while the genotypes of hepatitis E and G viruses, very scarce in WAEMU countries[57], were reported respectively in Nigeria and Ghana.

HBV genotypes

Knowledge of hepatitis viruses genotypes is of great epidemiological and clinical interest. Indeed, genotypes are responsible for variable clinical manifestations with differences depending on the stage of the disease, mutations and response to treatments[58,59]. They are also an invaluable tool for mapping the molecular evolution and dynamics of infection transmission because the different genotypes have a distinct geographic distribution. The study of Archampong *et al*[59]

Table 1 Distribution of hepatitis B virus genotypes in West African Economic and Monetary Union countries, Ghana and Nigeria

Ref.	Year	Countries	Patients	Type of study	Samples	HBV genotypes (*n*)
Diarra *et al*[80]	2018	Burkina Faso	Occult HBV	Cross-sectional	21	E (17) and A3 (4)
Archampong *et al*[59]	2017	Ghana	HBV-HIV coinfected	Cross-sectional	63	E (58), A (4) and D (1)
Boyce *et al*[58]	2017	Ghana	HBV-HIV coinfected	Case reports	3	D/E (3)
Cella *et al*[63]	2017	Mali	Malian refugees	Cross-sectional	16	E (16)
Lawson-Ananissoh *et al*[81]	2017	Côte d'Ivoire	Chronic HBV	Prospective	33	E (27), A (6)
Dongdem *et al*[73]	2016	Ghana	Chronic Hepatitis B	Cross-sectional	58	E (47), A (8) and D (3)
Opaleye *et al*[82]	2016	Nigeria	HBV+	Cross-sectional	17	E (17)
Compaore *et al*[62]	2016	Burkina Faso	HIV-1+ and HIV-1-	Case-Control	120	E (120)
Candotti *et al*[83]	2016	Burkina Faso	Blood donors	Prospective	99	E (71) A3QS (28)
Brah *et al*[84]	2016	Niger	HBV infected	Prospective	23	E (21), A3E (1) and D/E (1)
Boyd *et al*[85]	2016	Côte d'Ivoire	HBV-HIV coinfected	Prospective	100	E (98) and A (2)
Ampah *et al*[61]	2016	Ghana	Randomized volunteers	Prospective	52	E (52)
Traore *et al*[55]	2015	Mali	Adults volunteers	Cohort study	90	E (82), D/E (5), D (1) and A (2)
Faleye *et al*[86]	2015	Nigeria	Pregnant women	Cross-sectional	6	E (6)
Faleye *et al*[87]	2015	Nigeria	Asymptomatic individuals	Cross-sectional	13	E (13)
Maylin *et al*[88]	2015	Senegal	Chronic HBV	Cohort study	87	E (65), A (22)
Honge *et al*[76]	2014	Guinea-Bissau	HIV+	Cross-sectional	26	E (25) and D (1)
De Paschale *et al*[12]	2014	Benin	Pregnant women	Prospective	19	E (19)
Forbi *et al*[56]	2013	West Africa[1]	Pregnant women and HIV+	Multicenter	83	E (74) and A (9)
Hübschen *et al*[89]	2011	Nigeria	Cohorts samples	Cohorts study	163	E (154) and A (9)
Geretti *et al*[90]	2010	Ghana	HIV+	Cross-sectional	86	E (82) and A (4)
Forbi *et al*[69]	2010	Nigeria	Asymptomatic volunteers	Cross-sectional	55	E (53) and A3 (2)
Chekaraou *et al*[32]	2010	Niger	Blood donors	Cross-sectional	24	E (20), D/E (4)
Candotti *et al*[91]	2007	Ghana	Pregnant women	Cross-sectional	70	E (69) and A (1)
Vray *et al*[92]	2006	Senegal	Blood donors	Cross-sectional	32	E (23) and A (9)
Huy *et al*[60]	2006	Ghana	Blood donors	Cross-sectional	12	E (12)
Candotti *et al*[66]	2006	Ghana	Blood donors	Cross-sectional	100	E (87), A (10) and D (3)
Fujiwara *et al*[65]	2005	Benin	Blood donors	Cross-sectional	21	E (20) and A (1)
Mulders *et al*[64]	2004	West Africa[2]	Measles or HIV+	Multicenter	79	E (78), and A (1)
Suzuki *et al*[70]	2003	Côte d'Ivoire	HBV carriers	Cross-sectional	48	E (42), A (3) and D (3)

[1]Ghana 13 (E = 100%), Côte d'Ivoire 70 (E = 87%); [2]Benin 13 strains, Burkina Faso 11 with 1 case of HBV-A genotypes (BFA-S121), Mali 18 strains, 15 strains from Nigeria and Togo 22 strains. WAEMU: West African Economic and Monetary Union; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus.

demonstrated that the majority of HBV-positive and patients co-infected with lamivudine 3TC resistance were infected with HBV genotype E. This review confirms the endemicity of HBV genotype E, with a prevalence of 90.6% (1468/1620, 95%CI: 0.891-0.920) and a predominance of serotype awy4. Indeed, some studies conducted in Ghana[60,61], Burkina Faso[62] and Mali[63] exclusively reported the HBV genotype E in

Table 2 Distribution of non-hepatitis B virus genotypes in West African Economic and Monetary Union countries, Ghana and Nigeria

Ref.	Year	Countries	Patients	Type of study	Samples	Others hepatitis genotypes (*n*)
Abubakar *et al*[93]	2017	Nigeria	HCV+	Prospective	173	HCV G1 (159) and G2 (14)
Ndiaye *et al*[94]	2015	Senegal	Drug users	Cohort study	25	HCV G1 (21), G2 (1), G3 (1) and G4 (2)
Henquell *et al*[95]	2016	Burkina Faso	woman	Case report	1	HCV G5 (1)
Opaleye *et al*[82]	2016	Nigeria	HBV+	Cross-sectional	14	HDV G1 (14)
De Paschale *et al*[12]	2014	Benin	Pregnant women	Prospective	6	HCV G1 (1), G2 (5)
Honge *et al*[76]	2014	Guinea Bissau	HIV+	Cross-sectional	8	HCV G2 (8)
Zeba *et al*[96]	2014	Burkina Faso	Blood donors	Cross-sectional	36	HCV G1 (4), G2 (22), G3 (8), G4 (2)
Forbi *et al*[56]	2013	Nigeria	Apparently healthy adult	Cross-sectional	12	HAV sub-G1A (12)
Diarra *et al*[97]	2013	Mali	Diabetic	Prospective	25	HCV G1 (7) and G2 (18)
Bouare *et al*[98]	2013	Mali	Old women	Prospective	14	HCV G1 (2) and G2 (12)
Forbi *et al*[47]	2012	Nigeria	Asymptomatic indigenes	Prospective	60	HCV G1 (51) and G2 (9)
Sombie *et al*[99]	2011	Burkina Faso	HCV+	Prospective	38	HCV G1 (10), G2 (27) and G5 (1)
Bengue *et al*[100]	2008	Côte d'Ivoire	Blood donors	Prospective	27	HCV G1 (21), G2 (5) and G5 (1)
Plamondon *et al*[101]	2007	Guinea Bissau	Adult volunteers	Cross-sectional	57	HCV G1 (1) and G2 (56)
Simpore *et al*[102]	2005	Burkina Faso	Pregnant women	Prospective	5	HCV G1 (2) and G2 (3)
Rouet *et al*[103]	2004	Côte d'Ivoire	HIV+/Pregnant women	Cross-sectional	6	HCV G1 (3) and G2 (3)
Agwale *et al*[104]	2004	Nigeria	HIV+ under ART	Prospective	12	HCV G1 (9) and G2 (3)
Candotti *et al*[45]	2003	Ghana	Blood donors	Cross-sectional	23	HCV G1 (3) and G2 (20)
Buisson *et al*[105]	2000	Nigeria	Acute hepatitis	Cross-sectional	7	HEV G3 (7)
Saito *et al*[79]	1999	Ghana	HIV+ and HIV-	Cross-sectional	9	HGV G1 (9)
Wansbrough-Jones *et al*[106]	1998	Ghana	Blood donors	Cross-sectional	7	HCV G1 (2) and G2 (5)
Oni *et al*[107]	1996	Nigeria	blood donors	Cross-sectional	5	HCV G1 (2) and G4 (3)

WAEMU: West African Economic and Monetary Union; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HEV: Hepatitis E virus; HGV: Hepatitis G virus; HIV: Human immunodeficiency virus; ART: Antiretroviral treatment.

their study populations.

In addition to the presence of other genotypes, including HBV genotypes A and D, studies have reported a strong predominance of genotype E[64-66]. Similar observations have led several authors to support further the common presence of HBV genotype E in West African populations[24]. Indeed, the predominance and almost exclusive circulation of genotype E in sub-Saharan Africa certainly indicates its West African origin[67-69]. Its distribution is limited to West Africa, unlike other HBV genotypes, despite the migration of slaves from West Africa to North America[66]. This review also reports low genetic diversity of HBV genotype E in West Africa (Figure 2)[64,66]. The low genome diversity and large distribution of genotype E in West Africa suggests a recent introduction of this genotype in the human host[64,70]. It is possible that it has been introduced relatively recently into an animal reservoir (the chimpanzee) as well as for HIV or that variant of genotype D (the closest to genotype E) has acquired an evolutionary advantage[66]. HBV genotypes A and D were also reported in this review with respective prevalence of 7.8% (126/1620, 95%CI: 0.065-0.092) and 0.74% (12/1620, 95%CI: 0.004-0.013). HBV genotype A, which is also found in sub-Saharan Africa, has been reported in eight of the 10 countries included in this review. Indeed, genotype E is predominant in West Africa, while genotype A has a relatively high prevalence in East Africa[71].

In 2006, Candotti *et al*[66] reported a prevalence of 10% and 3%, respectively, for HBV genotypes A and D in blood donors in Ghana. Similar results have also highlighted the cocirculation of genotypes A and D in Ghana, Mali, Côte d'Ivoire and Nigeria[59]. The majority of genotypes A identified in Burkina Faso are quasi-A3 genotypes (A3Q) documented in West African populations[72]. Indeed, data from previous studies suggest a predominance of the A1 genotype in East Africa and the A3 genotype in West and Central Africa, while the A2 subgenotype has a high frequency in North Africa where the genotype D is predominant. Africa has a high diversity of HBV genotypes and subgenotypes displaying distinct geographical distributions. Genotype A is found mainly in south-eastern Africa, genotype E in western and central Africa and genotype D prevails in northern Africa. Genotype E is rarely found outside Africa, except in individuals of African descent.

Characterization of HBV genotypes allows clinicians to determine patients' response to treatment and potential risks of complications[58,73]. Flink *et al*[74] have indeed reported that genotype A responds better to interferon alpha and pegIFNα than genotype D. Genotype recombination occurs in areas where multiple genotypes are in co-circulation, thus facilitating diversification between individuals within the general population. Our review reports a recombination prevalence of HBV genotype E with genotypes A and D of 0.87% (14/1599, 95%CI: 0.005-0.015). A/B, A/C, A/E, C/E, D/E and D/E/A recombination have been reported in West Africa[58]. Recombination requires co-infection with more than one genotype in the same patient. Appropriate treatment and elimination of risky

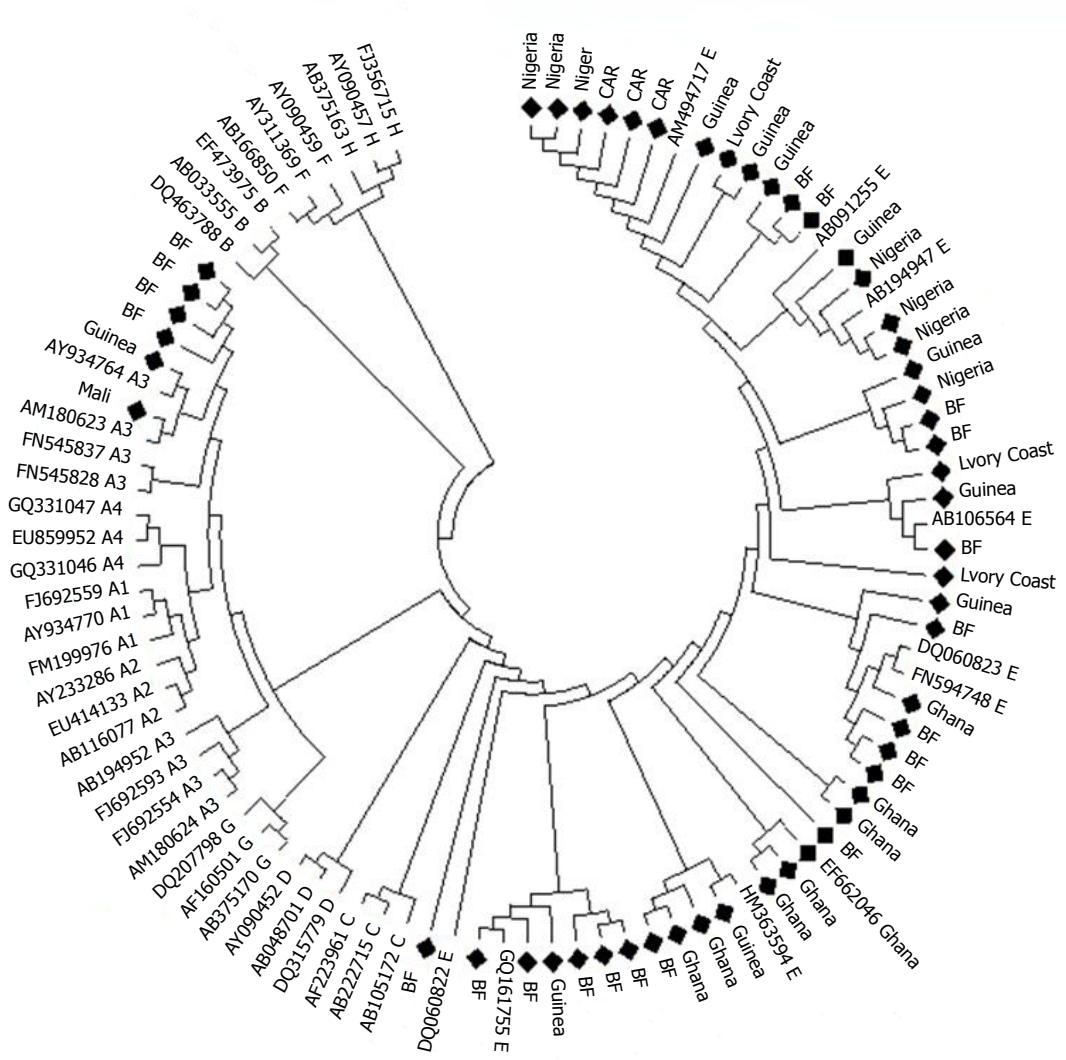

Figure 2 Phylogenetic tree of 53 hepatitis B virus genotype E and A sequences identified in West African Economic and Monetary Union countries including Ghana and Nigeria (indicated ♦). Phylogenetic analysis was performed with the neighbor-joining algorithm based on the Kimura two-parameter distance estimation method. Only bootstrap values of > 80% are shown (1.000 replicates). Reference hepatitis B virus sequences (44) recovered from GenBank are denoted with their accession numbers and genotypes/sub-genotypes are indicated.

behavior in people infected with the virus are therefore necessary for a considerable reduction in the spread of recombinant viruses.

Genotypes of HCV and other hepatitis viruses

The data in this review report five circulating HCV genotypes in the WAEMU countries, Ghana and Nigeria, with an overall predominance of genotype 1 (56.4%, 298/528). The prevalence of HCV genotype 1 has been reported by several authors in Nigeria, Senegal and Côte d'Ivoire, who record the most data presented in this review. Genotype 2 with a general frequency of 40.0% (211/528) was the main genotype found in Benin, Burkina Faso, Ghana, Guinea-Bissau and Mali (Figure 2). Indeed, several previous studies have reported a predominance of HCV genotype 2 in West Africa[75]. Most authors suggest a West African genotype 2 origin of HCV in the region, including The Gambia and Guinea-Bissau[46]. Indeed, HCV genotyping data in Guinea-Bissau report an almost exclusive predominance of genotype 2[76]. Candotti *et al*[45] reported 87.0% of genotype 2 was associated with chronic HCV infection with 13% of cases for genotype 1. In a study in Ghana in HCV/HIV coinfected patients, HCV sequences were phylogenetically assigned genotype 2 and subtypes 21 and 2r[77]. Although no published data on HCV genotypes were found in Togo, genotypes 2 and 1 were the most frequently isolated, with respective prevalence 73.2% and 17.1%, in a study conducted in 2014 in the Togolese general population.

Genotype 2 is, therefore, predominant in Togo, as it is in most parts of West Africa (unpublished data). In Martinique, where three quarters of the slaves sent in the 17th and 18th centuries came from West Africa, there is a great diversity of genotype 2[78]. The majority of molecular and epidemiological studies suggest that HCV genotype 2 has been present in West Africa for several centuries.

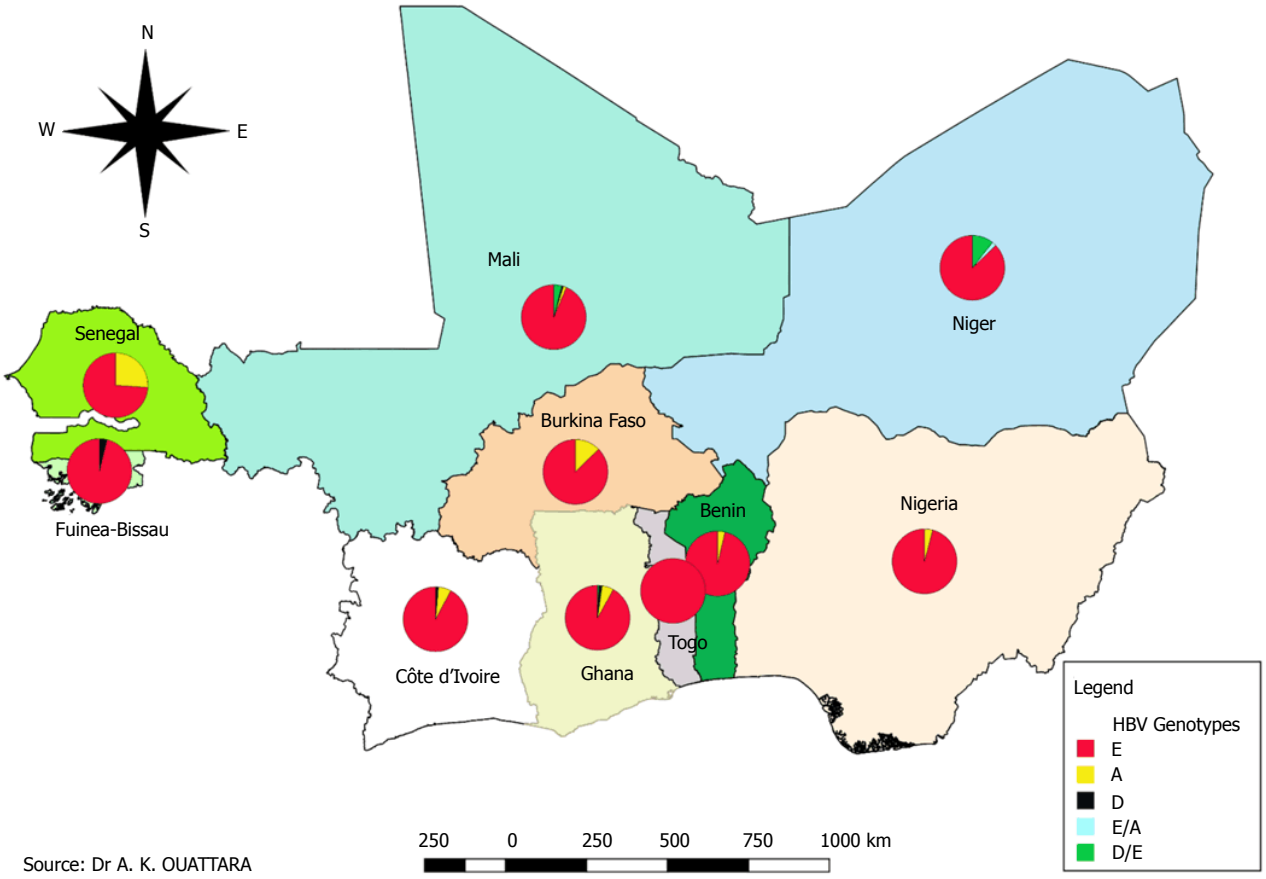

Figure 3 Hepatitis B virus genotypes reported in West African Economic and Monetary Union countries, Ghana and Nigeria. Pie charts show the proportion of different hepatitis B virus genotypes in West African countries according to the data in Table 1.

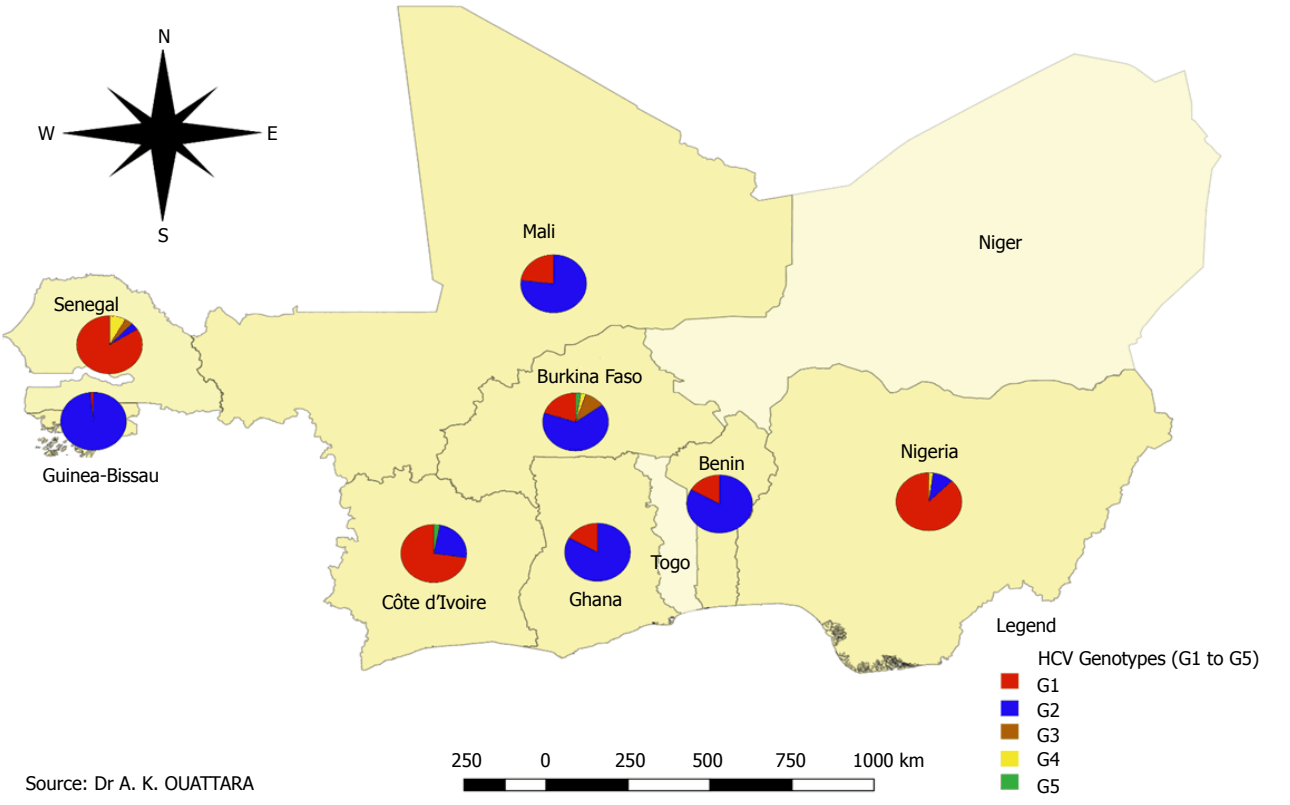

Figure 4 Hepatitis C virus genotypes reported in West African Economic and Monetary Union countries, Ghana and Nigeria. Pie charts show the proportion of different hepatitis B virus genotypes in West African countries according to the data in Table 2.

Data on the genotypes of other hepatitis viruses that are very infrequent or with relatively high frequencies in some areas have also been reported in this review. HAV genotype 1 and HEV and HDV were reported in Nigeria, while genotype 1 of HGV was found in Ghana. HAV, whose transmission is closely associated with lack of clean drinking water, unsuitable food, inadequate sanitation and poor personal hygiene, is prevalent in parts of Nigeria (World Health Organization). HDV is a satellite virus of HBV, because HDV only infects people with HBV. Limited data is available on circulating HDV genotypes. In a study in Togo, it was reported that 94.3% of the general population was infected with genotype 1, and 5.7% was infected with genotype 5. Studies on HGV are very limited[57,79]. The analysis of the 5% untranslated region nucleotide sequence of the genome of the HGV shows that the nine Ghanaian isolates of the HGV belong to genotype 1, the West-African type of the HGV[79].

CONCLUSION

The complexity of hepatitis virus genotypes often leads to a specificity of treatment associated with the genotype. The present review reporting a mapping of genotypes of hepatitis viruses A, B, C, D, E and G in the WAEMU, Ghana and Nigeria, reveals that the majority of studies conducted in Ghana and Nigeria have very little information on hepatitis D and G. In the WAEMU area including Ghana and Nigeria, HBV strains were classified as genotypes E, A, D with a predominance of genotype E and serotype ayw4. Genotype E is characterized by a high prevalence, low genetic diversity and wide geographical distribution.

The majority of HCV genotype data came from Nigeria, Senegal and Côte d'Ivoire were characterized by a predominance of genotype 1, while a high prevalence of genotype 2 was found in Benin, Burkina Faso, Ghana, in Guinea-Bissau and Mali. Further studies on the clinical implications of HBV genotype E are needed for the development of an effective treatment for HBV in West Africa. Monitoring the distribution of the different genotypes is also needed to reduce recombination levels and prevent the emergence of other viral strains. There is a diversity of genotypes and subtypes of hepatitis viruses with risks of recombination and emergence of even more virulent forms. Hepatitis viruses do not need a passport or visa to move from one country to another, and they have preceded us in WAEMU or ECOWAS. It is therefore appropriate for us to develop the adequate means to prevent, treat and even eradicate these viral infections using a vaccine covering all variants.

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REVIEW

Bioengineered functional humanized livers: An emerging supportive modality to bridge the gap of organ transplantation for management of end-stage liver diseases

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Abstract

End stage liver diseases (ESLD) represent a major, neglected global public health crisis which requires an urgent action towards finding a proper cure. Orthotropic liver transplantation has been the only definitive treatment modality for ESLD. However, shortage of donor organs, timely unavailability, post-surgery related complications and financial burden on the patients limits the number of patients receiving the transplants. Since last two decades cell-based therapies have revolutionized the field of organ/tissue regeneration. However providing an alternative organ source to address the donor liver shortage still poses potential challenges. The developments made in this direction provide useful futuristic approaches, which could be translated into preclinical and clinical settings targeting appropriate app-

lications in specific disease conditions. Earlier studies have demonstrated the applicability of this particular approach to generate functional organ in rodent system by connecting them with portal and hepatic circulatory networks. However, such strategy requires very high level of surgical expertise and also poses the technical and financial questions towards its future applicability. Hence, alternative sites for generating secondary organs are being tested in several types of disease conditions. Among different sites, omentum has been proved to be more appropriate site for implanting several kinds of functional tissue constructs without eliciting much immunological response. Hence, omentum may be considered as better site for transplanting humanized bioengineered *ex vivo* generated livers, thereby creating a secondary organ at intra-omental site. However, the expertise for generating such bioengineered organs are limited and only very few centres are involved for investigating the potential use of such implants in clinical practice due to gap between the clinical transplant surgeons and basic scientists working on the concept evolution. Herein we discuss the recent advances and challenges to create functional secondary organs through intra-omental transplantation of *ex vivo* generated bioengineered humanized livers and their further application in the management of ESLD as a supportive bridge for organ transplantation.

Key words: Bioengineered liver; Omentum; Secondary organ; Transplantation; End stage liver diseases

Core tip: The concept of bioengineering functional humanized neo-organs relies on finding more appropriate immunologically tolerable transplantation site. We have experienced omentum as more appropriate ectopic site with excellent properties of angiogenesis, regeneration, fibrotic reconstruction, and immunological compatibility which together endorse vascularisation, promote tissue healing, and minimize rejection of foreign body. However, regeneration of liver tissue in omentum is still unknown. Despite the amazing breakthroughs in the bioengineered organs, there is much work left to do. The approach described herein harbours enormous potential to overcome the limitations of organ transplantation and may support failing liver through ectopic transplantation as secondary organ.

Vishwakarma SK, Lakkireddy C, Bardia A, Paspala SAB, Tripura C, Habeeb MA, Khan AA. Bioengineered functional humanized livers: An emerging supportive modality to bridge the gap of organ transplantation for management of end-stage liver diseases. *World J Hepatol* 2018; 10(11): 822-836 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i11/822.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i11.822

INTRODUCTION

End stage liver diseases (ESLD) have become the major reason for the increasing deaths worldwide. According to the World Health Organisation, the total deaths caused by cirrhosis and liver cancer have increased by 50 million/year since 1990[1]. Liver transplantation is the only standard treatment available so far. However, more than 20% patients die on the waiting list due to a shortage of organ donors[2]. In order to expand the supply of livers available for transplantation, transplant surgeons and physicians have explored several new approaches including split liver transplants, living-related partial donor procedures[3] and the increasing use of "marginal" organs such as older donors, steatotic livers, non-heart-beating donors, donors with viral hepatitis, and donors with non-metastatic malignancy[4]. Despite these medical and surgical developments, it is unlikely that the availability of good liver grafts will ever be sufficient to meet the increasing demand of patients with end stage liver disease.

In order to overcome these limitations, various other treatment options are being explored among which hepatocytes transplantation has been described as the first supportive modality in regenerative medicine. But major challenges with such treatment is its limited availability of therapeutic dose from surgical samples, liver grafts or biopsies and their maintenance *in vitro* which requires cell-to-cell and cell-to-matrix interactions for proper functioning of anchorage dependent hepatocytes[5]. Usage of hepatocytes from xenogenic sources such as rabbit, porcine or canine, pose the risk of immunogenicity and transmission of zoonosis. This limitation can be addressed to certain extent by the usage of cell lines which can be maintained for longer time with higher growth rates under *in vitro* culture conditions but modification of gene expression under culture conditions might lead to problems and has issues related to its clinical applicability[6].

The first land mark study to bring hepatocyte transplantation into clinics was by Mito *et al*[7] in cirrhotic patients. In line with this study, our centre has treated seven acute liver failure patients by intra-peritoneal transplantation of human primary hepatocytes extracted from human foetus's which showed clinical improvement and support to the failing liver[8]. Following this, various other studies have reported successful transplantation of primary hepatocytes in treating various metabolic diseases[9,10]. Although higher successful rate has been reported using hepatocyte transplantation, yet use of fetal hepatocytes poses major hurdle of ethical issues for its wider clinical applicability. Other potential treatment alternatives discovered in recent years included induced pluripotent stem cells, Mesenchymal stromal cells (MSC) which have the ability to differentiate into hepatocytes but still they couldn't completely mimic the fully functional hepatocytes pointing towards a need to identify better niche for functional utilization of these cells[11-14].

Other alternative of direct cellular transplantation includes the use of extracorporeal liver support devices which can support a failing liver for a short period of time before organ transplantation[15]. But all these above mentioned treatment strategies may not fulfil the requirements to treat ESLD and may not provide immediate support for a failing liver to maintain normal functions. Hence, there is a need to develop bioengineered transplantable liver grafts which can retain the natural three-dimensional extra cellular matrix (3D-ECM) components and intact vascular networks similar to the native liver with repopulated functional hepatocytes or human hepatic progenitor cells. Rapid progress in the area of stem cell research and organ bioengineering paved a way in generating alternatives to liver transplantation.

After addressing all these limitations next comes the question of choosing an exact transplantable site where in these bioengineered organs can be easily acceptable and can able to perform the function. Recently omentum has been discovered as a wonderful ectopic site for transplantation with excellent properties like remarkable angiogenic[16], stem cell[17,18], fibrotic[19], and immune activities[20], which together endorse vascularization, promote wound healing, and minimize infection. Several studies have already demonstrated the importance of intra-omental transplantation in diabetic animal models[21,22]. However, the regeneration of liver tissue in ectopic sites is still unknown. Few studies have shown the omentum as a reservoir for proliferating renal, pancreatic, splenic[23-25] cells and as a site for hepatocytes engraftment which can be used in tissue engineering[26]. Hence, opting omental transplantation of bioengineered liver may offer development of secondary liver for the treatment of ESLD. This particular approach should offer promising treatment strategy in future and may rule out above mentioned limitations to answer for shortage of organ donors for ESLD.

CURRENT STATE OF REGENERATIVE STRATEGIES IN ESLD

Since last two decades, significant developments have been made to overcome the limitations of liver transplantation in ESLD. Among these strategies cell transplantation, use of extra-corporeal devices and transplantable bioengineered organs have been explored extensively.

CELL TRANSPLANTATION

In cell transplantation strategies, hepatocytes transplantation has been the most preferred cell types for infusion into liver due to their ability to perform major liver specific functions. However, getting therapeutic dose of human hepatocytes represents major limitation towards its wider clinical application[27,28]. Although several studies have reported use of 10% liver tissues to isolate enough number of hepatocytes post-*in vitro* expansion which can provide required clinical response in both animal models and human[5,29]. The *in vitro* enrichment of hepatocytes is challenging due to their contact-dependent growth, long-term survival and function and maintenance of normal phenotype without de-differentiation[5,30]. Therefore alternative strategies are highly desirable to overcome these limitations.

Recent studies have reported use of embryonic and adult pluripotent stem cells to generate desired number of functional hepatocytes for therapeutic applications. However, use of embryonic stem cells (ESCs) represents ethical hurdles and immune incompatibility for the transplant recipients[31-33]. Moreover, use of induced pluripotent stem cells (iPSCs) has been reported for effective differentiation into functional hepatocytes, however poses potential issues related to genetic instability and lack of functional transplantation studies[11,12]. Mesenchymal stromal cells (MSCs) represents another alternative type of pluripotent cells to generate functional hepatocytes and support liver regeneration[13,14]. However, multi-lineage differentiation of MSCs represents major challenge to control the effective trans-differentiation into desired number of functional hepatocytes while restricting other default lineage cells. Although, stem cell transplantation strategies have showed potential in liver regeneration through various mechanisms, still it has not been considered as durable solution to completely support the lost liver functions[15]. Hence, alternative strategies are highly desirable to generate therapeutic number of functional heaptocytes under controlled conditions.

MAJOR SOURCES OF REGENERATIVE CELLS FOR THE TREATMENT OF ESLD

Liver-derived stem sells

These are the stem cells that are derived from adult or fetal livers. Adult stem cells are known as oval cells which play an important role in liver regeneration when replication capacity of hepatocytes is impaired[34]. Fetal liver stem cells are known as bipotent hepatoblasts that has ability to differentiate into bile duct cells or hepatocytes[35-37]. Fetal liver stem cells have been used to repopulate liver in animal models[38,39] and cultured hepatoblasts transplanted into immunodeficient mice showed greater *in vivo* engraftment and differentiation[40]. But the limitation in use of liver derived stem cells is their low number around 0.3% to 0.7% of oval cells in adult liver[41], whereas fetal liver mass comprises only 0.1% of hepatoblasts[42] and has associated ethical issues. Thus isolation and expansion of these cells and usage for transplantation is challenging.

Bone marrow-derived stem cells

Stem cells derived from bone marrow comprise hematopoietic and MSCs[43]. Among these, mesenchymal stem cells consists greater potential in liver

regeneration[44] with immunosuppressive and immunomodulatory properties[45]. But they always pose problem with low rates of liver repopulation[46] and have low trans-differentiation ability to hepatoblasts which limits to restore normal liver function[47].

Annex group of stem cells

Stem cells derived from human umbilical cord, human placental tissue, amniotic fluid and human umbilical cord blood constitutes Annex group of stem cells. These are pluripotent stem cells with higher proliferation and differentiation rates than adult stem cells[46,48,49] and are not known to cause teratomas or teratocarcinomas formation in humans. Di Campli *et al*[50] study on diabetic severe combined immunodeficient mice after acute toxic liver injury when treated with intraperitoneal administration of human umbilical cord stem cells showed rapid liver engraftment, differentiation into hepatocytes, improved liver regeneration, and reduced mortality rates[50].

IPSCs

These are similar to ESCs and the limitation of ethical issue can be overruled by *in vitro* generation of iPSCs from somatic cells avoiding the usage of embryonic tissue or oocytes[51]. However, the use of these cells in clinical practice is limited due to major hurdles with the genomic instability of these cells.

EXTRA-CORPOREAL LIVER SUPPORT SYSTEMS

Extracorporeal liver support devices have been designed with a goal to carry out normal liver function in patients with end-stage chronic, acute-on-chronic and acute liver failure for a short period of time until donor organ gets available. Two types of liver support systems have been designed: (1) Non-biological; and (2) Bio-artificial liver support devices.

Non-biological liver support devices

Designed to filter and adsorb accumulated toxins that are not cleared by non-functional liver[52]. Three major types of such devices have been explored as follows:

Molecular adsorbent recirculating system: Molecular adsorbent recirculating system (MARS) has been well explored device which is a hollow fiber membrane hemodialyzer which removes soluble and protein-bound substances against albumin-rich dialysate. This device was approved by FDA in 2012 for the treatment of hepatic encephalopathy. However, the major limitation of such devices represents: (1) Short-term detoxification function; (2) Chance of getting sepsis; (3) Cost issues; (4) Can remove only albumin-bound toxins or drugs which are excreted in circulation; (5) Safety and efficacy of MARS has not been demonstrated in controlled, randomized trials; and (6) The effectiveness of MARS in patients that are sedative could not be established in clinical studies and therefore can't be predicted in sedated patients.

Promethus fractionated plasma separator and adsorption system: Other type of devices includes, promethus fractionated plasma separator and adsorption system (FPSA) which is an artificial device which removes both albumin-bound and water soluble toxins from blood more effectively than MARS. However, its wide applicability has been limited due to following reasons: (1) Direct contact between fractionated plasma and the Prometh anion exchanger causes significant adsorption of procoagulant and anti-coagulant factors, associated with clinically relevant adverse events; (2) Broad disturbances of the coagulation system have been confirmed in FPSA treated liver failure patients; and (3) An *ex vivo* recirculation model demonstrated nonspecific adsorption of coagulation factors protein S and protein C on the anion exchange cartridge.

Single-pass albumin dialysis: Moreover, to overcome on the limitations of above mentioned extracorporeal liver assist device, single-pass albumin dialysis (SPAD) system was evolved which functions as one-pass dialysis against albumin solution to remove albumin-bound toxins and water-soluble substances. Detoxification system in SPAD is similar to or greater than MARS and is less expensive than MARS and FPSA. However, again the suitability and wide clinical applicability of SPAD is limited due to following limitations: (1) Only albumin bound or water soluble toxins can be removed; (2) Lipid soluble toxins can't be removed by SPAD; (3) Bleeding risk from acquired coagulopathy; (4) Albumin solution is discarded after a single passage of membrane without being recycled; and (5) Absence of clinical data.

Bioartificial liver support systems

These are the bioreactors containing viable hepatocytes in a 3D network of hollow fibers. These are designed to achieve plasma perfusion and enhance the activities of living liver cells. Conversely, the membranes separating cells from plasma are not capable of achieving enough *in vivo* perfusion rates, and lack sources of safe, reliable, strongly proliferating and functionally active human cells. Still following major challenges remain to resolve: (1) Bio-artificial livers should be able to provide at least 10% of liver functioning; (2) Very difficult acquiring this many hepatocyte cells; (3) Controversy over the use of porcine cells due to possible transmission of infections; (4) Hepatocytes and plasma have very different physio-chemical properties; (5) Hepatocytes do not perform well when in contact with plasma; (6) Have a very high oxygen uptake rate; (7) Hepatocytes undergo a lot of stress inside of bio-artificial liver; (8) Any stress above 5 dyn/cm^2 renders cells useless; (9) Limited volume of the bioreactor;

(10) Maximum blood/plasma that can be safely drawn out of liver failure patient is one liter; (11) Difficult to achieve 10% of liver functioning within one liter; and (12) Makes Bio-artificial liver designing very difficult.

TRANSPLANTABLE BIOENGINEERED ORGANS

Owing to the hurdles in above mentioned devices, there is need to develop transplantable biological systems to provide: (1) Suitable three-dimensional organ architecture; (2) Organ specific intact vasculature for homogeneous supply of oxygen and nutrients; (3) Long-term cell survival and function within the natural organ specific niche; and (4) Metabolic, synthetic and detoxification functions similar to native liver.

MAJOR COMPONENTS OF HUMAN LIVER FOR BIOENGINEERING

Major components of human liver for bioengineering includes (1) Organ specific 3D-bioscaffolds; (2) Organ capsule; (3) Organ vasculature; (4) Cellular distribution in spatial anatomical organization of liver; (5) Biomolecules and growth factors for enhanced survival and function to transplanted cells; (6) Types of cells required for long-term support; and (7) Long-term functional response.

To provide these crucial components recently two major technological advancements have been made: (1) Organ bio-printing; and (2) Humanized neo-organ development.

Organ bio-printing

With the advancements in tissue engineering it is possible to construct complex parenchymal organ structures along with intact vascular network by 3D bio-printing[53]. 3D bio-printing is one of the prevalent examples of bioengineered organs in the science world today, and it is growing and advancing quickly. This jaw dropping technology is one of the hot topics in bioengineering. It still fascinates that we have the potential to build organs from the push of a button. 3D bio-printing is a form of tissue engineering which utilizes inkjet printers and builds the scaffolding of a particular organ, layer by layer[54]. These inkjet printers allow the use of multiple cell types for printing. Robbins *et al*[55] developed a metabolically active 3D hepatic tissue where they identified increased liver specific function lasting for up to 135 h, and compartment-specific organization, along with a primitive hepatocyte microanatomy of hepatic stellate cells and endothelial cells. Researchers have also build bone repair constructs by coating the 3D printed scaffold with stem cells, which can grow into tissues over time[54]. The mild conditions used for bio-printing and material sintering have allowed viable cells and active therapeutic proteins to be incorporated into the construct production process. Today, this particular technology has been emerged only for *ex vivo* and its application *in vivo* has not been experienced which needs to be validated further.

Humanized neo-organ development

The recent concept of bioengineering functional humanized neo-organs has given a hope towards finding permanent cure as an alternative support to the failing organ. This concept of artificial organs was first originated in the radiation field post-World War Ⅱ, and was executed in the first bone marrow transplant in the 1970s[56]. According the Llames S tissue engineering has three main constituents: The *ex vivo* expansion of cells, seeding of these expanded cells in three dimensional structures that mimic physiological conditions and grafting the prototype. The technology relies on the development of whole intact organ scaffolds through whole organ perfusion acellularization procedure which retains extra-cellular matrix and circulatory networks of the native organ post-acellularization[57]. This important phenomenon allows three-dimensional intact acellular organ specific scaffold for efficient repopulation of desired cell population further to generate functional neo-organ system.

With advancement in regenerative medicine it has been possible to create bioengineered functional tissues or organs that can be used clinically[58,59]. So far several successful studies have been published in generating various organs and tissues based on these acellularization and stem cell repopulation[59-61] that can be used for treating patients. Significant progress in generating several types of complex organ biological scaffolds has led to development of an efficient acellularization protocols for whole organs through perfusion based techniques[62-66] (Tables 1 and 2). These acellularized whole organs combined with an efficient recellularization process[67-70] have made it possible to use these bioengineered organs for *in vivo* preclinical studies in small animal models[71-73].

Our centre has well expertise in generating various types of acellularized whole organ bioscaffolds including xenogeneic liver through detergent-based perfusion. So far, we have successfully generated acellularized and repopulated humanized whole liver and demonstrated its applicability as better natural 3D-drug testing model system[74]. Apart from liver, we have also generated acellularized kidney[75], heart[76], spleen, meninges, and many more. Still various other studies are in pipeline in generating humanized bioengineered organs from our centre.

WHOLE LIVER BIOENGINEERING

Highly specialized thick and complex organs like liver can be subjected to acellularization technology to obtain intact 3D-ECM. Due to delayed co-morbidity beyond marginal criteria or because of delayed ischemic time,

Table 1 Method adopted for whole complex organ acellularization techniques for different organisms

Organ	Acellularization agent	Perfusion method	Animal model	Reference
Heart	SDS, PEG, Triton X-100, and enzyme-based protocols deoxycholic acid	Antegrade coronary perfusion	Rat	[98]
	Trypsin, EDTA, NaN3, Triton X-100, and deoxycholic acid	Retrograde aortic perfusion	Pig	[65]
Lung	0.1% and 0.5% SDS	Antegrade pulmonary arterial perfusion	Rat	[63]
	CHAPS	Pulmonary artery and tracheal perfusion	Rat	[66]
	Triton X-100 and sodium deoxycholate	Right ventricle and tracheal perfusion	Mouse	[99]
Liver	Triton X-100 plus 0.1% SDS	Portal vein perfusion	Rat	[100]
	SDS		Rat	[70]
	1% Triton X-100 and 0.1% ammonium hydroxide		Mouse, rat, ferret, rabbit and pig	[69]
	0.25% and 0.5% SDS		Pig	[101]
	Sodium citrate + SDS + Triton-X-100	Hepatic artery perfusion	Rat	[74]
Kidney	0.5, 3, 6, 10% Triton X-100, 5 mM	Renal artery perfusion	Rat	[71]
	calcium chloride, 5 mM magnesium sulfate, 1 M sodium chloride, DNase, and 4% sodium deoxycholate		Rat	[72]
	3% Triton X-100, DNase, and 4% SDS			
	1% SDS and 1% Triton X-100			
	1% Triton X-100 and 0.1% ammonium hydroxide		Pig	[68]
	Heparin and antibiotic-containing physiological saline, 0.1-1.0% SDS, 0.1% Triton-X-100 and 0.0025% deoxyribonuclease 1		Goat	[75]

Table 2 Study outcome and major limitations of different types of acellularization techniques adopted for different types of whole organ scaffold development

Organ	Acellularization Method	Study out come	Limitation	References
Rat liver	Perfusion with detergents (SDS, Triton X-100)	Perfusion with SDS removes most of cells, damages the ECM when treated with Triton X-100 and removes 97 % of DNA	SDS damages the ECM	[69,74]
Porcine liver	Mechanical perfusion (electroporation)	Most of the cells are removed, preserves the blood vessels	Disruption of microfilament and microtubule	[102]
Mouse heart	Enzymatic, detergents, Acids	Cells are removed	Damages the ECM proteins, poorly maintains the 3D architecture	[103]
Porcine trachea	Enzymatic (trypsin) non-enzymatic (EDTA), detergent (Triton X-100) and deionized Water	Cells are removed, clear the cell debris	Disruption of glycosaminoglycan, reduce the laminin and fibronectin	[104]
Rat kidney	Perfuse with SDS, deionized water, dTriton X-100 and PBS along with antibiotics	Twice filtration is observed	Loss of cell-mediated functions like transport of solutes	[105]
Rat heart	Perfused with detergents		Long-term cell survival, oxygen tension and continuous rhythmic beating	[63,98]
Goat kidney	Perfused with Trypsin-EDTA in PBS, perfuse antibiotics and then with SDS in PBS	Cells are removed, pore to pore interconnection in the scaffold		[75,106]

ECM: Extra cellular matrix.

in United Kingdom livers offered for transplantation are usually discarded[77]. This act offers a way to use this kind of livers for acellularization. The liver is the largest gland in the body and carries out numerous essential functions such as metabolism, maintaining homeostasis, and the synthesis of amino acids[57]. Therefore, acellularization is extremely beneficial to the liver because it not only maintains the microstructure but also its bio signals such as extracellular matrix proteins and adhesion peptides[57].

Since extracellular matrices are similar from species to species, whole organ scaffolds have become possible for livers. Several recent studies have been reported for efficient acellularization of livers obtained from various xenogeneic sources[78-81] and the resulting 3D-ECM structure has become an outstanding source for generating highly functionalized liver cells *in vitro*[82,83]. As these extracellular matrices are conserved between species, the process of recellularization with human cells into an animal scaffold is easier[57] and this kind of approach does not elicit any kind of immune rejection, cross contamination and zoonosis. In our recent study, we have demonstrated development of humanized whole liver using human hepatic progenitor cells repopulation through hepatic artery infusion into acellularized liver scaffolds[74]. These humanized livers perform detoxification and metabolic functions similar to the native liver. However, the complete recellularization of a fully function human liver has not yet been accomplished[57]. Recent advances in isolating and culturing both native cells and stem cells, as well as the development of acellularized organ scaffolds and biocompatible synthetic biomaterials, suggest that we are making rapid progress towards providing new alternatives to donor livers for transplantation[56].

CHALLENGES NEED TO BE ADDRESSED IN GENERATING COMPLETE BIOENGINEERED FUNCTIONAL LIVER

Despite the amazing breakthroughs in the bioengineered organs, there is much work left to do. Simply reconstructing the whole organ will not be sufficient to replace organ transplantation. The approaches described above are fairly new and are still in the developmental stages. There has been only handful of successful transplantation of bioengineered organs into actual humans. Scientists are still working on ways to engineer more complex organs such as the liver. There are also long-term issues to resolve, such as the preservation of the overall function of these bioengineered organs. However, little is known about the mechanisms by which these grafts may integrate and maintain function. When more complex organs are involved, the scenario is completely different, as investigations are still in very early stages and clinical translation is not foreseeable on the basis of current knowledge and available data[84]. The following major critical issues are yet to be resolved to make these approaches a clinical reality: (1) Liver is a complex organ with various cell types, hence rebuilding liver micro architectures with these cells is yet to be addressed; (2) The optimal cell source that can meet the criteria for recellularization of acellularized liver scaffolds still remains unclear; (3) The first and foremost challenge is the need to address the reconstruction of complete and functional uniform endothelial cell layer throughout acellularized liver scaffolds; (4) It is necessary to reconstruct biliary system which is needed for bile acid excretion to develop a fully functional bioengineered liver; (5) Assessing the functionality of these bioengineered livers after *in vivo* transplantation for long term needs to be studied clearly; (6) For organ functionality, maintaining its vascular structure is much more important. As hepatocytes require higher amounts of oxygen for their functionality, it is necessary to maintain hierarchical vascular network structure in acellularized liver scaffolds[85]. Critical step in engineering a transplantable liver is the creation of a functional vasculature capable of long-term perfusion following anastomosis. Without an appropriate endothelial lining of the vessels, continuous blood perfusion of the graft in the absence of anticoagulation quickly results in thrombosis; and (7) Finding an appropriate site for providing enough support to the failing liver has been one of the most challenging issue to use the bioengineered organs as secondary liver.

OMENTUM AS BETTER ECTOPIC SITE FOR TRANSPLANTATION TO GENERATE SECONDARY ORGAN *IN VIVO*

The major question for applying these humanized bioengineered livers relies on finding an exact and more appropriate transplantable site where in these bioengineered organs can be easily acceptable and are able to perform the function. Recently omentum has been discovered as a potential ectopic site for transplantation with excellent properties like remarkable angiogenic[16], stem cell[17,18], fibrotic[19], and immune[20] activities, which together endorse vascularization, promote wound healing, and minimize infection (Table 3). Several studies have already demonstrated the importance of intra-omental transplantation in diabetic animal models[22,86].

The omentum is a visceral adipose tissue derived from mesothelial cells[87] connected to the spleen, stomach, pancreas, and colon[88,89]. Although well known as a visceral fat depot, the role of the omentum in peritoneal immunity was not recognized until the early 1900s, when a British surgeon referred to it as 'the police man of the abdomen' due to its ability to attenuate peritonitis and promote surgical wound healing[90]. In fact, omentum was noted to move about the peritoneal cavity and occlude sites of inflammation, such as ruptured ovaries, inflamed

Table 3 List of recent studies reporting use of omentum as transplantation site to support the lost organ function from ectopic transplantation of engineered tissues or grafts

Animal model	Site of transplantation	Mode of graft used	Results	Reference
Femoral bone of New Zealand rabbit was	Greater omentum on the left side	Free transplant of the greater omentum	Process of the callus formation and its mineralisation are much quicker and thicker on the defect that was covered with the free transplant of the greater omentum.	[107]
Pancreatectomized dogs	Spleen or Omentum	Islet auto-transplantation	Beta cell response to mild non-insulin induced hypoglycemia was normal, whereas the alpha cell response was not.	[108]
Murine carotid artery injury model	Omentum was applied to the injured vessel	Omentum + Omental progenitor cells	Omentum can directly contribute reparative progenitor cells to injured tissues upon treatment with Tβ4.	[109]
Nondiabetic nude rats	Omentum/kidney capsule	Perinatal porcine islet cell grafts	In both sites, the A-cell volume increased fourfold between weeks 1 and 10 reflecting a rise in A-cell number. In the omental implants, however, the cellular insulin reserves and the percent of proliferating cells were twofold higher than in kidney implants. In parallel, the blood vessel density in omental implants increased twofold, reaching a density comparable with islets in adult pig pancreas.	[110]
Diabetic rat and nonhuman primate (NHP) models	Intra-omental	*In situ*-generated adherent, resorbable plasma thrombin biologic scaffold	Improved metabolic function and preservation of islet cytoarchitecture, with reconstitution of rich intrainsular vascular networks in both species.	[21]
Adult male Sprague Dawley rats	Omental transposition	Hepatic tissue sutured into the omentum mobilization of the omentum and transposition onto the left hepatic lobe	Omental transposition provided adequate microcirculation for proliferation of ectopic hepatic cells after liver resection.	[111]

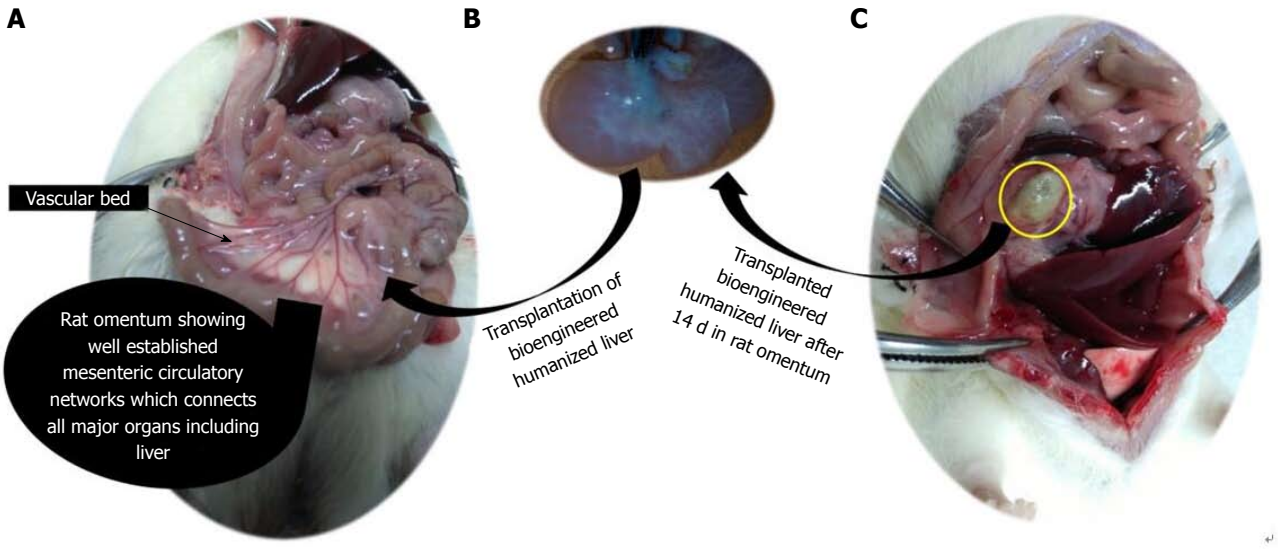

Figure 1 Intra-omental transplantation of bioengineered humanized livers showing development of secondary liver after 14 d. A: Anatomy of rat omentum showing well-established web of circulatory networks which connected with major organs; B: Developed bioengineered humanized liver in our lab *ex vivo*; C: Intra-omentally transplanted bioengineered humanized liver showing well engraftment with the surrounding tissue.

appendices, ulcerated intestines, or wounds due to trauma or surgery[90]. Consistent with this observation, the omentum has remarkable angiogenic[16], fibrotic[19], regenerative[17,18] and immune[20] activities, which together promote vascularization, accelerate wound healing, and limit infection. However, these same activities are also likely involved in pathological responses, such as the rapid growth of omental tumour metastases[91].

Once thought of as just a large amount of redundant fat overlying the intestines, surgeons' attitudes towards the omentum have changed. It is recognized as an organ in its own right, with many diverse functions ranging from its ability to attenuate the

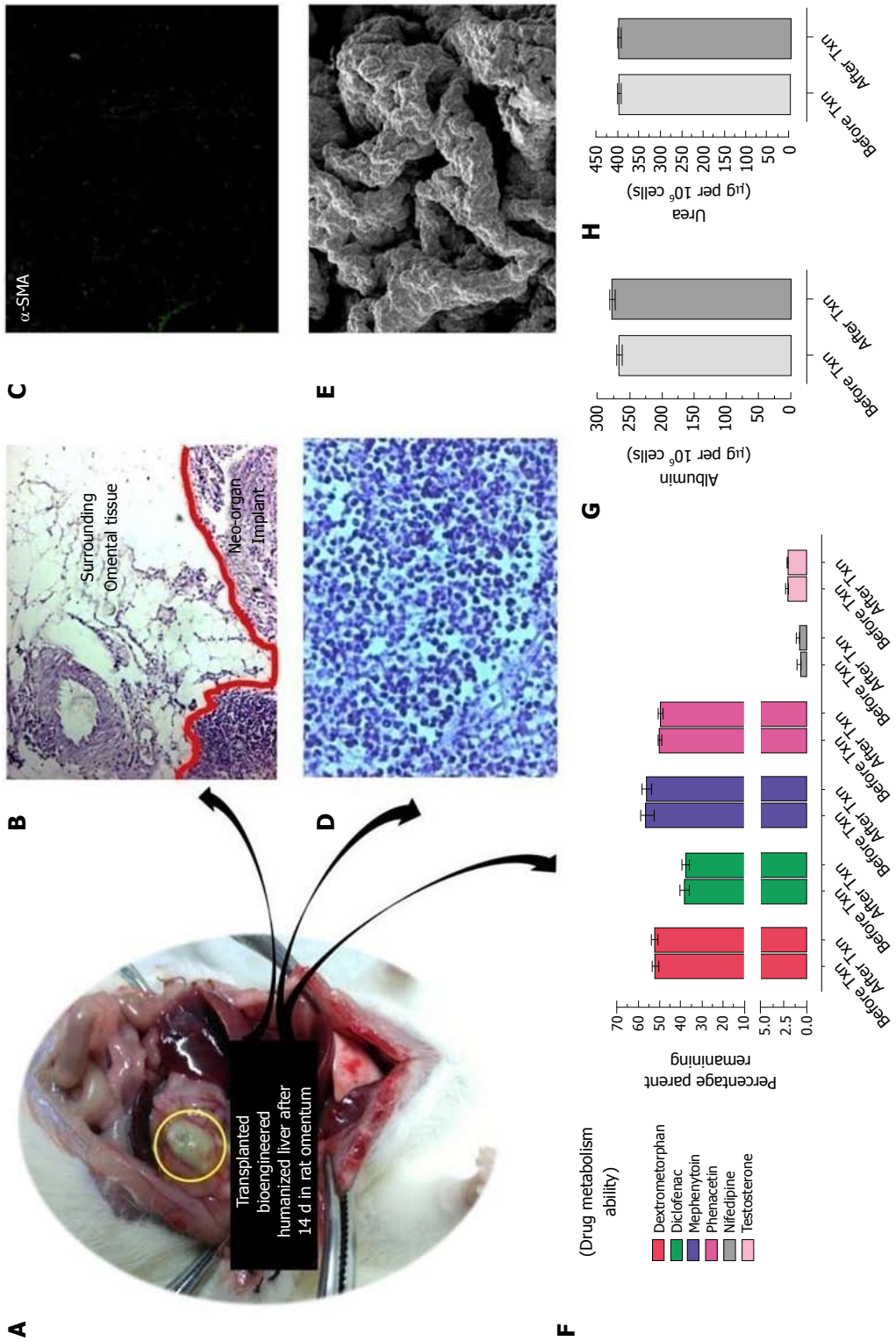

Figure 2 Intra-omental transplantation of bioengineered humanized livers showing no sign of fibrosis or immunological response at transplantation site. A: Optical image of transplanted implant at intra-omental site; B: Hematoxyeleine and eosin (HE) staining of the transplanted implant along with surrounding tissues showing no sign of immunological cells infiltration or tissue damage. Moreover, neo-vascularization was seen into nearby surrounding tissues which connects with the implant; C: Immunocytochemical staining using α-SMA showed no sign of fibrotic reactions to implant; D: HE staining showed well organized distribution and proliferation of hepatic cells into the implant post-transplantation; E: Scanning electron microscopy (SEM) image of retrieved graft at day 15 post-transplantation showing almost similar anatomy of bioengineered livers with natural liver; F: Retrieved livers at day 15 post intra-omental transplantation showed almost similar metabolic activity to before transplantation ($P > 0.05$). The other two important liver cell functions such as G: Albumin synthesis and H: Ammonia detoxification (*i.e.* urea production) is almost similar to the bioengineered humanized livers prior to transplantation ($P > 0.05$).

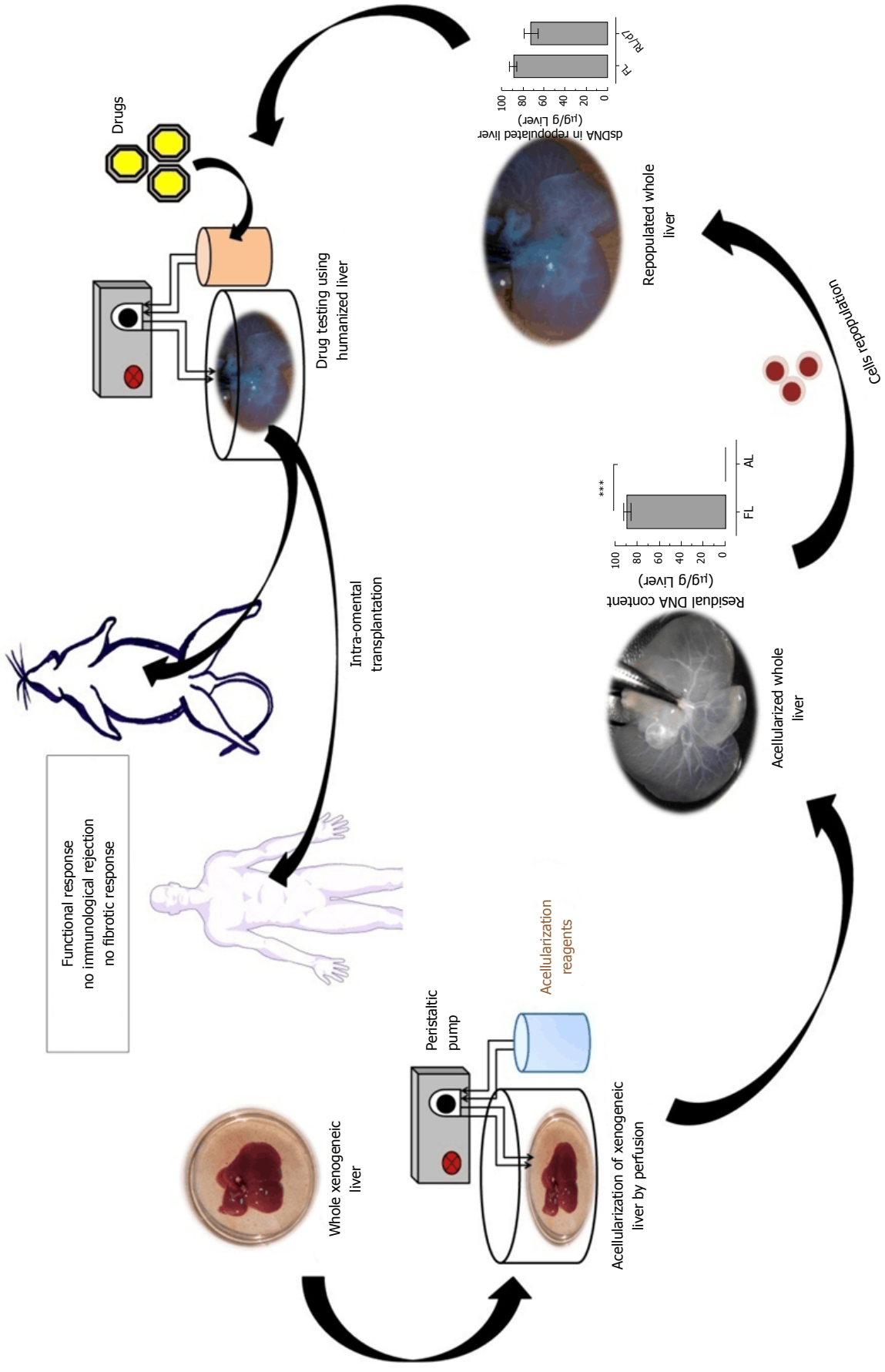

Figure 3 Brief overview of strategy for the development of immune-competent bioengineered humanized liver using acellularization and repopulation technology for future biomedical applications.

spread of sepsis in peritonitis to acting as a source of angiogenic and hemostatic factors involved in tissue healing and repair. The omentum has been identified as a source of adult stem cells which may have future prospects in the fields of tissue engineering and the synthesis of vascular grafts. Its regenerative properties have been exploited in virtually every field of surgery from the reconstruction of complex wounds to the protection of gastrointestinal anastomosis.

The regenerative properties of the omentum have been exploited by surgeons for over a century, ranging from the protection of anastomosis in gastrointestinal surgery, revascularization of arterial ulcers, to the reconstruction of head and neck deformities[92]. The advantage of the omentum is that it is an accessible and versatile source of growth factors, angiogenic factors, and leukocytes. It can be lengthened considerably by careful dissection to produce a mobile organ[93].

The regeneration of liver tissue in ectopic sites is still unknown. It has been discovered that the omnetum is a reservoir for proliferating renal, pancreatic, splenic tissues[23-25] and as a site for hepatocytes engraftment which can be used in tissue engineering[26]. Hepatocyte transplantation has been done in various tissues like spleen, pancreas and omentum[26,72,94-96]. With advancements in tissue engineering hepatocytes seeded onto polymer scaffolds and have been transplanted into omentum wherein engraftment of hepatocytes occurred due to elevated rates of angiogenesis into cell-polymer constructs within the omentum[96].

Thus intra-omental transplantation of bioengineered livers may provide adequate microcirculation for proliferation of ectopic hepatic cells repopulated within the bio-artificial liver. It has been observed that portal vein ligation does not affect the ectopic liver regeneration[97]. In our preliminary experiences, we have observed that intra-omental transplantation of bioengineered liver lobes gets easily accommodated into the site without eliciting immunological responses while maintain their biological functions and communicates blood borne growth factors for survival and function of the graft (Figure 1). We also observed that these bioengineered liver grafts survive at omental site in long-term and functions as secondary liver (Figure 2). These findings are well supported by earlier studies wherein other types of grafts have been transplanted into the omentum[21]. Future efforts at understanding mechanisms to regulate ectopic liver regeneration may assist the pursuit for liver tissue/organ bioengineering to support the failing liver functions in long-term.

CONCLUSION

Engineers and researches have been making monumental breakthroughs in the area of bioengineered organs. These bio-artificial organs may redefine transplants for human applications in future with more critical advancements. The introduction of cells into the human body is designed to stimulate regeneration, promote vascularization and/or supplement the production of hormones and growth factors[56]. Consequently, bioengineered biological substitutes present a new way to restore damaged tissue and maintain their functions. Not only does this provide a new source of organs, but probably even more reliable organs at that. Not only would people not need an organ donation, but their body will more readily accept a bioengineered organ through intra-omental transplantation, most likely reducing recovery time as well (Figure 3). In near future these potential strategies can overcome the limitation of organ donors and these bioengineered organs can even serve as a best natural 3D-drug testing models[74] and investigating precise molecular mechanisms in biomimetic natural organ system[112] and could support failing liver through ectopic transplantation as secondary organ in ESLD.

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ORIGINAL ARTICLE

Retrospective Study

African Americans are less likely to receive curative treatment for hepatocellular carcinoma

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Author contributions: All authors helped to perform the research; Sobotka LA conceived and designed the study, interpreted the data, drafted the article, and approved the final version of the article to be published; Hinton A acquired data, analyzed data, made critical revisions related to important intellectual content of the manuscript, and approved the final article to be published; Conteh LF conceived and designed the study, interpreted data, drafted the article, made critical revisions related to important intellectual content of the manuscript, and approved the final version of the article to be published.

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Abstract

AIM

To determine if racial disparities continue to exist in the treatment of hepatocellular carcinoma (HCC).

METHODS

A retrospective database analysis using the Nationwide Inpatient Sample was performed including patients with a primary diagnosis of HCC. Univariate and multivariate analyses were utilized to determine racial disparities in liver decompensation, treatment, inpatient mortality, and metastatic disease.

RESULTS

A total of 62604 patients with HCC were included consisting of 32428 Caucasian, 9726 African-American, 8988 Hispanic, and 11462 patients of other races. Caucasian patients were more likely to undergo curative therapies of liver transplant (OR: 2.66, 95%CI: 1.92-3.68), resection (OR: 1.82, 95%CI: 1.48-2.23), and ablation (OR: 1.77, 95%CI: 1.36-2.30) than African-American patients. Hispanic patients were more likely to undergo transplant (OR: 2.18, 95%CI: 1.40-3.39) and ablation (OR: 1.46, 95%CI:

1.05-2.03) than African-American patients. Patients of other races were more likely to receive a liver transplant (OR: 2.41, 95%CI: 1.62-3.61), resection (OR: 1.79 95%CI: 1.39-2.32), and ablation (OR: 2.03, 95%CI: 1.47-2.80) than African-American patients. There are no differences in the rates of transarterial chemoembolization between races.

CONCLUSION

Racial disparities in HCC treatment exist despite emphasis to support equality in healthcare. African-American patients are less likely to undergo curative treatments for HCC.

Key words: Racial disparity; Hepatocellular carcinoma; Liver transplantation; Resection; Ablation

Core tip: Racial disparities in the treatment of hepatocellular carcinoma (HCC) have been noted previously. This study investigated continued disparities in healthcare utilizing the Nationwide Inpatient Sample. African-American patients were less likely to undergo curative treatments, such as liver transplantation, liver resection, ablation, and transarterial chemoablation for HCC despite having less features of liver decompensation.

Sobotka LA, Hinton A, Conteh LF. African Americans are less likely to receive curative treatment for hepatocellular carcinoma. *World J Hepatol* 2018; 10(11): 849-855 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i11/849.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i11.849

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) has increased about 50% since 2003[1,2]. An increasing incidence across all races and ethnic groups has been noted, however the incidence in African-American and Hispanic patients has had the largest increase over the past ten years[1]. The incidence of HCC is two times higher in African-American, American-Indian, Alaskan-Native, and Hispanic patients compared to Caucasian patients[2]. Unlike other malignancies such as prostate cancer, where despite increases in incidence, mortality has actually declined, we have seen a concurrent increase in the mortality of HCC as the incidence increases. The mortality rates are twice as high in African-American patients compared to Caucasian patients[2,3]. A recent study completed using the Surveillance, Epidemiology, and End Results (SEER) database showed the median overall survival of all patients with HCC was 11 mo. However, African-American patients had a significantly worse prognosis compared to Caucasian patients with only a nine-month survival rate[4].

Racial disparities in the treatment of HCC have been highlighted in previous studies. African-American patients who presented with localized disease were less likely to undergo curative therapy with liver transplantation, surgical resection, and ablation compared to Caucasian patients with the same tumor burden[5,6]. Previous studies have also noted that African-American patients were more likely to present with metastatic HCC at the time of diagnosis and were therefore no longer candidates for specific curative treatments[7].

This study aims to investigate continued disparities in the treatment of HCC. We hypothesize that racial disparities will continue to be present despite recent emphasis for equal treatment in healthcare.

MATERIALS AND METHODS

Data source

Utilizing the (Nationwide) Inpatient Sample, which is part of the Healthcare Cost and Utilization Project (HCUP), we performed a retrospective database analysis. The HCUP is one of the largest publically available inpatient databases. Information obtained included primary and secondary diagnoses and procedures, patient demographics, expected payment source, total charges, discharge status, and length of stay[8]. This study is exempt from review from The Ohio State University Institutional Review Board because patient information is de-identified.

Study sample

Utilizing International Classification of Diseases, Ninth Revision, Clinical Modification codes, patient with a primary diagnosis of HCC (ICD-9 155.0) were included in this study. Patients were excluded if they were under the age of 18, or if they had a malignancy in the liver that was not hepatic in origin.

Outcomes of interest

Primary outcomes of interest included treatment disparities in HCC based on race, which was defined as Caucasian, African-American, Hispanic, or other. Specific treatments for HCC that were evaluated included liver transplantation, liver resection, ablation, and transarterial chemoablation (TACE). Secondary outcomes included differences in inpatient mortality, liver decompensation, and metastatic disease.

Covariates

Other variables evaluated included gender, age, insurance provider, region where treatment was received, etiology of cirrhosis, features of liver decompensation, metastatic disease, and comorbidities, defined by the Elixhauser Comorbidity (Table 1)[9] . Modification of the Elixhauser Comorbidity score was performed to exclude liver disease. Features of liver decompensation included ascites, jaundice, and hepatic encephalopathy as previously defined in other studies[10]. These variables

were determined by the appropriate ICD-9 code.

Statistical analysis

Association between race and factors of interest were evaluated using chi square tests. Multivariate regression models were fit for the presence of metastatic HCC, liver decompensation, mortality, and treatment. Terms in each module were determined through backwards selection where hepatitis C, hepatitis B, alcohol, non-alcoholic steatohepatitis (NASH), primary sclerosing cholangitis, primary biliary cirrhosis, autoimmune liver disease, metastasis, Elixhauser comorbidity score, and treatment were eligible for inclusion where appropriate. Data was analyzed using SAS software (version 9.4).

RESULTS

Demographics

There were 62604 patients with a primary diagnosis of HCC included in this study. The majority of the patients were Caucasian (32428, 52%) followed by African American (9726, 16%), Hispanic (8988, 14%), and patients of other races (11462, 18%).

Liver severity, metastatic HCC, and inpatient mortality

Upon univariate analysis, features of liver decompensation were significantly different between races ($P < 0.001$) (Table 1). Multivariate analysis demonstrated that Caucasian and Hispanic patients were more likely to have decompensated liver disease than African-American patients [(OR: 1.16, 95%CI: 1.03-1.30), (OR: 1.28, 95%CI: 1.10-1.30)] (Table 2).

Univariate analysis concluded the presence of metastatic disease was significantly different between races ($P = 0.007$) (Table 1). Upon multivariate analysis, Caucasian patients were less likely to have metastatic disease then African-American patients with HCC (OR: 0.82, 95%CI: 0.71-0.94). There was no statistical difference between other races (Table 2).

Inpatient mortality was significantly different between races upon univariate analysis ($P = 0.017$) (Table 1). Upon multivariate analysis, Caucasian patients were less likely to have inpatient mortality compared to African-American patients (OR: 0.78, 95%CI: 0.65-0.93). There was no statistical difference between other races (Table 2).

Inpatient treatment of HCC

There was a significant difference in treatment between races in the univariate analysis ($P < 0.001$) (Table 1). Upon stepwise multivariate analysis, Caucasian, Hispanic, and patients of other races were more likely to undergo liver transplantation compared to African-American patients [(OR: 2.66, 95%CI: 1.92-3.68), (OR: 2.18, 95%CI: 1.40-3.39), (OR: 2.41, 95%CI: 1.62-3.61)]. Caucasian patients and patients of other races were also more likely to undergo surgical resection than African-American patients (OR: 1.82, 95%CI: 1.48-2.23), (OR: 1.79, 95%CI: 1.39-2.32). Caucasian, Hispanic, and patients of other races were more likely to undergo ablation compared to African-American patients (OR: 1.77, 95%CI: 1.36-2.30), (OR 1.46, 95%CI: 1.05-2.03), (OR: 2.03, 95%CI: 1.47, 2.80)]. There was no significant difference in the rates of TACE between races (Table 3).

DISCUSSION

African-American patients are less likely to undergo curative treatments for HCC, and this study confirms that treatment disparities continue to exist despite efforts to reduce healthcare disparities. TACE was the only treatment without a disparity in utilization between races. TACE, however, is not considered to be curative for HCC. It is a means of controlling the malignancy and is often used to downstage tumor burden for liver transplantation or to keep tumor burden within transplant criteria. Differences in treatment exist despite African-American patients being less likely to present with decompensated disease compared to Caucasian patients. We know that patients whose disease is better compensated have a better tolerance of liver directed therapies for HCC. African-American patients have increased rates of metastatic disease and higher inpatient mortality. There are multiple factors that contribute to racial disparities in the management of HCC, including disease progression at time of diagnosis and social determinants of health. It is crucial to recognize these factors and their associations in order to formulate interventions to reduce racial disparities in treatment given the direct effect on patient survival and quality of life.

African-American patients are less likely to undergo curative treatments for HCC and many factors influence this disparity with the presence of metastatic disease being one major limitation to treatment[11]. African-American patients were more likely to have metastatic disease at the time of initial diagnosis[7]. This may be influenced by decreased HCC screening exams in African-American patients[12] and also by genetic differences between races. The presence of metastatic disease is not the only contributor to treatment discrepancies, however. Previous studies have found that African-American patients were less likely to undergo surgical treatment for HCC than Caucasian patients even when they presented with the same tumor burden and both groups were within Milan criteria[13].

Social factors, specifically the location in which patients receive therapy play a crucial role when considering treatment for patients with HCC. This study showed that the majority of African-American patients receive care in the Southern region of the United States, and patients living in the Southern United States are less likely to undergo curative therapies of liver transplantation and surgical resection[14,15]. Decreased access to providers who are able to provide timely diagnosis and treatment contributes to this disparity because of the increased rates of physician and hospital bed inequality in the South compared to the North. This makes it more challenging for patients in these areas

Table 1 Demographics and clinical parameters in patients with hepatocellular carcinoma grouped by race

	Caucasian (*n* = 32428)		African-American (*n* = 9726)		Hispanic (*n* = 8988)		Other (*n* = 11462)		*P* value
	n	%	*n*	%	*n*	%	*n*	%	
Age (yr)									< 0.001
⩽ 64	17695	54.6	6980	71.8	5357	59.6	6609	57.7	
65-79	10982	33.9	2287	23.5	2888	32.1	3762	32.8	
⩾ 80	3751	11.6	458	4.7	743	8.3	1090	9.5	
Sex									0.880
Male	23845	73.5	7172	73.7	6572	73.1	8319	72.7	
Female	8583	26.5	2554	26.3	2416	26.9	3122	27.3	
Primary payer									< 0.001
Medicare	15765	48.9	3471	35.9	3645	40.6	4520	39.5	
Medicaid	4155	12.9	2707	27.9	2206	24.5	2573	22.5	
Private insurance	9497	29.4	2236	23.1	2020	22.5	3143	27.5	
Other	2844	8.8	1267	13.1	1118	12.4	1202	10.5	
Geographic region									< 0.001
Northeast	7726	23.8	2603	26.8	1829	20.4	2395	20.9	
Midwest	5904	18.2	1785	18.4	536	5.9	3016	26.3	
South	13086	40.4	4454	45.8	3126	34.8	2102	18.3	
West	5712	17.6	883	9.1	3497	38.9	3950	34.5	
Discharge year									0.917
2010	8180	25.2	2194	22.6	2223	24.7	3162	27.6	
2011	8118	25.0	2457	25.3	2386	26.5	2980	26.0	
2012	7910	24.4	2490	25.6	2170	24.1	2660	23.2	
2013	8220	25.4	2585	26.6	2210	24.6	2660	23.2	
Hepatitis C	5056	15.6	2497	25.7	1518	16.9	1737	15.2	< 0.001
Hepatitis B	610	1.9	598	6.2	236	2.6	1975	17.2	< 0.001
Alcohol	5566	17.2	1278	13.2	2023	22.5	1157	10.1	< 0.001
NASH	10802	33.3	3436	35.3	3601	40.1	4140	36.1	< 0.001
Primary sclerosing cholangitis	326	1.0	81	0.8	35	0.4	140	1.2	0.046
Primary biliary cirrhosis	131	0.4	0	0.0	10	0.1	34	0.3	-
Autoimmune	91	0.3	25	0.3	60	0.7	⩽ 10	0.1	0.007
Other	14336	44.2	3818	39.3	2967	33.0	4566	39.9	< 0.001
Liver decompensation features									< .0010
Zero	18388	56.7	5690	58.5	4475	49.8	6827	59.6	
One	8968	27.7	2756	28.3	2846	31.7	3092	27.0	
Two	4074	12.6	1010	10.4	1340	14.9	1242	10.8	
Three or greater	998	3.1	270	2.8	328	3.6	301	2.6	
Metastasis									0.007
None	27328	84.3	7841	80.6	7497	83.4	9567	83.5	
Single site	3945	12.2	1453	14.9	1092	12.2	1516	13.2	
Two or more site	1155	3.6	433	4.5	399	4.4	379	3.3	
Elixhauser comorbidity score									< 0.001
< 3	15593	48.1	4177	43.0	4255	47.3	6642	57.9	
⩾ 3	16835	51.9	5549	57.1	4733	52.7	4821	42.1	
Treatment options									< 0.001
Transplant	1254	3.9	190	1.9	252	2.8	349	3.1	
Resection	4306	13.3	823	8.5	728	8.1	1644	14.3	
Ablation	2031	6.3	425	4.4	517	5.8	833	7.3	
TACE	2419	7.5	754	7.8	836	9.3	939	8.2	
Noninvasive treatment	22418	69.1	7534	77.5	6656	74.1	7697	67.2	
In hospital mortality	2924	9.0	1120	11.5	928	10.3	1151	10.1	0.017

TACE: Transarterial chemoablation; NASH: Non-alcoholic steatohepatitis.

to access a Hepatologist and a hospital that is better equipped to meet their needs[16].

Racial disparities in the utilization of TACE was not noted in this study, though previous studies have determined a discrepancy in Native-American patients and Hispanic patients compared to Caucasian patients[17]. This intervention may be considered more frequently in African-American patients because of increased frequency of the disease burden outside of Milan criteria. TACE is considered to be a first line treatment for large or multifocal HCC[18] and may be the ideal treatment for many African-American patients. However, it should be noted that TACE is not considered curative therapy for HCC. The question of whether it is being offered as the sole treatment option for patients who would otherwise be candidates for curative therapy should be raised.

The cost of intervention also influences treatment options. This study shows that African-American patients are the largest percentage of patients with Medicaid insurance compared to other races. Previous analysis on

Table 2 Multivariate logistic regressions comparing outcomes of hepatocellular carcinoma by race

Outcome	Race	Adjusted odds ratio	95%CI
Metastatic Hepatocellular Carcinoma[1]	African-American	Reference	
	Caucasian	0.82	0.71-0.94
	Hispanic	0.87	0.73-1.05
	Other/unknown	0.84	0.70-1.001
Liver Decompensation[2]	African-American	Reference	
	Caucasian	1.16	1.03-1.30
	Hispanic	1.28	1.10-1.51
	Other/unknown	1.14	0.995-1.31
Inpatient Mortality[3]	African-American	Reference	
	Caucasian	0.78	0.65-0.93
	Hispanic	0.82	0.66-1.03
	Other/unknown	0.86	0.69-1.07

[1]Model is adjusted for age, sex, race, geographic region, hepatitis C, alcohol, non-alcoholic steatohepatitis (NASH), liver decompensation features, and Elixhauser comorbidity score; [2]Model is adjusted for age, sex, race, geographic region, hepatitis C, alcohol, NASH, primary biliary cirrhosis, metastasis, and Elixhauser comorbidity score; [3]Model is adjusted for age, sex, race, geographic region hepatitis C, hepatitis B, alcohol, NASH, liver decompensation features, metastasis, and treatment.

Table 3 Multinomial logistic regression to evaluate disparities in treatment for hepatocellular carcinoma based on payer[1, 2]

Intervention	Race	Odds ratio	Confidence interval
Liver Transplant	African-American	Reference	
	Caucasian	2.66	1.92-3.68
	Hispanic	2.18	1.40-3.39
	Other/unknown	2.41	1.62-3.61
Resection	African-American	Reference	
	Caucasian	1.82	1.48-2.23
	Hispanic	1.24	0.94-1.64
	Other/unknown	1.79	1.39-2.32
Ablation	African-American	Reference	
	Caucasian	1.77	1.36-2.30
	Hispanic	1.46	1.05-2.03
	Other/unknown	2.03	1.47-2.80
TACE	African-American	Reference	
	Caucasian	1.15	0.93-1.41
	Hispanic	1.29	0.97-1.72
	Other/unknown	1.19	0.90-1.58

[1]Noninvasive treatment is treated as the reference category; [2]Model adjusts for age, gender, race, geographic region, hepatitis C, hepatitis B, alcohol, non-alcoholic steatohepatitis, liver decompensation features, and Elixhauser comorbidity score. TACE: Transarterial chemoablation.

cost of intervention for HCC has shown TACE to be one of the least costly interventions[19], and therefore may be the intervention most likely to be reimbursed from government funded insurance.

While it is important to recognize racial disparities in the treatment of patients with HCC, it is crucial to recognize the effect this has on an underrepresented patient's quality of life and life expectancy. Studies regarding quality of life in patients with chronic liver disease show decreased functional status and increased chronic, debilitating symptoms such as pain, edema, weakness, anorexia, and vomiting compared to patients without any liver disease[20]. These symptoms, specifically bodily pain and fatigue, are worse in patients with liver disease and HCC[21]. Patients with HCC are noted to have higher rates of depression compared to many other malignancies[22], therefore African-American patients with HCC that fail to undergo treatment are subject to increased complications and diminished quality of life compared to patients that undergo treatment. Life expectancy is also different between patients that undergo treatment for HCC compared to patients that are not treated[23]. If a patient were to undergo treatment, life expectancy and quality of life are improved[24,25].

Multiple interventions could be utilized to reduce disparities in the treatment of HCC. For example, early recognition of liver disease and risk factors for HCC are key to initiate and continue HCC screening in all patients, but specifically in minority patients that may have reduced access to care. This could potentially lead to earlier diagnosis in patients, and therefore the patient would be a candidate for curative treatment.

Limitations in this study must be noted. This data was obtained through the NIS database through ICD-9 coding. Verification of ICD-9 codes could not be obtained for each patient included in the study given patient privacy restrictions. However, these codes have been verified in previous studies. Other factors, specifically

size and number of lesions that affect the treatment for HCC were not included in this study. We were not able to obtain laboratory values and were unable to determine MELD score. Therefore, disease severity was defined by features of liver decompensation. Despite limitations, this study has several strengths. The primary strength was the number of patients that were enrolled in the study over a wide geographic area. Using the NIS database allowed for the collection of a large number of patients that otherwise would not have been obtained in a single institution study.

Disparities in the treatment of HCC based on patient race continue to exist despite emphasis to decrease disparities in healthcare. Despite having decreased rates of liver decompensation, African-American patient have higher rates of inpatient mortality and are less likely to undergo curative treatments, such as liver transplantation, surgical resection, or ablation. Because these patients are less likely to undergo these interventions, African-American patients with HCC are prone to a decreased quality of life and increased mortality rates. Further research needs to be conducted to find ways to decrease this disparity.

ARTICLE HIGHLIGHTS

Research background

Rates of hepatocellular carcinoma (HCC) continue to increase. Despite new treatment options, mortality rates are also increasing specifically in minority patients.

Research motivation

Given recent emphasis to minimize health care disparities, we aimed to determine if racial disparities in the treatment of HCC were decreasing.

Research methods

We performed a retrospective database analysis utilizing The Nationwide Inpatient Sample including patients with a diagnosis of HCC. Univariate and multivariate analyses were utilized to determine racial disparities in liver decompensation, treatment, inpatient mortality, and metastatic disease.

Research results

This large database analysis included 62604 patients with HCC, including 32428 Caucasian, 9726 African-American, 8988 Hispanic, and 11462 patients of other races. Despite having decreased rates of liver decompensation, African-American patient have higher rates of inpatient mortality and are less likely to undergo curative treatments, such as liver transplantation, surgical resection, or ablation than Caucasian patients.

Research conclusions

Racial disparities in HCC treatment exist despite emphasis to support equality in healthcare. African-American patients are less likely to undergo curative treatments for HCC.

Research perspectives

Further emphasis should be placed on determining why disparities continue to exist and hypothesize ways to reduce them in order to facilitate equality in healthcare.

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ORIGINAL ARTICLE

Observational Study

Factors associated with DAA virological treatment failure and resistance-associated substitutions description in HIV/HCV coinfected patients

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Abstract

AIM

To describe factors associated with treatment failure and frequency of resistance-associated substitutions (RAS).

METHODS

Human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfected patients starting a first direct-acting antiviral (DAA) regimen before February 2016 and included in the French ANRS CO13 HEPAVIH cohort were eligible. Failure was defined as: (1) non-response [HCV-RNA remained detectable during treatment, at end of treatment (EOT)]; and (2) relapse (HCV-RNA suppressed at EOT but detectable thereafter). Sequencing analysis was performed to describe prevalence of drug class-specific RAS. Factors associated with failure were determined using logistic regression models.

RESULTS

Among 559 patients, 77% had suppressed plasma HIV-RNA < 50 copies/mL at DAA treatment initiation, 41% were cirrhotic, and 68% were HCV treatment-experienced. Virological treatment failures occurred in 22 patients and were mainly relapses (17, 77%) then undefined failures (3, 14%) and non-responses (2, 9%). Mean treatment duration was 16 wk overall. Post-treatment NS3, NS5A or NS5B RAS were detected in 10/14 patients with samples available for sequencing analysis. After adjustment for age, sex, ribavirin use, HCV genotype and treatment duration, low platelet count was the only factor significantly associated with a higher risk of failure (OR: 6.5; 95%CI: 1.8-22.6).

CONCLUSION

Only 3.9% HIV-HCV coinfected patients failed DAA regimens and RAS were found in 70% of those failing. Low platelet count was independently associated with virological failure.

Key words: Human immunodeficiency virus; Hepatitis C virus; Direct-acting antiviral; Treatment virological failure; Resistant associated mutations

Core tip: In co-infected human immunodeficiency virus-hepatitis C virus (HCV) patients, after adjustment for age, sex, ribavirin use, HCV genotype and treatment duration, low platelets count was the only factor significantly associated with a higher risk of failure.

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INTRODUCTION

The treatment of hepatitis C virus (HCV) infection had been revolutionized with the recent development of direct-acting antiviral (DAA) combinations. Cure rates of over 90%, similar to those in HCV monoinfected patients, can now be achieved in human immunodeficiency virus (HIV)/HCV coinfected patients. This has been documented in clinical trials[1-5] as well as in real-life cohorts[6-9]. For the few patients failing treatment, resistance-associated substitutions (RAS) can emerge and emerging resistant strains appearing at viral rebound are a consequence rather than a cause of failure[10,11].

The real causes of failure to all-oral DAA regimens can be multiple. Several social and medical factors can jeopardize treatment adherence. Some first-generation regimens may not be optimal to treat difficult cases of hepatitis C, such as decompensated cirrhosis or genotype 3 HCV infection. In rare circumstances, especially for genotype 1a viruses, baseline mutations in the non-structural-5A (*NS5A*) gene can preexist in the viral species before treatment introduction and may have a potentially deleterious impact on sustained virological response (SVR)[12]. Drug-drug interactions between DAA and ARV therapy or other commonly prescribed medications in HIV/HCV coinfected patients are frequent and can decrease drug levels, thereby reducing the efficacy of therapy. Finally, adverse events, although rare with new DAA combinations, can occur and lead to treatment interruption and thus to treatment failure.

We aimed to describe the characteristics of patients failing first-line DAA treatment in the real-life French nationwide ANRS CO13 HEPAVIH cohort of HIV/HCV coinfected patients. Furthermore, we described the emergence of clinically relevant RAS to DAA classes upon DAA treatment failure, and report pharmacological drug monitoring results. Finally, we identified factors associated with the occurrence of virological treatment failure.

MATERIALS AND METHODS

Study population

The ANRS CO13 HEPAVIH cohort (ClinicalTrials.gov Identifier: NCT03324633) is a national multi-centre prospective hospital-based observational study of patients coinfected with HIV and viral hepatitis C that received approval by an Institutional Review board [Comité de Protection des Personnes (CPP) Ile de France Ⅲ, Paris, France].

All patients included in the cohort gave their consent

for study participation. In addition, patients from the 29 centers participating in the ANRS CO13 HEPAVIH cohort, who weren't included in the cohort but who gave their consent for specific follow-up during and after DAA treatment, were also eligible. For this sub-study, patients were included if they had started an all-oral DAA-based regimen before January 2016 (3 mo treatment), February 2016 (2 mo treatment) or October 2015 (6 mo treatment). Patients who participated in completed and published clinical trials were included in the analysis. We did not include patients who were participating in an ongoing clinical trial (including those completed but not yet published), patients who were treated with combinations including Peg-interferon (PegIFN), or with the sofosbuvir (SOF) + ribavirin (RBV) combination. Patients with premature treatment interruption for intolerance or death were also excluded because we were specifically interested in a virological outcome. The DAA regimen was at the discretion of the patient's physician[13-15].

Data collection and definitions

The following data were collected prospectively by each participating center, using an eCRF: Age, sex, risk factors for both HIV and HCV infections, HCV genotype, previous anti-HCV treatment, HIV-related characteristics, start and end dates of DAA treatment, initial doses of anti-HCV and anti-HIV drugs, any changes during follow-up, and HCV-RNA at each time point [baseline, week (W)2, W4, W8, W12 if treatment duration was 24 wk, EOT, follow-up W4 (FU-W4) and FU-W12]. Virological treatment failures were categorized as: (1) Non-response: HCV-RNA never undetectable during treatment; (2) Relapse: HCV-RNA undetectable at EOT and then detectable within the following 12 wk; and (3) Undefined failure: HCV-RNA unknown at end of treatment (EOT) and positive thereafter, without premature discontinuation of treatment. Cirrhotic status was based on liver biopsy (METAVIR fibrosis stage F4), liver stiffness $\geqslant$ 12.5 kPa (FibroScan®; Echosens, France), a FibroTest® value $\geqslant$ 0.75 (Biopredictive, France) or physical and biological signs of end-stage liver disease, as previously published[16,17].

Sequencing analysis

Patients with virological treatment failure, who provided specific consent for HCV genotype testing and who had HCV-RNA > 1000 IU/mL at the sequencing time point were included for HCV testing. Prevalence of drug class-specific RAS was evaluated at failure. The HCV NS3, NS5A and/or NS5B domains were amplified by reverse transcriptase nested polymerase chain reaction (PCR) using genotype and subtype-specific PCR primers to ensure successful amplification of the target gene(s). PCR products were purified and analyzed by population sequencing using an automated sequencer (ABI-3500xL Dx). The cutoff frequency for detecting variants with Sanger sequencing was approximately 15%. Sanger-derived sequences were aligned with Clustal_W, version 1.74 (Conway Institute UCD, Dublin, Ireland). NS3, NS5A and NS5B RAS were defined as clinically relevant when inducing > 10-fold resistance to DAA[13,18-20].

Drug concentrations

Plasma drug concentrations for DAA and RBV were collected, when available, for patients included in the cohort as part of routine therapeutic drug monitoring performed in several centers. Drug concentrations were measured using liquid chromatography coupled with the tandem mass spectrometry method[21]. Data were considered interpretable if concentrations were determined at steady-state and information regarding the time of the last drug intake was available.

A suboptimal concentration was defined as below the 2 μg/mL threshold for RBV[22,23], and when concentrations were below the reported expected range for DAA[24-27].

Statistical analysis

We included all patients who met the inclusion criteria, as described in the study population section. Variables are described as number and percentages, or median and IQR [or mean (SD)], as appropriate. Patient characteristics are reported upon initiation of DAA treatment. The Wilcoxon-Mann-Whitney test and Fisher's exact test were used to compare quantitative and qualitative variables between groups, respectively. Factors associated with virological treatment failure were determined using logistic regression models. In order to identify new independent predictors of virological treatment failure, we systematically adjusted for a fixed set of potential confounders based on literature reports. The following variables were thus forced in all models: age, sex, RBV use, and prescribed treatment duration[28]. We then tested the following variables in the model containing the forced variables: HCV genotype (3 *vs* others), cirrhosis (Yes *vs* No), severe cirrhosis (Yes *vs* No, and defined by a B or C or an elastometry value $\geqslant$ 20 kPa), plasma HIV-RNA (detectable *vs* undetectable), and platelet count (< 100 Giga/L *vs* $\geqslant$ 100 Giga/L). The effect of RBV on virological treatment failure and other potential factors was assessed by a marginal structural model (MSM) in order to consider a potential indication bias for the prescription of RBV. Sensitivity analyses, including patients with premature treatment discontinuations for intolerance/death, were also performed. The statistical methods of this study were reviewed by Linda Wittkop from Bordeaux Population Health Research Center, Bordeaux. SAS software version 9.4 (SAS Institute Inc., Cary, North Carolina) was used for all analyses.

RESULTS

General characteristics at DAA initiation

Among 877 patients treated with DAA-combination, 559 met the inclusion criteria and were included in the analysis (318 were not included for the following reasons: Treatment with PegIFN (n = 30), inclusion

Table 1 Patient characteristics at treatment initiation according to virological response

	Overall (n = 559)	SVR (n = 537)	Virological treatment failure (n = 22)	P value
Male sex	431 (77)	414 (77)	17 (77)	0.985
Age (yr)	52 (49-56)	52 (49-56)	53 (51-57)	0.586
CD4 (/mm^3) (n = 557)	618 (426-850)	619 (429-861)	527 (346-704)	0.040
Undetectable HIV-RNA (n = 558)	486 (87)	469 (88)	17 (77)	0.186
ARV treatment	549 (98)	527 (98)	22 (100)	1.000
PI[1]	127 (23)	122 (23)	5 (23)	
NNRTI[2]	98 (18)	95 (18)	3 (14)	
II[3]	204 (37)	197 (37)	7 (32)	
Others	120 (22)	113 (21)	7 (32)	
Active tobacco consumption (n = 263)	153 (58)	148 (58)	5 (71)	0.703
Active alcohol consumption (n = 266)	135 (51)	132 (51)	3 (43)	0.719
Active drug consumption (n = 257)	7 (3)	7 (3)	0 (0)	1.000
HCV genotype (n = 558)				0.475
1 without precision	26 (5)	24 (5)	2 (9)	
1a	232 (42)	221 (41)	11 (50)	
1b	64 (12)	64 (12)	0 (0)	
2	6 (1)	6 (1)	0 (0)	
3	62 (11)	60 (11)	2 (9)	
4	165 (30)	158 (30)	7 (32)	
5	1 (0)	1 (0)	0 (0)	
6	2 (0)	2 (0)	0 (0)	
Cirrhosis (n = 555)	209 (38)	200 (38)	9 (41)	0.748
Child Pugh, if cirrhosis (n = 189)				0.537
A	172 (91)	165 (91)	7 (88)	
B/C	17 (9)	16 (9)	1 (12)	
FIB-4 (n = 405)	2.1 (1.4-3.7)	2.1 (1.4-3.7)	3.3 (1.9-7.3)	0.313
FIB-4 > 3.25 (n = 405)	120 (30)	113 (29)	7 (50)	0.132
Elastometry (kPa) (n = 115)	9 (6-14)	9 (6-14)	10 (6-17)	0.942
Elastometry ⩾ 12.5 kPa (n = 115)	32 (28)	30 (27)	2 (50)	0.309
Elastometry ⩾ 20 kPa (n = 115)	17 (15)	16 (14)	1 (25)	0.478
HCV treatment history				0.570
Naïve	210 (38)	203 (38)	7 (32)	
Pretreated	349 (62)	334 (62)	15 (68)	
HCV viral load (log_{10} IU/mL) (n = 558)	6.09 (5.59-6.51)	6.09 (5.59-6.51)	6.04 (5.72-6.49)	0.886
Prothrombin rate (n = 298)	99 (89-100)	99 (89-100)	92 (82-100)	0.116
Prothrombin rate < 85% (n = 298)	54 (18)	50 (17)	4 (40)	0.087
Platelets (Giga/L) (n = 408)	171 (131-219)	171 (133-219)	148 (97-184)	0.168
Platelets < 100 Giga/L (n = 408)	57 (14)	51 (13)	6 (43)	0.007
Albumin (g/L) (n = 301)	41 (38-44)	41 (38-44)	42 (37-45)	0.939
Albumin < 35 g/L (n = 301)	26 (9)	24 (8)	2 (25)	0.146
DAA-combination				NA[5]
SOF + DCV ± RBV[4]	240 (43)	231 (43)	9 (41)	
SOF/LDV ± RBV	271 (49)	261 (49)	10 (46)	
SOF + SMV ± RBV	26 (4)	23 (4)	3 (14)	
Others[4]	22 (4)	22 (4)	0 (0)	
Mean (SD) DAA treatment durationa	16 (6)	15 (5)	16 (6)	

Results are presented as number (as percentages in brackets) or median (IQR in brackets) unless stated otherwise. [1]PI was boosted in 98 patients with SVR and in five patients with treatment failure; [2]NNRTI molecule was rilpivirine in 60 patients with SVR and three with failure, and was efavirenz in 25 patients with SVR; [3]II molecule was raltegravir in 153 patients with SVR and four patients with failure, and was dolutegravir in 38 patients with SVR and two with treatment failure; [4]Initial doses of DCV were 30, 60, 90 mg/d in respectively 57, 159 and 21 patients. The dose was unknown for the five other patients; [5]NA: not applicable, no formal statistical comparison was performed as the prescription of the DAA regimen was chosen by each patient's physician. SVR: Sustained virological response; ARV: Antiretroviral; PI: Protease inhibitor; NNRTI: Non-nucleoside reverse-transcriptase inhibitor; II: Integrase inhibitor; DAA: All-oral direct-acting antiviral; SOF: Sofosbuvir; RBV: Ribavirin; DCV: Daclatasvir; LDV: Ledipasvir; SMV: Simeprevir.

in an ongoing clinical trial (n = 2), treatment after the period of analysis (n = 190), no available treatment result (n = 32), treatment with SOF + RBV (n = 60), premature treatment interruption for intolerance (n = 3), and one patient died while on treatment). Mean treatment duration was 16 wk overall (15 wk in patients who failed DAA therapy and 16 wk in those with SVR). The characteristics of the 559 patients are summarized in Table 1.

Virological treatment failure

The virological treatment failure rate was 3.9% (95%CI: 2.5-5.9). Overall, 22 virological treatment failures were observed: Two non-responses, 17 relapses and three undefined virological treatment failures (HCV-RNA unknown at EOT). By univariate analysis (Table

Table 2 Adjusted logistic regression for factors associated with virological treatment failure

Covariables	Model 1		Model 2		Model 3		Model 4	
	OR (95%CI)	*P* value	OR (95% CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
	n = 538		*n* = 538		*n* = 526		*n* = 395	
Age at treatment initiation (per 10 yr)	1.2 (0.6-2.4)	0.58	1.3 (0.7-2.5)	0.48	1.2 (0.6-2.4)	0.53	1.6 (0.7-4.0)	0.29
Ribavirin *vs* no ribavirin	1.0 (0.3-3.0)	0.97	1.1 (0.3-3.2)	0.93	1.0 (0.3-3.0)	0.97	1.4 (0.4-5.5)	0.61
Male sex *vs* female	1.0 (0.4-2.8)	0.98	0.9 (0.3-2.7)	0.92	1.0 (0.3-2.8)	0.97	0.8 (0.2-2.7)	0.69
Treatment duration 24 wk *vs* 12 wk	0.4 (0.1-1.4)	0.15	0.5 (0.2-1.5)	0.21	0.4 (0.1-1.4)	0.16	0.2 (0.0-1.0)	0.05
Platelet count < 100 Giga/L *vs* ⩾ 100							6.5 (1.8-22.6)	0.004
Cirrhosis *vs* no cirrhosis	1.4 (0.5-3.9)	0.51						
HIV-RNA detectable *vs* undetectable			2.1 (0.7-5.9)	0.17				
Severe cirrhosis *vs* no severe cirrhosis					2.1 (0.4-10.3)	0.35		
HCV genotype 3 *vs* others							0.9 (0.1-7.5)	0.91

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

1), patients with virological treatment failure had a significantly lower CD4 cell count (median 527 cells/mm^3) compared to patients with SVR (619 cells/mm^3; P = 0.040). They also more frequently had a platelet count below 100 Giga/L (P = 0.007) and a trend for more frequently having a prothrombin time < 85% (40% *vs* 17%, P = 0.087) and albumin < 35 g/L (25% *vs* 8%, P = 0.146). They also had a non-significant trend for less frequent HIV-RNA suppression (77% *vs* 88%, P = 0.186).

Factors associated with treatment failure

In adjusted models (Table 2), platelet count < 100 Giga/L was significantly associated with a higher probability of virological treatment failure (Model 4). However, clinical cirrhosis status (Model 1), severe cirrhosis status (Model 3) or blood albumin (data not shown) were not associated with a higher probability of failure. Neither HIV-RNA (Model 2) nor CD4 cell count (data not shown) were associated with virological treatment failure. In addition, in the model containing platelet count, a prescribed treatment duration of 24 wk was associated with a lower risk of virological treatment failure (Model 4). RBV use was not associated with outcome in adjusted logistic regression models, and this result was confirmed by an analysis using MSMs (data not shown).

Sensitivity analyses, including patients with premature treatment discontinuations for intolerance/death, showed similar results (data not shown).

HCV resistance at virological treatment failure

The results of RAS analysis in the 14 patients with virological treatment failure, in whom either mutation NS3, NS5A or NS5B could be sequenced, are presented in Table 3. Almost three quarters of patients with available data (10/14; 71%) had at least one detectable RAS at the time of virological treatment failure. In patients receiving an NS5A inhibitor-based regimen, 55% (6/11) had NS5A RAS upon virological treatment failure. Common substitutions detected at failure included Q30R/H, 30E, 58D and/or Y93C/N, all found in patients with HCV genotype 1. In patients treated with daclatasvir (DCV) and an available NS5A RAS result (n = 5), four patients developed resistance to DCV. Furthermore, among six treated with ledipasvir (LDV) with available NS5A RAS result, two developed resistance to LDV. Overall, in all patients with available genotype (n = 14), six (43%) presented at least one NS5A RAS, leading to a high level of resistance to NS5A inhibitors (> 10-fold resistance). In patients receiving NS3 protease inhibitors, 2/2 patients with available data had NS3 RAS upon virological treatment failure. The substitutions detected at failure were 80K, 170T, 174N and 168V, leading to a high level of resistance to most protease inhibitors.

Multiple RAS conferring a higher level of resistance were detected in three (21%) patients, including two with NS3 + NS5A RAS and one with NS3 + NS5A + NS5B RAS. These three patients were previously treated with PegIFN + RBV and were exposed to NS5A inhibitors but not NS3 inhibitors.

Pharmacological data

Nine of the 22 (41%) patients who had DAA therapy failure had measurements of DAA and/or RBV concentration at W2 or W4 of treatment, seven of which were interpretable. Among these seven patients, suboptimal concentrations were reported in two (29%). These low concentrations concerned either DCV (in a patient treated with SOF + DCV, whose ARV treatment was rilpivirine + raltegravir), or RBV (in a patient treated with SOF/LDV + RBV who was taking rilpivirine + dolutegravir).

DISCUSSION

In this cohort of HIV/HCV coinfected patients, who were treated with an interferon-free DAA regimen with or without RBV, we report a low virological treatment failure rate of 3.9%. Our results are similar to those observed in clinical trials[28] or previous real-world studies of HIV/HCV coinfection[6,7]. Most of these virological treatment failures were due to relapse (77%) followed by non-response (9%), while 14% were due to undefined virological treatment failures (HCV-RNA unknown at EOT).

Due to very high rates of SVR, it has been difficult to identify factors associated with virological treatment failure of DAA in real-world studies, and no study to date has focused on HIV coinfection. In studies of HCV

Table 3 Resistance-associated substitution results in 14 patients with virological treatment failure for whom sequencing was performed in routine care

Pat	HCV treatment history	Treatment received	HCV genotype		Cirrhosis	ARV treatment	RAS		
			Before treatment	After treatment			NS3	NS5A	NS5B
A	Pretreated	SOF + SMV 12 wk	1a	1a	Yes	II	Q80K, I170T, S174N	Abs	Abs
B	Pretreated	SOF + SMV 12 wk	1a	1a	Yes	II	D168V	Abs	Abs
C	Pretreated	SOF/LDV 12 wk	4	4a	No	PI	Abs	Abs	Abs
G	Pretreated	SOF/LDV + RBV 12 wk	4	4d	No	Others	Abs	Abs	Abs
H	Pretreated	SOF + DCV 10 wk[3]	4	4	No	Others	Abs	ND	Abs
I	Naive	SOF/LDV 12 wk	1a	1a	No	PI	Abs	Abs	Abs
J	Pretreated	SOF/LDV 12 wk	1a	1a	No	Others	ND	Y93C	Abs
L	Naive	SOF + DCV 13 wk[2]	1a	1a	Yes	NNRTI	Q80K	Abs	Abs
M	Pretreated	SOF/LDV 12 wk	4	4a	No	PI	ND	Abs	A421V, M414L
N	Pretreated	SOF/LDV + RBV 12 wk	1a	1a	Yes	II	A168V	30E, 58D	Abs
P	Pretreated	SOF + DCV + RBV 12 wk[1]	1a	1a	No	PI	Abs	Y93N	Abs
Q	Pretreated	SOF + DCV + RBV 24 wk[1]	1a	1a	No	Others	T54S	Q30R	Abs
R	Pretreated	SOF + DCV 24 wk[2]	1a	1a	Yes	II	Q80K	Y93C	Y448H
W	Pretreated	SOF + DCV 24 wk[1]	1	1a	Yes	II	Abs	Q30H	Abs

[1]Initial dose of DCV: 30 mg/d; [2]Initial dose of DCV: 60 mg/d; [3]Initial dose of DCV: 90 mg/d. Pat: Patient; ARV: Antiretroviral; RAS: Resistance-associated substitution; NS3: Non-structural-3; NS5A: Non-structural-5A; NS5B: Non-structural-5B; SOF: Sofosbuvir; RBV: Ribavirin; DCV: Daclatasvir; LDV: Ledipasvir; SMV: Simeprevir; ND: Not done; PI: Protease inhibitor; NNRTI: Non-nucleoside reverse-transcriptase inhibitor; II: Integrase inhibitor; Abs: No RAS found.

monoinfected patients, however, several factors have been found to be associated with virological treatment failure: severity of cirrhosis (assessed by presence of ascites), low albumin, low platelet count/high total bilirubin[29-35], male sex[30,31], and the preexistence of baseline RAS[34,36].

In our study, we found that low platelet count was significantly associated with a higher rate of virological treatment failure. It is likely that low platelet count is a surrogate marker of cirrhosis, since we found an association between low albumin levels and low PT time by univariate analysis. However, we failed to observe a significant relationship between severe cirrhosis and failure. This might be due to the fact that in cases of severe cirrhosis, physicians adapted the treatment to each complex situation by extending the duration or by adding RBV (76% of the patients with Child Pugh B or C cirrhosis received treatment of 24 wk duration *vs* 29% of the other patients in our study), and this might be explained by unreported events of decompensation.

In the first randomized phase 3 clinical trials, which assessed the efficacy and safety of DAA, decompensated cirrhosis was an exclusion criterion, which precluded the possibility of assessing this factor as a potential predictor of failure. More recently, several trials have clearly demonstrated that patients with Child Pugh B or C cirrhosis and those with genotype 3 infection have a lower rate of SVR with DAA alone and need the addition of RBV. This was the case for the SOF/LDV combination and for a combination[37,38] of velpatasvir/SOF[39].

We observed a trend (by univariate analysis only) toward a higher rate of detectable HIV-RNA in patients with virological treatment failure *vs* in those with SVR (P = 0.19). This might reflect suboptimal adherence, with patients who are non-compliant for their HIV treatment while possibly also non-adherent to their HCV treatment. Nonetheless, this result did not remain significant by multivariable analysis and thus may also simply reflect a biased estimate.

Moreover, among seven patients with failure and interpretable pharmacological data, suboptimal blood concentrations of DAA were measured in two of them. These results could reflect different situations (drug interactions, suboptimal dosing errors, suboptimal adherence) and warrant both further investigation and wider-scale assessment of pharmacological data. Regarding RAS in our study, we did not determine pretreatment RAS and we cannot exclude the possibility that some failures may be due to pre-existing RAS. However, at a population level, the effects of baseline RAS in *NS5A*, although not rare, are minimal[10,36,40,41]. This prompted EASL experts[18,20] to recommend that genotyping should not be performed for naïve patients but instead considered when retreatment is anticipated with a *NS5A* inhibitor regimen in patients who have previously failed NS5A treatment.

In most of our patients who failed DAA-treatment, RAS was investigated. We found RAS in 50% of those failing NS5A-based therapy and in the two patients failing NS3, but no major RAS S282T to NS5B. This high prevalence of *NS5A* and -3 RAS failure in our study confirms the EASL recommendation to evaluate HCV resistance to NS5A inhibitors (spanning amino acids 24 to 93) if resistance testing is available, as these analyses can guide decisions for further treatment[18,20].

There are several limitations to this study. Firstly, since the study was an observational cohort, our results must be interpreted with caution, since treatment

prescriptions were dependent on drug availability (with variations over time) and known efficacy with regards to HCV genotypes. Those results were obtained with second generation DAA (LDV, DCV, elbasvir/grazoprevir), and those results may not be entirely applicable to the newer, pangenotypic regimens such as velpatasvir/SOF or pibentasvir/glecaprevir. Our analysis is limited by the small number of subjects with virological treatment failure, and thus likely has limited power to identify all potential risk factors. All patients with virological treatment failure could not be explored by genotyping to investigate the emergence of RAS due to the need to obtain patient consent. Furthermore, baseline genotyping was not available routinely, since this test is not recommended in France for treatment-naïve patients. Finally, Sanger sequencing was used for the detection of RAS, which may not be sensitive enough to detect minor populations of RAS (< 15%). The strengths of our study include prospective data collection with regular monitoring and high quality data.

In conclusion, our study identified that low platelet count is associated with a higher probability of DAA failure. This parameter likely reflects hepatic insufficiency, and our results are concordant with previously published findings on HCV monoinfected patients. We also speculate that some degree of low adherence could explain some cases of failure, since suboptimal drugs levels were observed in 29% of the cases that could be explored, and HIV viral load was often detectable in patients with virological treatment failure to DAA. This study confirms the very low rate of treatment failure with all-oral DAA in HIV/HCV coinfected patients, as well as the high risk of the emergence of non-structural NS3 or NS5A RAS in patients with virological DAA failure.

ARTICLE HIGHLIGHTS

Research background

In human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfection, all-oral direct-acting antiviral (DAA) regimens achieve virological cures in > 95% of patients.

Research motivation

Risk factors for failure are mainly related to severity of cirrhosis in HCV monoinfected patients, but are unknown in the population of HIV HCV coinfected patients. We wanted to know whether additional factors related to non-adherence or HIV status could be involved in the occurrence of failures. We believed that identifying the risk factors for failure would allow for the adaptation of treatment to patients with higher risk of failure.

Research objectives

The main objectives were to determine the risk factors for virological treatment failure to DAA in HIV/HCV coinfected patients and to describe the frequency of RAS.

Research methods

HIV/HCV coinfected patients who started the first DAA regimen before February 2016 and who were included in the French ANRS CO13 HEPAVIH cohort were eligible. Failure was defined as: i) Non-response (HCV-RNA remained detectable during treatment, at end of treatment (EOT)), ii) relapse (HCV-RNA suppressed at EOT but detectable thereafter). Sequencing analysis was performed to describe prevalence of drug class specific RAS. Factors associated with failure were determined using logistic regression models.

Research results

Research findings: Among 559 patients, 77% had suppressed plasma HIV-RNA < 50 copies/mL at DAA treatment initiation, 41% were cirrhotic, and 68% were HCV treatment-experienced. Virological treatment failures occurred in 22 patients and were mainly relapses (17, 77%) then undefined failure (3, 14%) and non-responses (2, 9%). Mean treatment duration was 16 wk overall. Post-treatment NS3, NS5A or NS5B RAS were detected in 10/14 patients with samples available for sequencing analysis. After adjustment for age, sex, RBV use, HCV genotype and treatment duration, low platelet count was the only factor significantly associated with a higher risk of failure (OR: 6.5; 95%CI: 1.8-22.6); Contributions to the field: In HIV/HCV coinfected patients, the risk factors of failure were more related to the severity of cirrhosis than to HIV immunovirological status or non-adherence issues. Problems that remain to be solved: It remains to be determined whether the low platelet count associated with a higher probability of failure reflects the severity of cirrhosis.

Research conclusions

In our study of HIV/HCV patients receiving all-oral DAA, only 3.9% HIV-HCV coinfected patients failed DAA regimens. RAS were found in 70% of those failing. Low platelet count was independently associated with virological failure. We think that this low platelet count reflects the severity of cirrhosis.

Research perspectives

As the treatment failure number is low, it would be useful to build international collaborations and gather data for several cohorts in order to gain significance power. The results obtained with first generation all-oral DAA could be compared with the newer, pangenotypic drug regimen.

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ORIGINAL ARTICLE

Observational Study

Cross-sectional study to determine viral hepatitis knowledge in different urban populations in Brazil

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Abstract

AIM

To evaluate viral hepatitis knowledge among individuals from different resource areas and health conditions to identify possible gaps.

METHODS

A cross-sectional, descriptive study was carried out among 447 individuals from five distinct populations in Brazil: Southeast Viral Hepatitis Ambulatory (n = 100), South (n = 89) and Northeast (n = 114) Health Center, Southeast (n = 77) and Northeast (n = 67) low resource areas. All individuals answered a questionnaire assessing sociodemographic characteristics and viral hepatitis awareness. The perception was scored based on the average number of correct answers of all participants and categorized as "low" (0-28 correct answers) or "desirable" (29-46 correct answers). Associations between sociodemographic characteristics and perception were also evaluated.

RESULTS

A low level of knowledge was observed in individuals from Northeast Health Center, Northeast and Southeast low resource areas while desirable knowledge was observed in individuals from Viral Hepatitis Ambulatory and South Health Center. According to sociodemographic characteristics, desirable scores were more common among those with secondary education (47.1%), those who declared themselves as white (46.3%), and those who lived in houses with three individuals (25.5%). Multivariate analysis showed an association between viral hepatitis perception and type of population.

CONCLUSION

The results demonstrated high level of knowledge among study participants from health clinics from the Southeast region of Brazil and the importance of education programs in increasing the level of knowledge in low resource areas.

Key words: Viral hepatitis; Knowledge; Perception; Urban population; Brazil

Core tip: This study evaluated viral hepatitis knowledge among individuals from five different resource areas and health conditions in Brazil. Participants responded to a questionnaire and the perception was scored as "low" or "desirable". Individuals from Northeast Health Center and Northeast and Southeast low resource areas exhibited low perception, while Southeast and South Health Center exhibited a desirable perception. A positive association was observed between perception and education level, race, number of individuals living in the same house and population type. The results showed the importance of prevention campaigns, especially among individuals living in low resource areas.

Cruz HM, Barbosa JR, Baima Colares JK, de Moraes Neto AH, Alencar MF, Bastos FI, da Mota JC, Carvalho-Costa FA, Ivantes CA, Lewis-Ximenez LL, Villar LM. Cross-sectional study to determine viral hepatitis knowledge in different urban populations in Brazil. *World J Hepatol* 2018; 10(11): 867-876 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i11/867.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i11.867

INTRODUCTION

Hepatitis is the name given to liver inflammation resulting from autoimmune disease, excessive consumption of alcohol or drugs, bacteria and viruses. Viral hepatitis is a group of viruses (hepatitis A, B, C, D, E, G known as HAV, HBV, HCV, HDV, HEV, HGV) that are etiologically and epidemiologically distinct[1-3].

Ingestion of contaminated food or water transmits HAV and HEV; in this fashion, washing food and hands and treating water are methods of prevention. On the other hand, HBV, HCV and HDV can be transmitted by contact with infected bodily fluids (transfusion of blood or blood products, or invasive medical procedures), unsafe sexual practices, or from transmission from mother to child. Prevention of HBV, HCV and HDV is made by blood and organ donor selection, using disposable or sterilized materials and the use of condoms in sexual intercourse[1,4-6].

There are vaccines to prevent HAV and HBV that are safe and effective; one vaccine for HEV is commercialized only in China, but there are no Federal Drug Administration-approved vaccines for HCV and HDV[7]. The clinical course of hepatitis viruses can be acute and chronic for HBV, HCV, HDV and HEV. The clinical manifestations of hepatitis can be absent or appear when the disease is advanced, with cirrhosis or liver cancer[1,2]. Viral hepatitis laboratory diagnosis is performed through the detection of specific antigens, antibodies and viral genome, mainly by enzyme immunoassays and molecular assays such as the polymerase chain reaction[8].

HBV and HCV occur chronically in 257 and 71 million people respectively, causing more than 1.2 million deaths annually[2]. Approximately 15 million people are infected with HDV[2]. Annually, there are an estimated 126 million new cases of HAV and 3.3 million new cases of HEV[2,9,10]. In 2016, 61297 deaths were related to viral hepatitis in Brazil. HEV prevalence in Brazil varies from 2% to 29%[11-15].

The evaluation of knowledge is assessed to verify how far community knowledge corresponds to biomedical concepts[5]. Some factors, such as education, health literacy, family income, age, and access to information, could be associated with gaps in knowledge[16,17].

Around the world studies have been conducted in order to evaluate viral hepatitis perception among health professionals and students, viral hepatitis patients or other risk groups[17-21]. There are still few reports regarding viral hepatitis knowledge in low resource areas[5,22-24]. In view of these gaps, the aim of the present study is to evaluate the viral hepatitis knowledge among individuals from different resource areas and health conditions in Brazil to identify possible gaps and help authorities in the development of prevention and education programs.

MATERIALS AND METHODS

Population studied

This was a cross-sectional study conducted from March 2015 to November 2015, wherein a minimum sample size of 50 participants per group was defined. A nonprobability sampling method with consecutive sampling was used in which every subject meeting the criteria of inclusion was selected until the required sample size was achieved in this setting.

Individuals were previously informed about the study and participant eligibility criteria were: Both genders, more than 18 years of age, free from psychoactive drug use, agreement to inclusion, and signed, informed consent. The local ethical committee approved the study (CAEE 38846914.5.0000.5248).

The final sample was made up of a total of 447 questionnaires about hepatitis knowledge obtained from five groups belonging to different geographic regions in Brazil, as follows: (1) Southeast Viral Hepatitis Ambulatory, comprising 100 individuals living in the Rio de Janeiro state, both in nearby cities and in different districts of the city of Rio de Janeiro, who were referred to the outpatient clinic. These individuals included not only those with acute, chronic or suspected cases of viral hepatitis but also those accompanying patients to the Brazilian Referral center for viral hepatitis diagnosis. The recruitment was performed prior to medical consultation. The Rio de Janeiro state is situated in the Southeast region of Brazil, with a human development Index (HDI) of 0.761[25]; (2) South Health Center, comprising 89 individuals residing in the city of Curitiba (Paraná State) that were recruited in the Guidance and Monitoring Center prior to medical consultation. This center performs anonymous testing for hepatitis, syphilis and human immunodeficiency virus. Curitiba is situated in the South region of Brazil with an estimated population of 1908359, an HDI of 0.823, and poverty rate of 31.71%[25]; (3) Northeast Health Center, comprising 114 individuals resident in the city of Fortaleza (Ceará State) and who were users of the Brazilian Unified Health System seeking care in Medical Care Center integrated to the University of Fortaleza. Recruitment was carried out prior to medical consultation. Fortaleza is situated in the Northeast region of Brazil, with an estimated population of 2627482, an HDI of 0.754, and a poverty rate of 43.17%[25]; (4) Southeast low resource areas, comprising 77 individuals living in low resource communities from the Southeast region of Brazil (Complex of Manguinhos district of Rio de Janeiro city). Interviewers visited residents in their homes and only applied the questionnaires to those who agreed to participate. Rio de Janeiro city is situated in the Southeast region of Brazil, with an estimated population of 6520266, an HDI of 0.799, and a poverty rate of 23.85%. Manguinhos complex exhibited the fifth worst HDI (0.726) among the 126 neighborhood groups in the city of Rio de Janeiro, and the average family income in population was below a minimum Brazilian income[25]; and (5) Northeast low resource areas, comprising 67 individuals resident in a low-resource community from the Northeast Region of Brazil (Nossa Senhora de Nazaré city, Piauí State). This city had approximately 5000 inhabitants and residents had a low income. Interviewers visited residents in their homes and only applied the questionnaires to those who agreed to participate. Nossa Senhora de Nazaré is situated in the Northeast region of Brazil, with an estimated population of 4786, an HDI of 0.586, and a poverty rate of 56.6%[25].

To assess knowledge scores, five populations were further aggregated into three groups, which were categorized as Southeast Viral Hepatitis Ambulatory (n = 100), medical centers (n =203, South and Northeast) and under-privileged communities (n = 144, Southeast and Northeast low resource areas).

Data collection instrument

The questionnaire was composed of two parts: (1) Social-demographic characteristics; and (2) viral hepatitis perception. Social-demographic characteristics included gender, age, education level, race, monthly family income, marital status and number of people in-house. Monthly family income was considered in relation to the Brazilian minimum salary and classified as "low" (< US $276.00 approximately), "intermediate" (US $276.00 to $828.00 approximately) or "high" (> US $828.00 approximately).

Viral hepatitis perception was assessed by the participants' understanding of the proposed questions. The questionnaire was composed of nine groups of questions covering aspects about viral hepatitis including general information (questions 1 to 4), transmission (question 5), prevention (question 6), clinical manifestations (question 7), risk factors (question 8), and

complications (questions 9). All questions except items 3 and 4 had subdivisions (*i.e.* 1a, 1b, 1c and 1d); thus, a total of 46 answers could be correctly pointed out. Additionally, in items 3, 4, 5 and 6, individuals were asked to report which type of hepatitis virus related to their response.

The initial version of the questionnaire was structured in the Brazilian Portuguese language and developed from a questionnaire applied in a previous study[24] and through literature review about knowledge in viral hepatitis[5,16,17]. The questionnaire was then piloted with 30 respondents for its acceptability and consistency, including 15 self-administered and 15 interviewed. From the self-administered questionnaire, three of them had many unfilled questions, and one of them entirely unfilled. The questionnaire was modified after the pilot study and the interview format was chosen for data collection. After this evaluation, the questionnaire was made available for data collection. Data from the pilot study was not included in the final analysis. Participants were interviewed face-to-face in a confidential setting. At the end of the interview, the correct answers were shown to each volunteer.

Score of knowledge

The viral hepatitis perception score was created based on the average of correct answers of all participants' responses (28.7). The perception was divided in two scores: "low" (0-28 correct answers) and "desirable" (29-46 correct answers). Associations between sociodemographic characteristics and perception were also evaluated.

Statistical analysis

Descriptive statistics were generated for the responses and the chi-squared test for independence or for trend and Kruskal-Wallis test were used to compare categorical and continuous variables respectively, among the perception score groups. The variables that were associated with perception score categories were inserted into the logistic regression model, using a forward stepwise method. The 95%CIs of the estimated odds ratios were also calculated, and a *P*-value was calculated using the Statistical Package for the Social Sciences (SPSS for Windows, release 20.0; IBM Corp., Armonk, NY, United States).

RESULTS

Demographic characteristics

Most of the participants were female (269/60.2%), aged over 40 years (254/56.8%), had secondary education (186/41.6%), received intermediate monthly family income (250/55.9%), and declared themselves as non-white (225/50.3%), married (224/50.1%) and living in houses with three individuals (128/28.6%). Only marital status was not significantly different between the five populations ($P = 0.909$) (Table 1).

Viral hepatitis perception

In the case of most categories, the majority of questions were correctly answered (varying from 56.4% to 77.3%), with the exception of the complications category where only 39.4% were answered correctly. Individuals from Southeast Viral Hepatitis Ambulatory showed the highest number of correct answers in general (66.4%), clinical manifestations (84.7%), complications (46.5%), transmission (81.4%) and prevention (80.6%). Participants from South Health Center showed the highest number of correctly answered questions regarding risk of acquiring hepatitis (68.1%) (data not shown).

Table 2 describes the correct responses towards viral hepatitis knowledge separated by populations. More than 70% of participants recognized that hepatitis is caused by viruses, the existence of HAV, HBV, HCV and the availability of vaccines for hepatitis. Additionally, more than 60% of individuals did not know that hepatitis can be caused by alcohol or medicines and that an individual cannot have the same type of hepatitis more than once, while more than 70% of participants were unaware of the existence of HDV and HEV.

Clinical manifestations and risk of acquiring hepatitis questions were correctly answered by most individuals. However, work in rural areas as a risk factor in the acquisition of hepatitis was incorrectly answered by more than 60% of participants. Less than 27% of interviewees were able to associate loss of body movements, blood through the mouth, loss of vision and blood in the stool as complications of hepatitis. In addition, more than 50% of participants incorrectly answered questions about transmission by seafood, the absence of transmission by mosquito bite, and modes of prevention, such as killing mosquitoes and using masks.

In general, correct answers were more common in Southeast Viral Hepatitis Ambulatory and less common in Northeast low resource areas. In questions such as "Does hepatitis D exist?", "Can hepatitis be transmitted by mosquito bite?", "Can killing mosquitoes prevent viral hepatitis?" and "Does using masks prevent hepatitis?" less than 50% were correctly answered by all participants but more than 50% of such questions were correctly answered in Southeast Viral Hepatitis Ambulatory (Table 2).

Less than 10% of correct answers were observed in questions such as "Do hepatitis D and E exist?" in Northeast Health Center, "Is loss of body movement a complication of hepatitis?" in Southeast and Northeast low resource areas, and "Is blood in the stool a complication of hepatitis?" in Northeast Health Center, Southeast and Northeast low resource areas (Table 2).

In bivariate analysis of answered questions, some were not significant, such as those informing whether hepatitis can be caused by medicines, whether jaundice, pale stools and dark urine are clinical manifestations of hepatitis, whether people working in laboratories are at risk of infection, and whether loss of blood through the

Table 1 Participants' sociodemographic characteristics of studies, *n* (%)

Item	Total, 447	Southeast viral hepatitis ambulatory, 100	South health center, 89	Northeast health center, 114	Southeast low resource areas, 77	Northeast low resource areas, 67	*P* value
Gender							
Female	269 (60.2)	55 (55.0)	33 (37.1)	76 (66.7)	51 (66.2)	54 (80.6)	0.000
Male	178 (39.8)	45 (45.0)	56 (62.9)	38 (33.3)	26 (33.8)	13 (19.4)	
Age groups by yr							
≤ 40	193 (43.2)	29 (29.0)	28 (31.5)	68 (59.6)	27 (35.1)	41 (61.2)	0.000
> 40	254 (56.8)	71 (71.0)	61 (68.5)	46 (40.4)	50 (64.9)	26 (38.8)	
Education							
Illiterate	136 (30.4)	28 (28.0)	11 (12.4)	27 (23.7)	38 (49.3)	32 (47.8)	0.000
Primary school	66 (14.8)	16 (16.0)	12 (13.5)	15 (13.2)	13 (16.9)	10 (14.9)	
Secondary school	186 (41.6)	42 (42.0)	48 (53.9)	51 (44.7)	25 (32.5)	20 (29.8)	
College	59 (13.2)	14 (14.0)	18 (20.2)	21 (18.4)	1 (1.3)	5 (7.5)	
Family income							
Low	38 (8.5)	3 (3.0)	1 (1.1)	5 (4.4)	7 (9.1)	17 (25.3)	0.000
Intermediate	250 (55.9)	62 (62.0)	25 (28.1)	72 (63.2)	55 (71.4)	41 (61.2)	
High	145 (32.5)	35 (35.0)	61 (68.5)	34 (29.8)	11 (14.3)	4 (6.0)	
Race							
White	211 (47.2)	47 (47.0)	67 (75.3)	33 (28.9)	42 (54.5)	22 (32.8)	< 0.0001
Non-white	225 (50.3)	51 (51.0)	20 (22.4)	74 (64.9)	35 (45.5)	45 (67.2)	
Marital status							
Married	222 (49.7)	46 (46.0)	44 (49.4)	59 (51.8)	40 (51.9)	33 (49.3)	0.909
Unmarried	224 (50.1)	54 (54.0)	45 (50.6)	55 (48.2)	36 (46.8)	34 (50.7)	
People in home							
1	39 (8.7)	14 (14.0)	9 (10,1)	8 (7.0)	7 (9.1)	1 (1.5)	0.000
2	97 (21.7)	23 (23.0)	28 (31.5)	16 (14.0)	24 (31.1)	6 (9.0)	
3	128 (28.6)	34 (34.0)	23 (25.8)	30 (26.3)	24 (31.2)	17 (25.4)	
4	94 (21.0)	14 (14.0)	17 (19.1)	32 (28.1)	8 (10.4)	23 (34.3)	
5	88 (19.7)	14 (14.0)	12 (13.5)	28 (24.6)	14 (18.2)	20 (29.8)	

mouth or blood in the stool are complications of infection (P = 0.110, P = 0.922, P = 0.054, P = 0.233 and P = 0.121, respectively) (Table 2).

Figure 1 shows the distribution of correct answers in each population; the highest number of correct answered were found in the Southeast Viral Hepatitis Ambulatory group and the lowest number in Northeast low resource areas. Also, it was possible to observe a larger dispersion of correct-answered in Northeast low resource areas.

In 19 questions, it was necessary to determine which hepatitis type was related to the participant's response; only in three of them were more than 50% of the participants able to correctly identify at least one of the related hepatitis types. The percentage of incorrect answers (*i.e.* did not know, did not respond, or did not associate the correct hepatitis type with the question) from these three questions were 14.5% for "Selecting uninfected donors is hepatitis prevention", 40.3% for "Can hepatitis be transmitted by air?" and 41.2% for "Which hepatitis types have a vaccine?". For the other questions, the percentage of wrong answers varies from 50.6% ("Can hepatitis be transmitted by hemodialysis?") to 88.1% ("Can hepatitis be transmitted by seafood?") (data not shown).

Perception about viral hepatitis

The average of correct answers from all individuals was 28.7 ± 6.1- which was considered as the cut off value in this analysis; in this way, scores from 0 to 28 were considered "low" and scores of 29 to 46 were considered "desirable". Only Southeast Viral Hepatitis Ambulatory and South Health Center demonstrated a desirable knowledge (30.5 ± 5.0 and 29.5 ± 5.6, respectively) (Table 3).

Regarding the rate of correct answers, 255 (57.0%) individuals scored above average, with 87 (87.0%) from Southeast Viral Hepatitis Ambulatory, 52 (58.4%) from South Health Center, 56 (49.1%) from Northeast Health Center, 34 (44.1%) from Southeast low resource areas, and 26 (39.4%) from Northeast low resource areas.

The caveats of gender, age, marital status and number of people in the home were associated with approximately the same average number of correct answers. The majority of the individuals with both low and desirable scores received an intermediate family income; however, a lower average number of correct answers was observed in individuals who received low family income.

Desirable perception was more common among females (58.4%), subjects aged over 40 years (60.0%), with a secondary education (47.1%), receiving intermediate family income (56.9%), declaring themselves white (51.8%), married (50.2%) and living in houses with three individuals (25.5%), and belonging to Southeast Viral Hepatitis Ambulatory (34.1%) (Table 3).

Perception was associated only with education level, race, individuals living in the same home and populations in bivariate analysis (Table 3). In multivariate analysis, population-type was found to be statistically significant (Table 4).

Table 2 Correct answers regarding viral hepatitis given by individuals from each group evaluated (*n* = 447) according to general aspects, clinical manifestations, risk of acquiring hepatitis, complications, transmission and prevention, *n* (%)

Sentence	Total, *n* = 447	Southeast viral hepatitis ambulatory, *n* = 100	South health center, *n* = 89	Northeast health center, *n* = 114	Southeast low resource areas, *n* = 77	Northeast low resource areas, *n* = 67	*P* value
General aspects							
Can hepatitis be caused by viruses	321 (71.8)	84 (84.0)	69 (77.5)	75 (65.8)	48 (62.3)	45 (67.2)	0.005
Can hepatitis be caused by bacteria	242 (54.1)	50 (50.0)	19 (21.3)	74 (64.9)	56 (72.7)	43 (64.2)	0.000
Can hepatitis be caused by alcohol	172 (38.5)	31 (31.0)	31 (34.8)	38 (33.3)	34 (44.2)	38 (56.7)	0.006
Can hepatitis be caused by medicines	154 (34.5)	45 (45.0)	29 (32.6)	32 (28.1)	24 (31.2)	24 (35.8)	0.110
Does hepatitis A exist	394 (88.1)	98 (98.0)	78 (87.6)	97 (85.1)	68 (88.3)	53 (79.1)	0.004
Does hepatitis B exist	410 (91.7)	99 (99.0)	88 (98.9)	95 (83.3)	73 (94.8)	55 (82.1)	0.000
Does hepatitis C exist	359 (80.3)	99 (99.0)	86 (96.6)	66 (57.9)	61 (79.2)	47 (70.1)	0.000
Does hepatitis D exist	121 (27.1)	56 (56.0)	18 (20.2)	10 (8.8)	18 (23.4)	19 (28.4)	0.000
Does hepatitis E exist	92 (20.6)	40 (40.0)	15 (16.9)	7 (6.1)	14 (18.2)	16 (23.9)	0.000
Does a vaccine for hepatitis exist	376 (84.1)	91 (91.0)	78 (87.6)	97 (85.1)	58 (75.3)	52 (77.6)	0.026
Can you have the same hepatitis more the once	132 (29.5)	37 (37.0)	23 (25.8)	37 (32.5)	13 (16.9)	22 (32.8)	0.040
Clinical manifestations							
No clinical manifestations	292 (65.3)	89 (89.0)	75 (84.3)	61 (53.5)	35 (45.5)	32 (47.8)	0.000
After years	311 (69.6)	81 (81.0)	75 (84.3)	61 (53.5)	47 (61.0)	47 (70.1)	0.000
Fever discomfort, nausea	369 (82.6)	76 (76.0)	67 (75.3)	99 (86.8)	64 (83.1)	63 (94.0)	0.008
Jaundice, pale stools and dark urine	410 (91.7)	93 (93.0)	81 (91.0)	103 (90.4)	72 (93.5)	61 (91.0)	0.922
People at risk of acquiring hepatitis							
People working in laboratory	235 (52.6)	61 (61.0)	39 (43.8)	67 (58.8)	38 (49.4)	30 (44.8)	0.054
People who work in hospitals	310 (69.4)	72 (72.0)	58 (65.2)	88 (77.2)	56 (72.7)	36 (53.7)	0.014
Not people who work in rural areas	157 (35.1)	36 (36.0)	41 (46.1)	46 (40.4)	18 (23.4)	16 (23.9)	0.006
People who work in the beauty areas	353 (79.0)	91 (91.0)	70 (78.7)	89 (78.1)	57 (74.0)	46 (68.7)	0.007
People who use drugs	393 (87.9)	98 (98.0)	85 (95.5)	96 (84.2)	64 (83.1)	50 (74.6)	0.000
People who receive tattoos or piercings	389 (87.0)	98 (98.0)	79 (88.8)	96 (84.2)	64 (83.1)	52 (77.6)	0.001
People who live indoors	253 (56.6)	46 (46.0)	45 (50.6)	66 (57.9)	57 (74.0)	39 (58.2)	0.004
Not people who work in offices	299 (66.9)	19 (19.0)	68 (76.4)	28 (24.6)	26 (33.8)	31 (46.3)	0.000
Complications							
Cirrhosis	361 (80.8)	91 (91.0)	82 (92.1)	79 (69.3)	62 (80.5)	47 (70.1)	0.000
Liver cancer	378 (84.6)	91 (91.0)	78 (87.6)	95 (83.3)	65 (84.4)	49 (73.1)	0.031
There is no loss of body movements	88 (19.7)	32 (32.0)	17 (19.1)	27 (23.7)	6 (7.8)	6 (9.0)	0.233
There is no loss of blood through the mouth	65 (14.5)	17 (17.0)	18 (20.2)	16 (14.0)	8 (10.4)	6 (9.0)	0.000
There is no vision loss	117 (26.2)	30 (30.0)	23 (25.8)	40 (35.1)	13 (16.9)	11 (16.4)	0.016
There is no blood in the stool	49 (11.0)	18 (18.0)	10 (11.2)	9 (7.9)	7 (9.1)	5 (7.5)	0.121
Transmission							
By transfusion and transplantation	386 (86.4)	94 (94.0)	85 (95.5)	91 (79.8)	67 (87.0)	49 (73.1)	0.000
By sex	310 (69.4)	96 (96.0)	76 (85.4)	64 (56.1)	42 (54.5)	32 (47.8)	0.000
By water and contaminated vegetables	318 (71.1)	88 (88.0)	49 (55.1)	79 (69.3)	60 (77.9)	42 (62.7)	0.000
By seafood	135 (30.2)	59 (59.0)	17 (19.1)	27 (23.7)	23 (29.9)	9 (13.4)	0.000
By tattoo and piercing	361 (80.8)	96 (96.0)	76 (85.4)	88 (77.2)	57 (74.0)	44 (65.7)	0.000
By cutting instruments	385 (86.1)	99 (99.0)	77 (86.5)	90 (78.9)	66 (85.7)	53 (79.1)	0.005
By hemodialysis	280 (62.6)	74 (74.0)	58 (65.2)	60 (52.6)	53 (68.8)	35 (52.2)	0.010
Cannot be by mosquito bite	221 (49.4)	58 (58.0)	49 (55.1)	60 (52.6)	31 (40.3)	23 (34.3)	0.000
Cannot be by air	268 (60.0)	69 (69.0)	69 (77.5)	68 (59.6)	34 (44.2)	28 (41.8)	0.000
Prevention							
Building cesspools	324 (72.5)	78 (78.0)	49 (55.1)	89 (78.1)	63 (81.8)	45 (67.2)	0.000
Channeling water	318 (71.1)	76 (76.0)	53 (59.6)	84 (73.7)	63 (81.8)	42 (62.7)	0.007
Selecting uninfected donors	363 (81.2)	90 (90.0)	71 (79.8)	105 (92.1)	58 (75.3)	39 (58.2)	0.000
Filtering water and treating drinks	372 (83.2)	88 (88.0)	57 (64.0)	101 (88.6)	71 (92.2)	55 (82.1)	0.000
Killing mosquitoes does not prevent hepatitis	189 (42.3)	53 (53.0)	41 (46.1)	41 (36.0)	33 (42.9)	21 (31.3)	0.029
Providing vaccine	405 (90.6)	94 (94.0)	80 (89.9)	107 (93.9)	70 (90.9)	54 (80.6)	0.030
Using masks does not prevent hepatitis	210 (47.0)	69 (69.0)	57 (64.0)	46 (40.4)	26 (33.8)	12 (17.9)	0.000
Using condoms	378 (84.6)	97 (97.0)	82 (92.1)	93 (81.6)	56 (72.7)	50 (74.6)	0.000

Southeast Viral Hepatitis Ambulatory showed a higher number of desirable scores while underprivileged communities showed a lower number of desirable scores compared to low scores in the same areas. Medical centers also present a larger proportion of desirable scores compared to low scores though this was less pronounced (Figure 2).

DISCUSSION

In the present study, knowledge level was scored according to the mean number of correct answers. Individuals from Southeast Viral Hepatitis Ambulatory and South Health Center showed a desirable knowledge in contrast to those recruited at Northeast Health Center,

Table 3 Sociodemographic characteristics according to knowledge scores for viral hepatitis, *n* (%)

Item	Mean of knowledge score (± SD)	Knowledge score		Bivariate analysis *P* value
		Low (0-28), *n* = 192	Desirable (29-46), *n* = 255	
Gender				
Female	28.49 ± 6.16	120 (62.5)	149 (58.4)	0.430
Male	29.04 ± 6.10	72 (37.5)	106 (41.6)	
Age in yr				
≤ 40	27.6 ± 6.6	91 (47.4)	102 (40.0)	0.120
> 40	27.5 ± 8.5	101 (52.6)	153 (60.0)	
Education level				
Illiterate	25.4 ± 8.3	74 (38.5)	62 (24.3)	0.002
Primary school	28.1 ± 5.1	32 (16.7)	34 (13.3)	
Secondary school	30.9 ± 5.7	66 (34.4)	120 (47.1)	
College	30.8 ± 5.4	20 (10.4)	39 (15.3)	
Family income				
Low	26.1 ± 6.9	21 (10.9)	17 (6.7)	0.200
Indeterminate	29.4 ± 7.0	105 (54.7)	145 (56.9)	
High	30.3 ± 5.5	57 (29.7)	88 (34.5)	
Race				
White	29.8 ± 7.1	79 (41.1)	132 (51.8)	0.030
Non-white	28.2 ± 6.3	107 (55.7)	118 (46.3)	
Marital status				
Married	28.4 ± 7.1	94 (48.9)	128 (50.2)	0.840
Unmarried	27.3 ± 8.0	97 (50.5)	127 (49.8)	
Individuals living in the same home				
1	30.5 ± 5.0	11 (5.7)	28 (11.0)	0.014
2	29.5 ± 5.6	41 (21.4)	56 (22.0)	
3	28.3 ± 6.3	63 (32.8)	65 (25.5)	
4	28.8 ± 6.6	31 (16.1)	63 (24.7)	
≥ 5	27.6 ± 6.4	46 (24.0)	42 (16.5)	
Population				
Southeast viral hepatitis ambulatory	33.1 ± 4.5	13 (6.8)	87 (34.1)	< 0.0001
South health center	29.1 ± 5.3	37 (19.3)	52 (20.4)	
Northeast health center	27.5 ± 5.0	58 (30.2)	56 (22.0)	
Southeast low resource areas	27.6 ± 4.7	43 (22.4)	34 (13.3)	
Northeast low resource areas	25.0 ± 8.5	41 (21.3)	26 (10.2)	

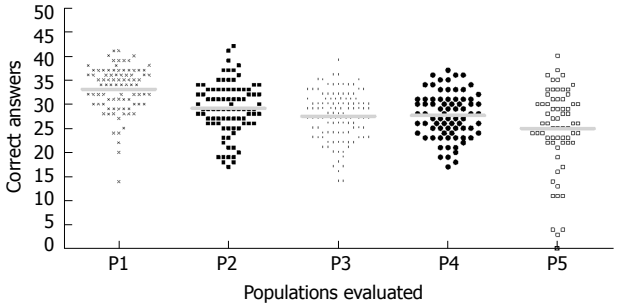

Figure 1 Distribution of correct answers plotted according to each population evaluated. The y-axis represents the number of correct answers. The solid lines represent the average for P1 (Southeast Viral Hepatitis Ambulatory), P2 (South Health Center), P3 (Northeast Health Center), P4 (Southeast low resource areas) and P5 (Northeast low resource areas), which were respectively: 33.1 ± 4.5; 29.1 ± 5.3; 27.5 ± 5.0; 27.6 ± 4.7; and, 25.0 ± 8.5.

Southeast and Northeast low resource areas. The findings of the current study are in line with previous findings[5,22,24]. However, the study in Egypt noted high baseline knowledge about HCV[23], likely due to the scale of the HCV epidemic in this country.

Complications arising from viral hepatitis was the worst set of questions evaluated in the current study. Although more than 80% of participants can correctly correlate cirrhosis and liver cancer with complications of viral hepatitis, most of them related complications that are not caused by hepatitis. In previous studies between health professionals, more than half of participants answered correctly to the questions about HCV complications[26]. However, an insufficient knowledge regarding HCV complications was observed in a study among health professionals[27]. Clinical manifestations of viral hepatitis were the best set of questions evaluated, contrary to previous observations[28].

In the present study, most individuals recognize the existence of HAV, HBV and HCV and do not recognize the existence of hepatitis D or E. The same finding has previously been observed in Brazil[24]. Another study[28] observed a very weak knowledge regarding the five hepatitis types among medical science students. Transmission and prevention modes were correctly answered in general; this data was also observed among medical and health science students in Ethiopia in the evaluation of HBV knowledge[21]. A large number of individuals do not know that viral hepatitis can be transmitted by seafood, as observed previously[24]. Since HAV and HEV can be transmitted in this way[29,30], the transmission may continue if preventive measures are not taken.

Viral hepatitis transmission by mosquito and forms to prevent it were incorrectly answered by most par-

Table 4 Final adjusted model of multivariate logistic regression for knowledge scores for viral hepatitis

Variable	Knowledge score			
	OR	95%CI		*P* value
		Lower	Upper	
Education level				
Illiterate	2.230	1.084	4.586	0.290
Primary school	1.799	0.807	4.009	0.151
Secondary school	1.028	0.528	2.002	0.936
College	1.000	-	-	-
Individuals living in the same home				
1	1.000	-	-	-
2	1.611	0.671	3.867	0.286
3	2.328	0.992	5.465	0.052
4	0.818	0.332	2.017	0.663
⩾ 5	1.832	0.748	4.486	0.185
Population				
Southeast viral hepatitis ambulatory	1.000	-	-	-
South health center	6.154	2.900	13.058	0.000
Northeast health center	8.617	4.177	17.777	0.000
Southeast low resource areas	7.491	3.508	15.994	0.000
Northeast low resource areas	11.262	5.007	25.327	0.000

CI: Confidence interval; OR: Odds ratio.

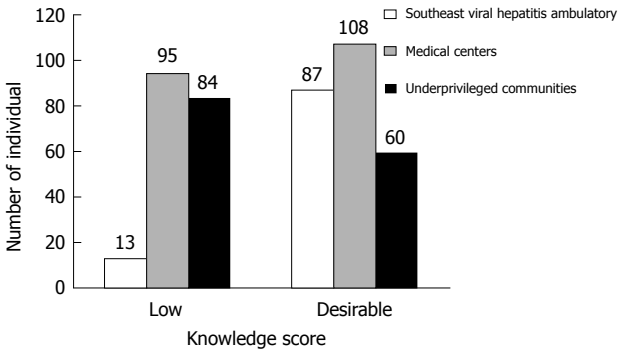

Figure 2 Number of individuals according to knowledge score in each group evaluated.

ticipants, probably due to the country-wide presence of Dengue virus, the transmission of which is widely understood by the public. Most individuals did not cite transmission by air but, curiously, masks to avoid airborne contamination were cited. These questions highlight the need for raising awareness among the public to reinforce knowledge related to the modes of transmission and prevention.

In present study, population type was the significant demographic factor associated with knowledge level in multivariate analysis, the same as found in other studies[5,24,31]. Contrary to a previous general population study in Brazil[24], monthly family income had no association with knowledge in the present study.

The results obtained in the present study can be used as a data source for the projection of intervention methods in health and public health policies, such as explanatory educational leaflet, educational booklets, lectures in schools, health campaigns, health fairs and others, in order to increase access to information of viral hepatitis and possibly to reduce the number of cases of these infections, especially among individuals from low-resource areas that showed a lower level of knowledge in the present study.

The present study has some limitations. The study did not assess the information regarding the neighborhood of each participant to observe the sociodemographic diversity. The study did not assess the occupation of participants to categorize and compare with studies in specific groups, such as health or beauty professionals. In Viral Hepatitis Ambulatory and in medical centers, it was not asked whether participants had previously consulted and whether they had any prior knowledge about hepatitis.

In conclusion, in general, desirable knowledge was observed among most participants. However, Northeast Health Center and under-privileged communities showed low knowledge. Knowledge levels were associated with education level, race and number of individuals living in the same home. The results of the present study should prove useful for information and prevention campaigns targeted at the general population, especially between neglected communities, in order to reduce the transmission of viral hepatitis.

ARTICLE HIGHLIGHTS

Research background

Viral hepatitis is an important public health problem in the world, causing more than 1 million deaths annually. It is important to evaluate viral hepatitis perception to identify the possible gaps and help public health authorities to create strategies to increase access to information about these infections.

Research motivation

Few studies have been done to evaluate viral hepatitis perception in uninfected individuals, particularly in Latin America.

Research objectives

The main aim of this study was to evaluate the viral hepatitis knowledge among

individuals from different resource areas and health conditions in Brazil to identify possible gaps and help authorities in the development of prevention and education programs.

Research methods

This was a cross-sectional study, wherein a questionnaire to evaluate viral hepatitis perception was applied among 447 individuals from five different populations in Brazil (Southeast low resource areas, Northeast low resource areas, South Health Center, Northeast Health Center, Southeast Viral Hepatitis Center). The viral hepatitis perception score was created based on the average of correct answers of all participants' responses (28.7), and associations between sociodemographic characteristics and perception were also evaluated.

Research results

High perception level about viral hepatitis was observed in Southeast Viral Hepatitis Ambulatory and South Health Center compared to Northeast Health Center, Southeast and Northeast low resource areas. According to sociodemographic characteristics, desirable scores were more common among those with secondary education (47.1%), those who declared themselves as white (46.3%), and those who lived in houses with three individuals (25.5%). Population type was associated with knowledge level in multivariate analysis.

Research conclusions

The study demonstrated a low level of perception about viral hepatitis among individuals from low resource areas. Identifying the knowledge gaps in this group could help to create strategies for increasing access to information and consequently reducing the transmission of these diseases.

Research perspectives

This study demonstrates that it is necessary to improve the access to health information about viral hepatitis, especially among residents of low-resource settings. It is important to conduct a random sampling evaluation of larger numbers of individuals to confirm the results observed. A questionnaire could help to conduct these studies, the same as was used in the present work.

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META-ANALYSIS

Cardiac stress testing and coronary artery disease in liver transplantation candidates: Meta-analysis

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Abstract

AIM

To evaluate the diagnostic value of dobutamine stress echocardiography (DSE) and myocardial perfusion scintigraphy (MPS) in predicting coronary artery disease (CAD) in cirrhotic patients listed for liver transplantation (LT), using invasive coronary angiography (ICA) as gold-standard.

METHODS

Retrieval of studies was based on Medical Subject Headings and Health Sciences Descriptors, which were combined using Boolean operators. Searches were run on the electronic databases Scopus, Web of Science, EMBASE, MEDLINE (PubMed), BIREME (Biblioteca Regional de Medicina), LILACS (Latin American and Caribbean Health Sciences Literature), Cochrane Library for Systematic Reviews and Opengray.eu. There was no language or date of publication restrictions. The reference lists of the studies retrieved were searched manually.

RESULTS

The search strategy retrieved 322 references for DSE and 90 for MPS. In the final analysis, 10 references for DSE and 10 for MPS were included. Pooled sensitivity was 28% and 61% for DSE and MPS and specificity was 82% and 74%, for diagnosis of CAD using ICA as gold-standard, respectively.

CONCLUSION
DSE and MPS do not have adequate sensitivity for determination of whether CAD is present, despite having significant specificity.

Key words: Myocardial perfusion imaging; Coronary angiography; Liver transplantation; Echocardiography; Stress

Core tip: The concept of cardiac involvement in cirrhotic patients has been changing as patients listed for liver transplantation (LT) have become older and sicker. We aimed to evaluate the diagnostic value of dobutamine stress echocardiography (DSE) and myocardial perfusion scintigraphy (MPS) in predicting coronary artery disease (CAD) in cirrhotic patients listed for LT, using invasive coronary angiography as gold-standard. A systematic review and meta-analysis was performed, including 10 references for DSE and 10 for MPS. We concluded that DSE and MPS do not have adequate sensitivity for determination of whether CAD is present, despite having significant specificity.

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INTRODUCTION

When liver transplantation (LT) programs were beginning three decades ago, it was believed that the systemic vasodilation that occurs in end-stage liver disease (ESLD) might be able to protect patients from coronary artery disease (CAD)[1]. Nevertheless, studies have shown that CAD is more prevalent in cirrhotic patients than previously suspected. In a cohort with high risk for CAD, 26% of the patients had previously unknown CAD on routine invasive coronary angiography (ICA)[2].

The cardiac profile for LT candidates has been changing, because they are now older and sicker[3]. Data from the United Network for Organ Sharing (UNOS) show that the proportion of LT recipients over the age of 65 years in the United States increased from 9.6% in 2003 to 16.3% in 2013[4]. This has been a cause for major concern regarding perioperative cardiac risk. For example, a publication from 1996 predicted that around 50% of patients with significant CAD would die from cardiac complications in the perioperative period[5]. However, in a more recent study, the presence of obstructive CAD did not significantly impact post-LT survival, when modern treatment of CAD pre-LT is taken into account[6]. Furthermore, patients with ESLD have a specific type of cardiovascular sickness, currently known as cirrhotic cardiomyopathy, whose role in LT survival is yet to be established[7].

These findings suggest a real need for protocols for cardiac evaluation of patients awaiting LT - particularly for cirrhotic patients. The American Association for the Study of Liver Diseases (AASLD) published a guideline in 2005 that recommends myocardial stress testing for every patient referred for LT[8]. Nevertheless, the guideline published in 2012 by the American Heart Association (AHA) and the American College of Cardiology (ACC)[9], suggested that myocardial stress testing should be reserved for patients with three or more CAD risk factors. A score has recently been published for evaluation of perioperative cardiac risk, but it has yet to be validated further[10].

The aim of this systematic review with meta-analysis is to summarize the evidence related to the diagnostic value of two non-invasive cardiac stress testing methods: Dobutamine stress echocardiography (DSE) and myocardial perfusion scintigraphy (MPS), for the diagnosis of CAD in cirrhotic pre-LT patients, using ICA as gold-standard.

MATERIALS AND METHODS

This study was carried out in accordance with the recommendations contained in the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA-P) guidelines[11]. Our systematic review was registered with the International Prospective Register of Systematic Reviews (PROSPERO), maintained by York University, on 17 August 2015 and was last updated on 5 April 2018 [registration No. 10.15124/CRD42015025391 (www.crd.york.ac.uk/prospero/)].

Data sources

Studies were retrieved using Medical Subject Headings (MeSH) and Health Sciences Descriptors (DeCS), which were combined with Boolean operators. Searches were run on the electronic databases Scopus, Web of Science, Embase, Medline (PubMed), BIREME (Biblioteca Regional de Medicina), LILACS (Latin American and Caribbean Health Sciences Literature), Cochrane Library for Systematic Reviews and Opengray.eu. There was no language or date of publication restrictions. The reference lists of the retrieved studies were submitted to manual search. The search strategies used for each test and each database are shown in Supplemental material. Databases were last searched between August and September of 2015.

Inclusion criteria and outcomes

Cohort or case-control studies were eligible for selection, hence it was analyzed the diagnostic accura-

cy of DSE and/or MPS in adult patients with cirrhosis submitted for pre-LT evaluation. The tests had to be performed as a part of cardiac evaluation before LT. Studies were excluded if they did not meet these inclusion criteria. If there was more than one study published using the same population, the most recent study was selected for the analysis. Studies published only as abstracts were included, as long as the data available made analysis possible. The outcome measured was a diagnosis of CAD using ICA as gold standard.

Study selection and data extraction

An initial screening of titles and abstracts was the first stage to select potentially relevant papers. The second step was the analysis of the full-length papers. Two independent reviewers (Jonathan Soldera, Fabio Camazzola) extracted data using a standardized data extraction form after assessing and reaching consensus on eligible studies. The same reviewers separately assessed each study and extracted data about the characteristics of the subjects, the diagnostic accuracy for DCE and MPS and the outcomes measured. A third party (Santiago Rodriguez) was responsible for divergences in data extraction, clearing them when required. Quality of evidence regarding diagnostic accuracy was evaluated according of the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2)[12].

Statistical analysis

In anticipation of possible heterogeneity between the populations of the studies, a random-effects DerSimonian and Laird model was used. Data regarding the tests' diagnostic accuracy was collected. The measures of diagnostic accuracy chosen were specificity, sensitivity, likelihood ratio and diagnostic odds ratio. Heterogeneity was assessed using the I^2 statistic. MetaDisc 1.4 was used for diagnostic accuracy. The small number of studies included made funnel plot analysis impossible.

RESULTS

Systematic review

The search strategy retrieved 322 references for DSE and 90 for MPS. After analyzing titles and abstracts, 111 references for DSE and 24 for MPS were excluded because they were duplicates and the full texts were retrieved for 60 references on DSE and 26 on MPS. In the final analysis, 10 references were included for DSE and 10 for MPS. Flowcharts illustrating the search strategies are shown in Supplemental Figures 1 and 2, respectively. Studies included were either a case-control study or a prospective or historical cohort study.

DSE

Data were collected after the conclusion of a systematic review of the 10 studies included in the diagnostic analysis that used ICA as the gold-standard. The data extracted are summarized in Table 1.

A minority of the patients included in these studies underwent ICA and they were generally higher risk patients with positive DSE findings or multiple risk factors. Data for risk factors specifically for the patients who underwent ICA were not available for most studies, therefore the data on risk factors described refer to the whole study population, as summarized in Supplemental Table 1.

The initial meta-analysis was performed including all studies. Global sensitivity was 28% [95% confidence interval (CI): 21.2%-35.6%] with high heterogeneity (I^2 = 69%) (Figure 1), specificity was 82.9% (95%CI: 78.5%-86.8%) with high heterogeneity (I^2 = 84.1%) (Figure 2) and the diagnostic odds ratio was 2.09 (95%CI: 0.96-4.58) with moderate heterogeneity (I^2 = 47.5%) (Supplemental Figure 3). The positive likelihood ratio was 1.7 (95%CI: 1.06-2.7) with moderate heterogeneity (I^2 = 51.4%) (Supplemental Figure 4) and the negative likelihood ratio was 0.92 (95%CI: 0.81-1.04) with little heterogeneity (I^2 = 18.8%) (Supplemental Figure 5). An asymmetrical Receiver Operating Characteristic (ROC) curve is provided in Supplemental Figure 6.

A meta-regression was performed using the subsets of patients from each of the study samples who had undergone ICA and no statistically significant association was detected between this variable and the diagnostic odds ratio (P = 0.0586).

In order to attempt to reduce heterogeneity between studies, a sub-analysis was performed of sensitivity and specificity according to the definition of a positive ICA result employed by each study. Studies that used a positive ICA defined as any number of lesions with at least one greater than 70%, had a sensitivity of 21% (95%CI: 13.4%-31.3%) with high heterogeneity (I^2 = 71%) and a specificity of 91.5% (95%CI: 86.8%-95%) with high heterogeneity (I^2 = 63.5%), while studies that defined positive ICA as any number of lesions, with at least one greater than 50%, had a sensitivity of 36.1% (95%CI: 25.1%-48.3%) with high heterogeneity (I^2 = 66.3%) and a specificity of 69.9% (95%CI: 61.4%-77.6%) with high heterogeneity (I^2 = 68%).

MPS

Data were collected after conclusion of a systematic review of the 10 studies included in the diagnostic analysis that used ICA as the gold-standard. The data extracted are summarized in Table 2.

As with DSE, a minority of the patients included in these studies underwent ICA, and they were generally

Table 1 Studies included in analysis - dobutamine stress echocardiography

Ref.	TP	FP	FN	TN	Total number of patients in the study	Proportion of patients who underwent ICA	Definition of patients included	Criteria for ICA indication	Lesion for definition of positive ICA	QUADAS-2 quality analysis criteria
Ibrahim *et al*[13]	5	8	5	22	366	10.9%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	NA	RB: P + I - R + F ? AC: P + I ? R +
Donovan *et al*[14]	3	6	1	8	190	9.5%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 50%	RB: P + I + R + F + AC: P + I + R +
Findlay *et al*[15]	1	6	0	0	117	6%	Cirrhotic patients in pre-LT evaluation	Transplanted patients	> 70%	RB: P + I + R + F + AC: P – I + R +
Harinstein *et al*[16]	2	7	14	41	105	61%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 70%	RB: P + I + R + F + AC: P + I + R +
Harinstein *et al*[16]	4	5	20	35	105	61%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 50%	RB: P + I + R + F + AC: P + I + R +
Plotkin *et al*[17]	2	0	0	19	40	52.5%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 70%	RB: P + I + R + F + AC: P + I + R +
Ramrakhiani *et al*[18]	4	10	0	0	201	7%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 70%	RB: P + I – R – F ? AC: P + I – R –
Tsutsui *et al*[19]	2	5	0	10	230	7.4%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 50%	RB: P + I + R + F + AC: P + I + R +
Umphrey *et al*[20]	0	0	0	9	157	5.7%	Cirrhotic patients in pre-LT evaluation	High risk patients	> 70%	RB: P + I + R + F + AC: P + I + R +
Snipelisky *et al*[21]	12	16	20	18	66	100%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 50%	RB: P + I + R + F + AC: P + I + R +
Patel *et al*[22]	15	10	56	124	420	48.8%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive DSE	> 70%	RB: P + I + R + F + AC: P + I + R +

TP: True positive; FP: False positive; FN: False negative; TN: True negative; ICA: Invasive coronary angiography; LT: Liver transplantation; DSE: Dobutamine stress echocardiography; NA: Not available; QUADAS-2: Quality assessment of diagnostic accuracy studies-2; RB: Risk of bias; P: Patient selection; I: Index text; R: Reference standard; F: Flow and timing; AC: Applicability concerns.

higher risk patients with a positive MPS result or multiple risk factors. As with DSE, data for risk factors specifically for the patients who underwent ICA were not available for most studies, therefore the data for risk factors described refer to the whole study population, as summarized in Supplemental Table 2.

The diagnostic data were used for meta-analysis. The initial meta-analysis was performed including all studies. Global sensitivity was 61.8% (95%CI: 50%-72.8%) with high heterogeneity (I^2 = 69.8%) (Figure 3), specificity was 74.3% (95%CI: 70.2%-78.2%) with high heterogeneity (I^2 = 77.1%) (Figure 4) and the diagnostic odds ratio was 4.74 (95%CI: 1.51-14.8) with high heterogeneity (I^2 = 61.9%) (Supplemental Figure 7). The positive likelihood ratio was 2.26 (95%CI: 1.47-3.48) with high heterogeneity (I^2 = 63.5%) (Supplemental Figure 8) and the negative likelihood ratio was 0.57 (95%CI: 0.32-1.02) with high heterogeneity (I^2 = 62.7%) (Supplemental Figure 9). An asymmetrical ROC curve is provided in Supplemental Figure 10.

A meta-regression was performed using the subsets of patients from each of the study samples who had undergone ICA and no statistically significant association was detected between this variable and the diagnostic odds ratio (P = 0.4984).

In order to attempt to reduce heterogeneity between studies, a sub-analysis was performed of sensitivity and specificity according to the definition of a positive ICA result employed by each study. Studies that used a positive ICA defined as any number of lesions with at least one greater than 70% had a sensitivity of 59.4% (95%CI: 46.4%-71.5%) with high heterogeneity (I^2 = 70.5%) and specificity of 76.3% (95%CI: 71.6%-80.5%) with high heterogeneity (I^2 = 80%). In another sub-analysis, including only the four studies in which ICA was performed for all patients, sensitivity was 57.1% (95%CI: 44%-69.5%) with high heterogeneity (I^2 = 71.1%) and specificity was 75.5% (95%CI: 71.4%-79.7%) with high heterogeneity (I^2 =

Table 2 Studies included for analysis - myocardial perfusion scintigraphy

Ref.	TP	FP	FN	TN	Total number of patients in the study	Proportion of patients who underwent ICA	Definition of patients included	Criteria for ICA indication	Lesion for definition of positive ICA	QUADAS-2 quality analysis criteria
Baker *et al*[23]	8	4	0	14	74	35.1%	Cirrhotic patients in pre-LT evaluation with cardiac risk factors	High risk patients/ positive MPS	> 70%	RB: P – I + R + F + AC: P – I + R +
Kryzhanovski *et al*[24]	0	1	0	0	63	1.6%	Cirrhotic patients in pre-LT evaluation with cardiac risk factors	High risk patients/ positive MPS	> 70%	RB: P – I + R + F + AC: P – I + R +
Senzolo *et al*[25]	0	2	0	0	24	8.3%	Cirrhotic patients in pre-LT evaluation	Positive MPS	> 70%	RB: P – I + R + F + AC: P – I + R +
Kandiah *et al*[26]	1	4	0	5	93	10.7%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive MPS	> 70%	RB: P – I + R + F + AC: P – I + R +
Oprea-Lager *et al*[27]	1	1	0	0	156	1.2%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive MPS	> 70%	RB: P – I + R + F + AC: P – I + R +
Davidson *et al*[28]	7	24	12	40	83	100%	Cirrhotic patients in pre-LT evaluation with cardiac risk factors	High risk patients/ positive MPS	> 70%	RB: P + I + R + F + AC: P + I + R +
Aydinalp *et al*[29]	6	34	0	64	389	26.7%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive MPS	> 50%	RB: P + I + R + F + AC: P + I + R +
Zoghbi *et al*[30]	2	11	2	12	87	31%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive MPS	> 70%	RB: P – I + R + F + AC: P – I + R +
Bezinover *et al*[31]	3	1	3	9	173	9.2%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive MPS	NA	RB: P + I + R + F + AC: P + I + R +
Bhutani *et al*[32]	20	46	12	215	414	70.7%	Cirrhotic patients in pre-LT evaluation	High risk patients/ positive MPS	> 70%	RB: P + I + R + F + AC: P + I + R +

TP: True positive; FP: False positive; FN: False negative; TN: True negative; ICA: Invasive coronary angiography; LT: Liver transplantation; MPS: Myocardial perfusion scintigraphy; NA: Not available; QUADAS-2: Quality assessment of diagnostic accuracy studies-2; RB: Risk of bias; P: Patient selection; I: Index text; R: Reference standard; F: Flow and timing; AC: Applicability concerns.

84.2%).

DISCUSSION

It is essential to understand the role of CAD in cirrhosis and LT patients. There is a need to improve pre-LT diagnostic tools because the age of LT candidates is rising and the proportion of NASH patients has been increasing. This systematic review is the largest current meta-analysis of diagnostic data for DSE and MPS in pre-LT patients. It increases the data available in a previous study of DSE as a diagnostic and prognostic tool for LT candidates, published by Nguyen *et al*[33], which found that DSE had a high negative predictive value for adverse outcomes post-LT.

Among the general population, a prior meta-analysis of five studies found that both DSE and MPS are accurate for detection of CAD, with sensitivity of 85% and specificity of 87%[34] for DSE and sensitivity of 83% and specificity of 77% for MPS[35]. However, this meta-analysis found much lower sensitivity values for diagnosis of CAD in patients awaiting LT, while specificity rates did not vary so much. This could have happened because results for stress testing might be false due to modifications in hemodynamics caused by ESLD, such as high-output cardiac failure, cirrhotic cardiomyopathy, anemia and the use of beta blockers[36,37].

Nevertheless, the most used method for pre-LT cardiac stress testing is DSE, since cirrhotic patients have a low tolerance of exercise[38]. When compared to ergometric cardiac stress testing, DSE has higher sensitivity (67% *vs* 88%) and specificity (71% *vs* 83%)[39-41]. The prognostic value of MPS has also been evaluated previously, with a hazard ratio of 3.17 for all-cause mortality for a group with reversible perfusion

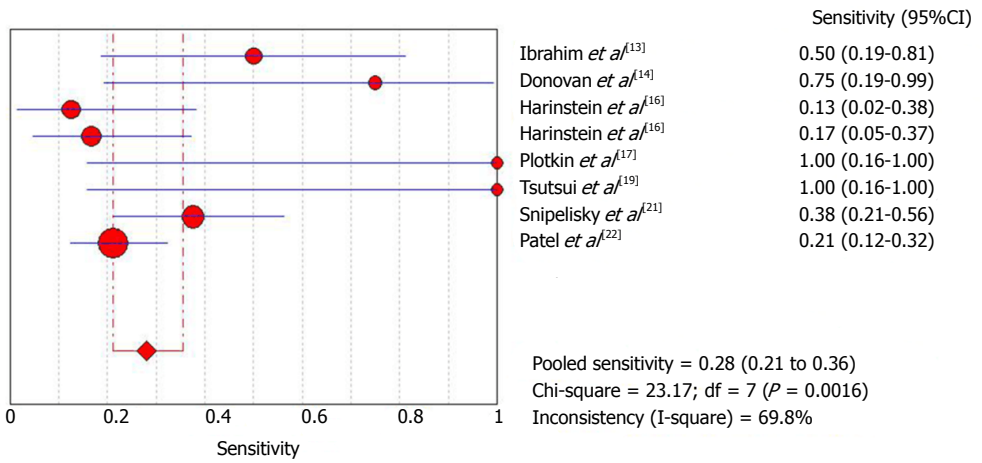

Figure 1 Forest plot for sensitivity meta-analysis - dobutamine stress echocardiography.

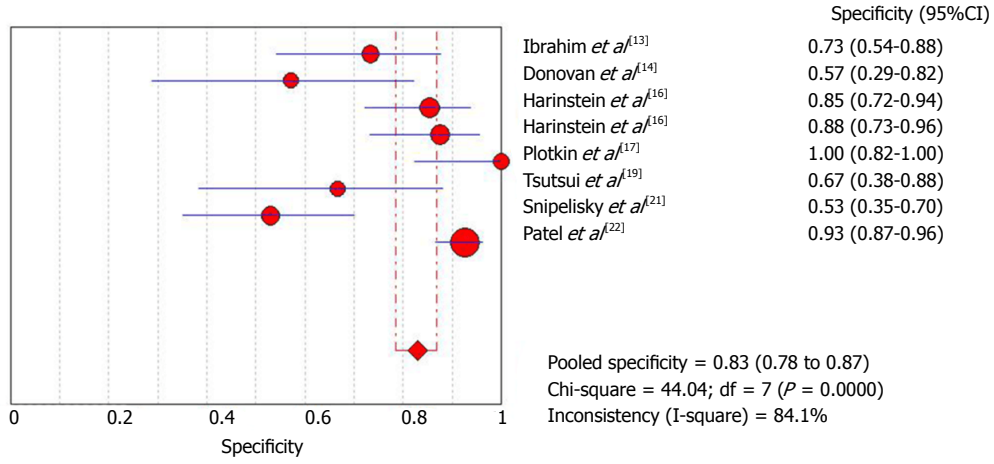

Figure 2 Forest plot for specificity meta-analysis - dobutamine stress echocardiography.

defect when compared to a group without perfusion defect[27].

The goal of both tests is to detect significant CAD prior to LT. In a high risk cohort in whom all patients underwent ICA and half had arterial systemic hypertension or diabetes, a 60% prevalence of CAD was found - one third with severe disease. Presence of moderate to severe CAD was associated with the presence of two or more cardiac risk factors[2]. If needed, ICA and stenting, seem to be safe in cirrhotic patients, taking precaution with the doubling of antiplatelet blockade in patients with esophageal varices[42]. The presence of CAD is associated with a poorer prognosis post-LT[43-45], although, Wray *et al*[6] did not detect a change in prognosis in the cohort they described. One must keep in mind also that pre-LT cardiac evaluation is costly and is not free from risks. In a previous study by Fili *et al*[46], the study protocol failed to demonstrate improvement in prognosis, but did raise costs.

One meta-analysis has found that DSE is superior to MPS among patients undergoing major vascular surgery - a positive DSE meant higher relative risk for perioperative MACE and all-cause mortality, when compared to MPS[47]. The prognostic role of DSE and MPS in patients undergoing kidney transplantation has been studied by two meta-analyses, which found these tests to be accurate in predicting outcomes, with DSE performing better than MPS in their analysis. Nevertheless, in this context, a normal non-invasive stress test did not necessarily exclude the possibility of adverse cardiac outcomes[48,49].

Analyzing the data collected and presented in this meta-analysis, it can be concluded that DSE and MPS offer limited accuracy for predicting CAD diagnoses. They both have low sensitivity and moderate specificity, which does not make them the ideal tests for pre-LT cardiac risk evaluation, as they also do

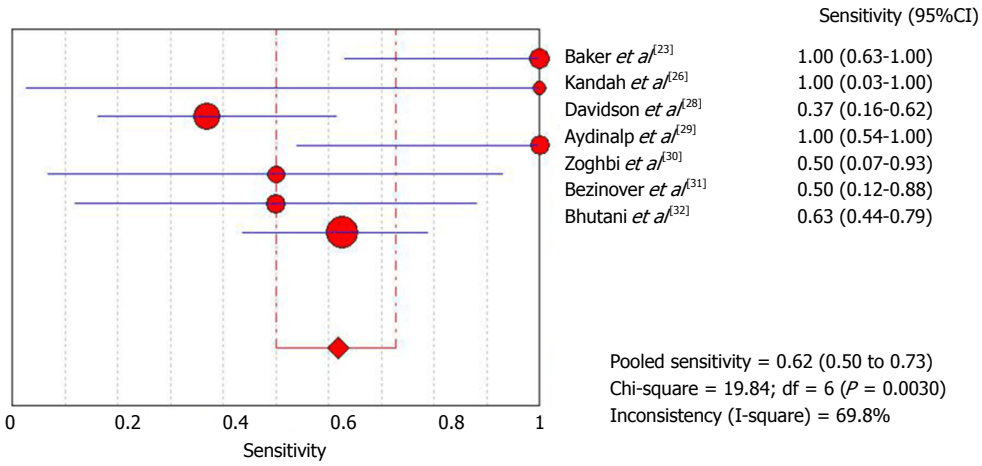

Figure 3 Forest plot for sensitivity meta-analysis - myocardial perfusion scintigraphy.

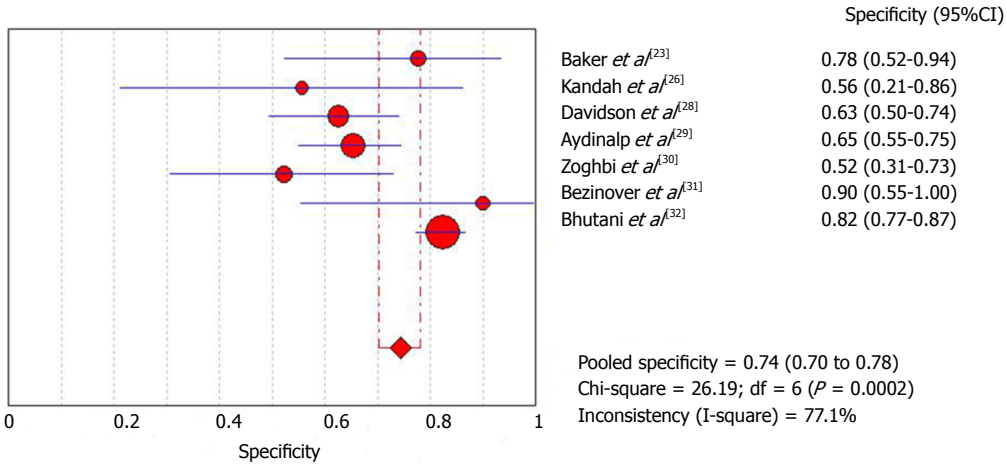

Figure 4 Forest plot for specificity meta-analysis - myocardial perfusion scintigraphy.

not predict adverse outcomes with accuracy[50]. This is consistent with the latest ACC/AHA guidelines, which describes non-invasive stress testing as of low sensitivity and specificity for detecting CAD in liver-transplant candidates[9]. Nevertheless, the high specificity found in this meta-analysis show that both DSE and MPS are useful for identifying patients with CAD. Notwithstanding, a negative stress test does not exclude the presence of CAD.

The element most likely to affect the results of this meta-analysis is selection of patients with indications for both LT and ICA. Generally, physicians happen to be more cautious in referring sicker and older patients for LT, which might mean that this group of patients is under-represented in this meta-analysis. Also, ICA is generally ordered only for high-risk patients with a positive DSE or MPS, and a positive ICA can lead to de-listing for LT, or even death before LT, due to advanced heart conditions.

This heterogeneity of indications for DSE and MPS as part of pre-LT evaluation is reflected in the heterogeneity found in this meta-analysis, which is high throughout. Sub-analyses and meta-regressions were attempted in order to minimize heterogeneity, but with no substantial success. A major limitation is that, in most studies, just a few patients were referred for ICA, generally those with higher risk or a positive non-invasive stress test, which might over represent the proportion of CAD in pre-LT patients.

The results of this meta-analysis call into question the AASLD rationale of recommending routine non-invasive stress testing in pre-LT cardiac evaluation, since DSE and MPS both have low sensitivity for detecting CAD and did not predict outcomes adequately. Nevertheless, further prospective studies with standardized and homogenous patient characteristics are necessary in order to arrive at a better understanding of the value of pre-LT cardiac evaluation and a better-grounded decision on whether it is more cost-effective to follow AASLD[8] or ACC/AHA reco-

mmendations[9]. Initiatives such as development of the CAR-OLT score might help clarify this problem[10]. This paper's strengths are its complete search strategy, performed in multiple databases. Nevertheless, results are just for pre-LT candidates; hence only patients referred for LT because of ESLD were reviewed.

The results of this systematic review and meta-analysis can also have been limited due to a post-referral bias, since patients with previously known serious cardiac conditions are generally not referred for LT. Early revascularization, in the general population, might lead to a significant change in the history of CAD and a better survival. This is somewhat unclear for ESLD patients. Because of the small number of studies and their limitations, the quality of evidence in the meta-analysis was low throughout, which might have negatively impacted this review.

In conclusion, this meta-analysis found that among few and limited studies, DSE and MPS are of limited value for predicting positive ICA. Their low sensitivity might make them inadequate for pre-LT cardiac evaluation. Prospective studies with larger samples are needed to better define an adequate test for predicting CAD in pre-LT patients.

ARTICLE HIGHLIGHTS

Research background

The concept of cardiac involvement with coronary artery disease (CAD) in cirrhotic patients has been changing as patients listed for liver transplantation (LT) have become older and sicker. A previous study of dobutamine stress echocardiography (DSE) as a diagnostic and prognostic tool for LT candidates, published by Nguyen *et al*, which found that DSE had a high negative predictive value for adverse outcomes post-LT. This study tries to elucidate the problem of CAD screening in pre-LT patients.

Research motivation

There is a real need for protocols for cardiac evaluation of patients awaiting LT - particularly for cirrhotic patients. The American Association for the Study of Liver Diseases (AASLD) published a guideline in 2005 that recommends myocardial stress testing for every patient referred for LT. Nevertheless, the guideline published in 2012 by the American Heart Association (AHA and the American College of Cardiology (ACC), suggested that myocardial stress testing should be reserved for patients with three or more CAD risk factors. Better understanding the use of these tools might lead to better choices for pre-LT patients and better prognosis post-LT.

Research objectives

To evaluate the diagnostic value of DSE and myocardial perfusion scintigraphy (MPS) in predicting CAD in cirrhotic patients listed for LT, using invasive coronary angiography (ICA) as gold-standard. This could help clinicians choose the best test for predicting adverse cardiac events post-LT.

Research methods

A systematic review and meta-analysis was performed. Searches were run on the electronic databases Scopus, Web of Science, EMBASE, MEDLINE (PubMed), BIREME (Biblioteca Regional de Medicina), LILACS (Latin American and Caribbean Health Sciences Literature), Cochrane Library for Systematic Reviews and Opengray.eu. There was no language or date of publication restrictions. The reference lists of the studies retrieved were searched manually.

Research results

The search strategy retrieved 322 references for DSE and 90 for MPS. In the final analysis, 10 references for DSE and 10 for MPS were included. Pooled sensitivity was 28% and 61% for DSE and MPS and specificity was 82% and 74%, for diagnosis of CAD using ICA as gold-standard, respectively.

Research conclusions

This study found that DSE and MPS do not have adequate sensitivity for determination of whether CAD is present, despite having significant specificity. There is a need for better tools in order to detect CAD in pre-LT patients. It is not feasible to determine whether AASLD or AHA/ACC is correct, hence both tests underperformed. It is proposed a hypothesis that new methods, tests or scores are need in order to clarify this question, which could impact pre-LT decisions in the future.

Research perspectives

It is possible to conclude that current evidence regarding pre-LT cardiac stress testing is lacking, and future research are bound to focus into solving this important clinical question. A comprehensive study, cohort or randomized, is necessary in order to gather more information on the utility and feasibility of the use of current and future tests in order to determine the presence of pre-LT CAD.

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CASE REPORT

Trapped vessel of abdominal pain with hepatomegaly: A case report

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Abstract

Abdominal pain with elevated transaminases from inferior vena cava (IVC) obstruction is a relatively common reason for referral and further workup by a hepatologist. The differential for the cause of IVC obstruction is extensive, and the most common etiologies include clotting disorders or recent trauma. In some situations the common etiologies have been ruled out, and the underlying process for the patient's symptoms is still not explained. We present one unique case of abdominal pain and hepatomegaly secondary to IVC constriction from extrinsic compression of the diaphragm. Based on this patient's presentation, we urge that physicians be cognizant of the IVC diameter and consider extrinsic compression as a contributor to the patient's symptoms. If IVC compression from the diaphragm is confirmed, early referral to vascular surgery is strongly advised for further surgical intervention.

Key words: Liver imaging; Abdominal pain; Hepatic circulation; Ischemia/reperfusion

Core tip: Common etiologies of abdominal pain with elevated transaminases are clotting disorders and trauma. In this article, we present a rare case of external compression of the diaphragm as the cause of these symptoms that requires surgical intervention to relieve the obstruction.

Grandhe S, Lee JA, Chandra A, Marsh C, Frenette CT. Trapped vessel of abdominal pain with hepatomegaly: A case report. *World J Hepatol* 2018; 10(11): 887-891 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i11/887.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i11.887

INTRODUCTION

Inferior vena cava (IVC) obstruction presenting with abdominal pain and hepatomegaly is generally seen in patients with venous thromboses, including Budd Chiari syndrome, infective phlebitis, or in iatrogenic cases such as post-liver transplant or vascular catheter placement[1-4]. In the realm of obstetrics and gynecology, IVC obstruction is more commonly seen as vessel compression in the late second trimester of pregnancy[5]. Occasionally, tumors, such as renal cell carcinomas, may be initially detected due to compression of the IVC. Herein, we present a unique case of abdominal pain due to IVC constriction from extrinsic compression of the diaphragm. Previously this has been documented in patients with congenital chest wall abnormalities, such as pectus excavatum; however, this patient described below is one of the first to have this pathology without this birth defect[6].

CASE REPORT

A 49-year-old female was referred for further evaluation of hepatomegaly, abdominal pain, and thrombocytopenia. On interview, she endorsed a several year history of right upper quadrant abdominal pain and very mild dyspnea with exertion. She reported that the abdominal discomfort was worse in a sitting position. At the time of initial evaluation she was feeling well with no symptoms of jaundice, pruritus, abdominal pain, nausea, vomiting, edema, or ascites. She also reported no constitutional symptoms.

Past medical history was notable for a 10-year history of mild thrombocytopenia (platelet count 90000-130000) of unclear etiology with negative laboratory workup. Past surgical history was remarkable for an enlarged and nodular appearing liver observed during laparoscopic cholecystectomy performed one year prior due to the same symptoms. The patient has been followed by a hematologist as an outpatient, and a recent liver spleen single photon emission computed tomography scan had confirmed hepatomegaly without splenomegaly. Abdominal ultrasound characterized the liver as 18.1 cm in size with no evidence of cirrhosis or portal hypertension. Patent vasculature was reported throughout with normal hepatopedal flow. The patient had also previously undergone a computer tomography-guided liver biopsy, which showed mild perivascular and pericellular fibrosis but no evidence of advanced fibrosis or cirrhosis.

Physical examination revealed hepatomegaly 4 cm below the costal margin but was otherwise unremarkable. Initial labs included a complete blood count (white count: 4.6, hemoglobin: 13.5, platelets: 112000), comprehensive metabolic panel (sodium: 141, potassium: 3.8, urea nitrogen: 11, creatinine: 0.8, alkaline phosphatase: 51, total protein: 7.3, aspartate aminotransferase: 30, and alanine aminotransferase: 29), and coagulation panel (international normalized ratio: 1.0). Additional workup revealed a ferritin of 28. Antimitochondrial antibody and actin IgG were negative. Ceruloplasmin level was 32. Patient also tested positive for antinuclear antibody titer (1:80, diffuse pattern) and epstein-barr virus IgG. An elevated transient elastography score of 9.6 was also noted.

Due to concern for early cirrhosis in the setting of thrombocytopenia and an elevated transient elastography score, the patient was advised to pursue a healthy lifestyle and abstain from alcohol. A magnetic resonance venography of the abdomen showed no evidence of thrombosis or obstruction.

At this point in time the patient reported worsening, intermittent, epigastric abdominal discomfort that radiated to the right upper quadrant of her abdomen, often waking her up at night and only improved with standing upright or walking. She occasionally felt nauseous but otherwise reported no jaundice, pruritus, edema, ascites, chest pain, or dyspnea. Physical examination showed a positive hepatojugular reflux, consistent with hepatic congestion. The patient was evaluated by a cardiologist, and a transthoracic echocardiogram showed pericardial thickening but no evidence of constrictive pericarditis or systolic or diastolic dysfunction.

The patient then underwent a transjugular liver biopsy with intravenous ultrasound and pressure measurements, which showed an elevated central venous pressure at 13-15 mmHg, wedged right hepatic vein pressure with occlusion balloon measuring 16-17 mmHg, and a dilated IVC of 3 cm cephalad to the patent veins prior to reentry into the right atrium. Significant respiratory variation involving near-collapse of the retrohepatic IVC at end-expiration was noted. There was question of intraluminal narrowing of the retrohepatic IVC down to approximately 10-15 mm, which had significantly improved upon Valsalva maneuver. Right heart catheterization showed hepatic congestion with normal intracardiac and pulmonary artery pressures.

Table 1 Laboratory data pre and post-venolysis

	Pre-venolysis	Post-venolysis
Sodium	137	137
Potassium	4.1	3.7
Chloride	109	104
Bicarbonate	24	31
Blood urea nitrogen	14	10
Creatinine	0.8	0.8
Glucose	111	98
Calcium	7.4	8.3
Alkaline phosphatase[1]	39	---
Albumin[1]	2.9	---
Total protein[1]	5.6	---
Aspartate aminotransferase[1]	49	---
Alanine aminotransferase[1]	43	---
Bilirubin, direct[1]	0.1	---
Bilirubin, total[1]	0.6	---

[1]Post-venolysis alkaline phosphatase, albumin, total protein, aspartate aminotransferase, alanine aminotransferase, bilirubin direct and total were unable to be obtained due to loss of insurance.

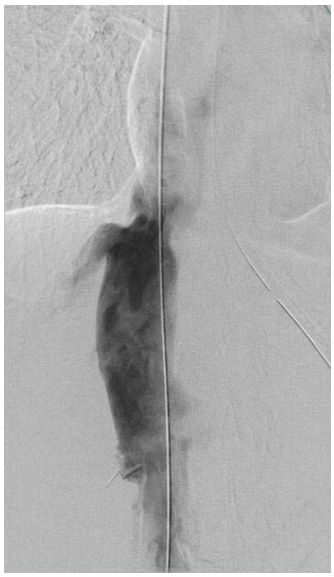

Figure 1 Venogram of the inferior vena cava (pre-lysis). Significant stenosis noted at level of diaphragm prior to exploratory laparotomy, inferior vena cava venolysis, and division of the diaphragmatic constriction.

A multidisciplinary conference among the hepatology, vascular surgery, and cardiology services was held. It was suspected that the diaphragm, *via* the diaphragmatic hiatus through which the IVC was passing, was causing extrinsic compression of the vessel, thereby eliciting symptoms of epigastric and right upper quadrant pain. Repeat transient elastography was still elevated and the patient underwent an exploratory laparotomy with IVC venolysis and division of the diaphragmatic constriction. After the above intervention, resolution of previously identified constriction was noted *via* repeat venogram (Figures 1 and 2) and intravascular ultrasound.

Intraoperative liver biopsy revealed sinusoidal congestion with dilatation in the perivenular areas, features consistent with extrahepatic venous outflow obstruction. The patient recovered remarkably well from the laparotomy. The available pre- and post-venolysis labs are presented in Table 1. At her four week postoperative follow up visit, she reported resolution of her abdominal discomfort and complete ability to perform her activities of daily living without the use of any pain medications. Unfortunately, follow-up liver enzymes were unable to be obtained due to losing her health insurance.

DISCUSSION

The most common etiologies of IVC obstruction are from hypercoagulable states, inflammation, trauma, or recent surgery. Budd Chiari syndrome and hepatic vena cava syndrome, more common conditions associated with hepatic vein outflow obstruction, and hepatic vena cava syndrome may present subacutely or even arise from congenital strictures of the hepatic segment of the IVC[1,2]. While the cause of IVC obstruction is usually due to the abovementioned causes, it is important to consider extrinsic compression from neighboring tumors or even native structures, such as the diaphragm, as in the case outlined above. To date, very few cases have been published attributing abdominal pain or hepatomegaly to compression of the IVC by the diaphragm[7].

Chronic IVC obstruction may be silent in presentation or manifest late with acute symptoms of abdominal pain, hepatomegaly, renal dysfunction, or even unilateral limb symptoms such as leg heaviness, pain, swelling, or even cramping[8,9]. These unusual features may be anatomically related to the extensive network of collateralization of the natural and tributary vessels near the IVC.

The symptoms that arise from extrinsic compression and intrinsic occlusion of the IVC can be explained by understanding the embryological development of the large vessel. As the IVC develops near the liver and diaphragm, new outgrowths from hepatic veins and

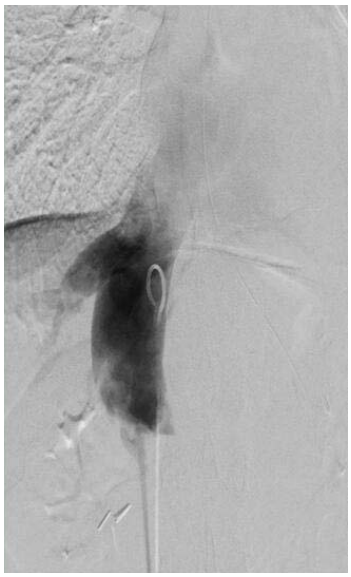

Figure 2 Venogram of the inferior vena cava (post-lysis). Resolution of the stenosis noted at level of diaphragm after exploratory laparotomy, inferior vena cava venolysis, and division of the diaphragmatic constriction.

the infrarenal IVC may make this site more prone to developmental anomalies such as strictures and webs[8]. The patency of the iliac vein is important to collateral function, and occlusion of this vessel usually precipitates acute symptoms of abdominal pain[9]. However, the extent of collateralization may actually prevent patients from developing significant hepatic or renal dysfunction. In addition to the rich vasculature, studies analyzing the interaction between the diaphragm and the IVC during inspiration are limited, but they all support the idea that the size and shape of the lumen of the IVC can be altered by the contraction or anatomy of the diaphragm[7].

The diagnostic workup of IVC obstruction includes a color Doppler sonography and contrast-enhanced computed tomography, magnetic resonance imaging, or venography[9]. Intravascular ultrasound with pressure measurements and cavography provide an additional assessment of hepatic and collateral vein obstructions and thromboses and may indicate if these obstructions are subacute or chronic in nature[4]. Our patient showed evidence of IVC obstruction based on venography and intravascular ultrasound. In terms of therapeutic intervention, endovascular management of hepatic vein outflow obstruction usually includes portocaval shunts or balloon angioplasties with stent implantation[9,10]. Stent implantation *via* balloon angioplasty has proven to be safer with fewer complications of restenosis compared to open surgery[9].

IVC obstruction continues to remain an infrequent cause of abdominal pain and chronic liver disease. While this condition may be rare, it may lead to chronic abdominal pain, cirrhosis, and portal hypertension if not recognized and treated appropriately. Nonetheless, whether from intravascular obstruction, thrombosis, or extrinsic compression from neighboring structures, it is important to keep a broad differential and consider atypical causes of this phenomenon once common etiologies have been ruled out. Referral to vascular surgery may be necessary for surgical intervention, which will ultimately provide symptomatic relief for these patients.

ARTICLE HIGHLIGHTS

Case characteristics

Patients who present with abdominal pain and hepatomegaly are commonly diagnosed as having Budd Chiari or another type of obstruction of the inferior vena cava (IVC) whether it is intrinsic due to thrombosis or an obstruction. However, extrinsic compression, although rare, can also be the culprit of the patient's symptoms.

Clinical diagnosis

Right upper quadrant and epigastric pain and hepatomegaly.

Differential diagnosis

Budd Chiari, infective phlebitis, intravascular obstruction, thrombosis, or external compression from neighboring structures including the diaphragm, kidney, or uterus.

Laboratory diagnosis

Complete blood count, comprehensive metabolic panel, coagulation panel, in addition to labs evaluating causes of cirrhosis including ferritin, anti-mitochondrial antibody, anti-smooth muscle antibody, antinuclear antibody, and ceruloplasmin.

Imaging diagnosis

Color doppler sonography and contrast-enhanced computed tomography, magnetic resonance imaging, or venography.

Pathological diagnosis

Sinusoidal congestion with dilatation in the perivenular areas, features consistent with extrahepatic venous outflow obstruction.

Treatment

Portocaval shunts or balloon angioplasties with stent implantation.

Related reports

A case of IVC compression from the diaphragm has been reported only once in the literature from Louisiana State University Health Science Center in a patient with Pectus Excavatum. Interestingly an article from 1992 demonstrated how radiography can help identify how the IVC can be obstructed, but never specifically discussed a case in which the IVC was externally compressed by the diaphragm.

Experiences and lessons

This case will guide clinicians to think of other etiologies that can cause abdominal pain and hepatomegaly in patients with unremarkable laboratory data. Biopsies are not necessary for this diagnosis. With consideration of this diagnosis, patient care will be expedited with quicker referrals, thereby minimizing the delay in treatment and resolution of symptoms.

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EDITORIAL

Unresolved issues in the prophylaxis of bacterial infections in patients with cirrhosis

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Abstract

Bacterial infections are highly prevalent and a frequent cause of hospitalization and short-term mortality in patients with cirrhosis. Due to their negative impact on survival, antibiotic prophylaxis for bacterial infections in high-risk subgroups of patients with cirrhosis has been the standard of care for decades. Patients with prophylaxis indications include those at risk for a first episode of spontaneous bacterial peritonitis (SBP) due to a low ascitic fluid protein count and impaired liver and kidney function, patients with a prior episode of SBP and those with an episode of gastrointestinal bleeding. Only prophylaxis due to gastrointestinal bleeding has a known and short-time duration. All other indications imply long-lasting exposure to antibiotics - once the threshold requirement for initiating prophylaxis is met - without standardized criteria for re-assessing antibiotic interruption. Despite the fact that the benefit of antibiotic prophylaxis in reducing bacterial infections episodes and mortality has been thoroughly reported, the extended use of antibiotics in patients with cirrhosis has also had negative consequences, including the emergence of multi-drug resistant bacteria. Currently, it is not clear whether restricting the use of broad and fixed antibiotic regimens, tailoring the choice of antibiotics to local bacterial epidemiology or selecting non-antibiotic strategies will be the preferred antibiotic prophylaxis strategy for patients with cirrhosis in the future.

Key words: Cirrhosis; Antibiotic Prophylaxis; Multi-drug resistant bacteria; Spontaneous bacterial peritonitis; Bacterial infections

Core tip: Antibiotic prophylaxis in patients with cirrhosis has proven to be effective in preventing new episodes of bacterial infections and reducing mortality. However, the

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broad and fixed indication of long-term antibiotic therapy in these patients has led to an increase in the emergence of multi-drug resistant bacteria. The development of new strategies for bacterial infection prevention is currently under debate, thus reflecting the need for randomized controlled trials and local epidemiological studies to improve prophylactic antibiotic choice in patients with cirrhosis.

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INTRODUCTION

During the natural history of cirrhosis, patients may suffer from complications that significantly increase their risk of short-term mortality. One of the main culprits of this outcome is bacterial infections[1], which are highly prevalent. One-third of patients with decompensated cirrhosis will develop an episode of bacterial infection during a one-year period[2], and 24% of patients admitted for an infection will develop a second episode during the same hospital stay[3]. Furthermore, bacterial infections are associated with a grim prognosis: patients with end-stage liver disease listed for liver transplantation who suffer an infection have a 42% risk of de-listing or death within 6 mo from admission[4].

For these reasons, antibiotic prophylaxis has been the standard of care in high-risk patients with cirrhosis for decades[5,6]. The rationale is accomplishing selective intestinal decontamination to decrease bacterial translocation, which is considered the trigger event for bacterial infections in cirrhosis. There is also evidence of an immunomodulatory effect caused by certain antibiotics such as fluoroquinolones that might have an additional beneficial impact on patients with cirrhosis survival[7,8].

Currently, there are two clinical scenarios for antibiotic prophylaxis in cirrhosis: prevention of spontaneous bacterial peritonitis (SBP) and prevention of bacterial infections after upper gastrointestinal bleeding.

Antibiotic prophylaxis for the prevention of SBP is currently recommended in most practice guidelines[1,9]. Primary prophylaxis with fluoroquinolones is indicated in patients with poor liver or renal function and a low ascitic fluid protein count, since these patients showed higher short-term survival under prophylaxis in randomized trials[10]. However, a meta-analysis of studies addressing primary prophylaxis in patients with SBP failed to confirm a benefit in survival[5].

The use of norfloxacin after a first episode of SBP to prevent its recurrence is the most robust and widely recognized indication of antibiotic prophylaxis in cirrhosis. Data to sustain secondary prophylaxis for SBP arose from two clinical trials performed in the 1990s and early 2000s. These studies showed that the cumulative incidence of SBP recurrence at one year reached 20%-26% in patients receiving norfloxacin compared to 68% in patients in the placebo group[11,12]. Notably, these randomized controlled trials included fluoroquinolones that were effective in treating Gram-negative bacilli, the predominant etiology of SBP at that time. Whether these antibiotics are useful in today's changing bacteria epidemiology has not been re-assessed; to our knowledge, no other studies evaluating secondary prophylaxis for SBP have been published.

Patients with cirrhosis undergoing an episode of acute gastrointestinal bleeding have also proven to benefit from antibiotic prophylaxis. In a robust meta-analysis conducted by Chavez-Tapia *et al*[13], treatment with antibiotics reduced bacterial infections, all-cause mortality, re-bleeding events and hospitalization length in patients with acute gastrointestinal bleeding.

PITFALLS OF ANTIBIOTIC PROPHYLAXIS IN CIRRHOSIS: EMERGENCE OF RESISTANT BACTERIA AND DRUG TOXICITY

Only prophylaxis due to gastrointestinal bleeding has a known and short-time duration. All other indications imply long-lasting exposure to antibiotics; once the

threshold requirement for initiating prophylaxis is met, without standardized criteria for re-assessing antibiotic interruption, the patient usually maintained antibiotic use until liver transplantation or death. Another usual scenario in clinical practice is the concomitant use of two types of antibiotics as prophylaxis in a single patient (*i.e.*, rifaximin for hepatic encephalopathy and norfloxacin for SBP prevention), with conflicting conclusions as to the efficacy of using only one drug for both objectives[14-16].

Consequences of long-term antibiotic use in patients with cirrhosis are now increasingly being reported. There has been a shift in the type of responsible microorganisms: initially, in the 1990s, Gram-negative bacilli caused two thirds or more of bacterial infections in patients with cirrhosis, whereas in the last 20 years, Gram-positive cocci have been identified in almost one-half of infections in this population[17-20].

Most importantly, the prevalence of resistant and multi-drug resistant bacteria (MDR: bacteria with acquired non-susceptibility to at least one agent in three main antibiotic families[21]) has significantly scaled. The prevalence of MDR bacteria increased by 100% when comparing two studies performed in 2002 and 2007-2011 that analyzed bacterial infections in patients with cirrhosis during hospitalization[19]. In the latter study, MDR infections accounted for 18%-23% of all identified bacteria[22]. Several authors have reported similar or even higher rates of MDR bacteria in different geographies and settings (up to one-half of bacterial infections in health-care acquired settings were caused by MDR bacteria)[23,24]. The main risk factors identified for the development of MDR infections were prior contact with the health-care system, a nosocomial or health-associated origin of infection, the use of norfloxacin prophylaxis, recent use of other antibiotics (cephalosporins or beta-lactams) or recent infection by MDR bacteria[22,23,25].

Higher rates of MDR bacterial infections are parallel with higher rates of inadequate initial empirical therapy. Initial empirical therapy has proven to be insufficient in as much as 90% of bacterial infections, but these rates are dependent on the origin of the infection and susceptibility pattern of the responsible bacteria. As expected, the extension of antibiotic resistance and failure of empirical therapy are an independent predictor of morbidity and mortality[23,26-28]. Currently, it is suggested that empirical antibiotic therapy should be based on the origin and type of infection, its severity, recent antibiotic use and the prevalence of MDR bacteria[29]. Thus, there is a growing need for conducting local studies to identify the epidemiology of MDR bacteria in patients with cirrhosis in each geography (for instance, with microbiological surveillance[29]), as well as exploring other prophylactic strategies for bacterial infections other than extended antimicrobial use.

Last but not least, the prolonged use of fluoroquinolones as prophylaxis may cause significant adverse events. This type of antibiotics has had prior warnings issued by the United States Food and Drug Admnistration referring to disabling and potentially permanent side effects involving tendons, muscles, joints, nerves and the central nervous system. Recently, this agency has strengthened its black box warning for fluoroquinolones, including a separate notice about the drug's potential mental side effects (disturbances in attention, disorientation, agitation, nervousness, memory impairment and delirium) and the risk of coma with hypoglycemia[30].

DIVERGENCE FROM ANTIBIOTIC PROPHYLAXIS TO PREVENT BACTERIAL INFECTIONS

Different alternatives to antibiotic prophylaxis have been suggested - whether replacing or complementing the use of antibiotics - such as the use of probiotics, fecal microbiota transplantation, statins, prokinetics and granulocyte colony-stimulating factor. Other suggested measures include restricting or suspending the use of other types of drugs, such as proton pump inhibitors or beta-blockers, that may influence bacterial infection incidence or outcome[31-39]. However, data regarding the efficacy of these strategies are contradictory or insufficient at the present time. Thus, non-antibiotic strategies are yet to be included in the standard of care practice guidelines.

UNANSWERED QUESTIONS ABOUT ANTIBIOTIC PROPHYLAXIS

The problem that may arise in the near future is the following: if current antibiotic prophylaxis regimens are sustained as the only strategy to prevent infections, and

physicians continue to choose wide-spectrum empiric antibiotics due to the increasing prevalence of MDR bacteria in patients with cirrhosis, what will happen when the available choices for antimicrobials run out? Another concern refers to the maintenance of standard antibiotic prophylaxis in a patient who suffered from an infection caused by quinolone-resistant MDR bacteria. In these cases, whether prophylaxis with the standard antibiotic choice would prevent new episodes of infection is uncertain. Perhaps these patients would benefit from another type of prophylaxis or even with the definite suspension of antibiotic prophylaxis?

CONCLUSION

Broad use of uninterrupted antibiotic prophylaxis in patients with cirrhosis has contributed to a shift in bacterial epidemiology and antibiotic resistance patterns. The emergence of MDR bacteria has negatively impacted the effectiveness of bacterial infection treatment. The need to conduct regional studies to detect the type and antibiotic susceptibility of bacteria causing infections appears to be clear. Perhaps antibiotic prophylaxis could also be tailored to local bacterial epidemiology in the future, to increase its effectiveness and decrease its deleterious effects. Thus, future studies are needed to better understand the role of antibiotic prophylaxis and to put in perspective the actual risks and benefits of current recommendations. If more rigorous and personalized use of antibiotic prophylaxis is not advocated, there may be a time in the near future when physicians run out of options to treat resistant bacterial infections in cirrhosis.

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EDITORIAL

Hepatitis C virus-associated hepatocellular carcinoma after sustained virologic response

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Abstract

The introduction of a direct-acting antiviral (DAA) for patients with hepatitis C virus (HCV) infection, could lead to higher sustained virologic response (SVR) rates with fewer adverse events, and it could shorten the treatment duration relative to the interferon era. Although most recent clinical studies have demonstrated that the occurrence rates of hepatocellular carcinoma (HCC) are decreased by SVR with both interferon-based and interferon-free-regimens, there are several reports about the unexpected observation of high rates of early tumor occurrence and recurrence in patients with HCV-related HCC undergoing interferon-free therapy despite SVR. Several mechanisms of HCC occurrence and rapid immunological changes, including cytokines and chemokines during and after DAA treatment, have also been reported. We focused on the possibilities that HCC occurs or recurs during and after DAA treatment, based on the reported clinical and basic studies. Further studies and observations will be needed to determine the short-term and long-term effects on hepatocarcinogenesis caused by the eradication of HCV with DAAs. New serum biomarkers and a follow-up system for HCV-patients with SVR should be established.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Sustained virologic response; Direct-acting antiviral agents

Core tip: The incidence of hepatocellular carcinoma (HCC) in hepatitis C virus (HCV) patients with sustained virologic response (SVR) after direct-acting antiviral (DAA)

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treatment is a serious health issue. We focused on the role of DAA treatment in hepatocarcinogenesis. DAAs may also lead to rapid changes in immune status through interactions between the host and HCV. Changes in the immune system may play a role in the progression of HCC. Further observations are needed to determine the effects on hepatocarcinogenesis caused by the eradication of HCV with DAAs.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer type[1] and the third most likely cause of cancer related deaths[2]. HCC is associated with chronic liver disease and cirrhosis in > 90% of cases[3]. In the West and Japan, hepatitis C virus (HCV) infection is one of the leading causes of chronic hepatitis, cirrhosis, and HCC. HCV affects approximately 130-210 million people worldwide, or 2%-3% of the world's population[4]. Approximately 20%-30% of chronically HCV infected patients show liver cirrhosis[5], and 1%-4% of cirrhotic patients develop HCC per year[6]. HCC is characterized by a 5-year survival rate of 10%-12%[7].

Recently, several regimens of direct-acting antiviral (DAA) combinations have been developed for the treatment of chronic HCV infection[8]. The introduction of DAA agents has improved sustained virologic response (SVR) rates to approximately 90% and shortened treatment duration[9]. DAAs also help to overcome interferon non-responsiveness[10]. SVR is associated with improved overall survival in HCV infected patients. Recurrence-free survival in HCV infected patients who have undergone resection or locoregional therapy for HCC is also improved by SVR[11].

According to studies from the interferon era, the survival benefit in HCC patients infected with HCV has been postulated to occur through anti-inflammatory, antiangiogenic, and antiviral properties, and interferon-based antiviral therapies were associated with improved outcomes in HCC patients who were infected with HCV during long-term observation[12,13]. However, treatment with DAA therapy can promptly eradicate serum HCV ribose nucleic acid (RNA), and liver failure, including HCC, may occur after the achievement of SVR[14]. In 2016, two articles suggested an unexpectedly higher rate of early occurrence and recurrence of HCC in HCV-infected patients who were treated with DAAs[15,16]. Both had relatively shorter-term follow-up periods after the end of treatment (EOT). However, several articles presenting the opposite data or the data from longer-term follow-up periods have been published. A conclusion has not been reached in this matter and several studies are still ongoing. Considering these circumstances, this report focuses on hepatocarcinogenesis after DAA treatment, which will be discussed based on clinical points of view.

HCC OCCURRENCE AND RECURRENCE AFTER SVR BY DAA TREATMENT

Interferon-free regimens with DAA combination can be used to treat HCV-infected individuals who cannot be treated with interferon-based regimens, such as older patients, patients with comorbidities, patients with cirrhosis, or patients with a history of HCC[17]. HCC recurrence or HCC occurrence, respectively, has been defined as the appearance of HCC in a patient with or without history of HCC [18].

In general, HCV infected patients with advanced liver fibrosis tend to develop HCC, compared to those with mild or moderate liver fibrosis[19]. Patients whose HCC has been curatively treated, also have a much higher risk of recurrence of HCC[20].

In 2016, Conti *et al*[15] reported that DAA therapy induced SVR in 91% of patients. During a 24-wk follow-up, HCC occurrence and recurrence, respectively, were detected in 9 of 285 patients (3.16%) and in 17 of 59 patients (28.8%); a total of 26 patients developped HCC. They also demonstrated that neither HCV genotype nor therapeutic DAA regimen correlated to HCC occurrence or HCC recurrence[15].

Similarly, Reig *et al*[16] reported an unexpectedly high rate and pattern of tumor recurrence coinciding with HCV clearance, suggesting the possible disruption of immune tumor surveillance. In their study[16], 8 (13.8%), 45 (77.6%), 2 (3.4%), 3 (5.2%) patients were HCV genotypes 1a, 1b, 3 and 4, respectively.

Guarino *et al*[18] extensively reviewed the association between DAA and HCC in patients with chronic HCV infection. They reported that, among 11 and 18 studies, the HCC occurrence and recurrence rates ranged from 0 to 7.4% and from 0 to 54.4%, respectively, although their observation periods were relatively shorter.

Li *et al*[21] reported that the short-term incidence of HCC is not increased after the eradication of HCV with DAA and mentioned that the previous reports about higher rates of HCC associated with DAAs may be related to the fact that those patients had a higher risk of developing HCC. Notably, this study also suggests that some patients have a higher risk of developing HCC after achieving SVR with DAA. It is important to elucidate the mechanism of the development of HCC after achieving SVR with DAA and to investigate the patients' characteristics. Thus, the rates of HCC occurrence or recurrence varied from a clinical point of views.

CLINICAL INDICATORS OF HCC OCCURRENCE AND RECURRENCE AFTER SVR BY DAA TREATMENT

HCV-infected patients have a decreased risk of HCC after achieving SVR by interferon treatment[11,22]. Previous studies reported that biomarkers including aspartate aminotransferase (AST), old age, liver cirrhosis and higher posttreatment alpha-fetoprotein (AFP) can predict HCC in patients after interferon therapy[23]. Toyoda *et al*[24] suggested that an elevated indicator of liver fibrosis, the FIB-4 index at SVR24, is also a predictor of HCC development in SVR patients. The FIB-4 index was a prediction of 5-year survival in HCV infected patients in the interferon-era[25].

Nguyen *et al*[26] suggested that AFP decreased significantly from pretreatment (median 7.2 ng/mL) to EOT (4.2 ng/mL) and at 12 wk after treatment (4.2 ng/mL) with DAAs. Liver inflammation increased AFP values in the absence of HCC. Of interest, they suggested that the pattern for normalization of AFP with entecavir showed a shorter period and gradual reduction compared to patients treated with pegylated-interferon.

Similarly, Nagaoki *et al*[27] showed that serum AFP levels decreased to similar levels at SVR24 both in the pegylated-interferon plus ribavirin and the DAAs treatment groups, and similar rates of HCC development existed in these two HCV genotype 1 infected patients groups (the cumulative HCC development rates after 1-, 3- and 5-years were 1.5%, 10% and 19% and 1.5%, 10% and 12%, respectively). These data suggested the possible reduced potential for HCC development by DAA treatment is as same as that of interferon-based treatment.

Moreover, Tag-Adeen *et al*[28] showed significant improvement in the FIB-4 index after achieving SVR by DAA in HCV genotype 4 infected patients. However, they also showed that achieving SVR did not guarantee improvement in cirrhosis (61% of cirrhotic patients showed liver stiffness > 12.5 kPa), and cirrhotic patients still had a risk for HCC development despite achieving SVR by DAA. Thus, from the clinical point of view, several liver fibrosis markers may be helpful for the early detection of HCC occurrence and recurrence.

CHANGE IN CYTOKINES AND CHEMOKINES IN HCC OCCURRENCE AND RECURRENCE AFTER SVR BY DAA TREATMENT

Previous reports suggested that DAA changes the cytokine/chemokine levels compared to the pretreatment levels, and it may be related to hepatocarcinogenesis. Sung *et al*[29] investigated the level of type I interferon, interferon-β in HCV genotype 1b infected patients. Type I interferons bind to a common cell surface receptor, resulting in the activation of the Jak-STAT signal transduction system[30]. Interferon-β may be important not only to prevent patients with acute hepatitis C from developing chronic infection[31] but also to reduce the risk of HCC[32]. After DAA treatment, the expression levels of interferon-β, interferon-induced protein 44 (IFI44) and C-X-C motif chemokine ligand 10 (CXCL10) significantly decreased and rapidly normalized at EOT in the peripheral blood mononuclear cells (PBMCs)[29]. IFI44 and CXCL10 correlated with the pretreatment expression level of interferon-β.

Carlton-Smith *et al*[33] exhibited similar interferon-stimulated gene results in PBMC

at treatment week four and EOT and showed a reduction in CXCL10, CXCL11 and macrophage inflammatory protein (MIP)-1β levels. Hengst *et al*[34] suggested that the expression level of 22 cytokines/chemokines including type I interferon and CXCL10 decreased significantly from baseline to 12 wk after treatment by DAA treatment in patients with HCV genotypes 1, 2, 3 and 4. CXCL10 level was increased in interferon-based therapies by the responsiveness to interferon. In contrast, the interferon system, including CXCL10, is not increased during DAA treatment. A similar effects were also observed in patients infected with HCV genotype 2 or 3 who were treated with DAA[35]. The interferon-stimulated intrahepatic and peripheral gene expression declines with HCV eradication[36].

DAA treatment increased the serum vascular endothelial growth factor (VEGF) level[37,38], and it remained stably elevated at the 3-mo follow-up[38]. These were observed in patients with HCV genotypes 1a, 1b, 2, 3 and 4. VEGF was significantly related to the serum angiopoietin-2 level, and angiopoietin-2 expression in HCC or in cirrhotic tissue before DAAs was related to the risk of HCC recurrence or occurrence. They[38] showed that the extremely high expression of angiopoietin-2 in recurrence of HCC and de novo HCC had its counterpart in the increased levels of circulating VEGF during DAA therapy. Therefore, they suggested that the interaction between the local overexpression of angiopoietin-2 by the slow blood flow of portal hypertension arising through the progression of chronic liver damage and circulating VEGF is a risk factor for developing HCC that is linked with the advanced stage of cirrhosis in patients treated with DAAs[38].

Tumor necrosis factor (TNF)-α, known as an important inflammatory mediator that induces immune responses, was originally found to induce tumor lysis. TNF induces the cellular apoptosis of hepatoma cell lines and HCV core and NS5A proteins block TNF-induced cellular apoptosis[39,40]. TNF-α related apoptosis-inducing ligand (TRAIL) also induces the apoptosis of human hepatic stellate cells and HCV blocks TRAIL-induced cellular apoptosis[41]. Spaan *et al*[42] reported that there is down-regulation in TRAIL-mediated killing by NK cells during DAA therapy in patients infected with HCV genotype 1b. Further studies will be needed. Thus, rapid changes in several cytokines and chemokines are observed during and after DAA treatment and may have several effects on HCC occurrence and recurrence.

IMMUNOLOGICAL MECHANISMS OF HCC OCCURRENCE AND RECURRENCE AFTER SVR BY DAA TREATMENT

Several reports showed that a rapid decreased or normalized immuno-surveillance causes early HCC recurrence or occurrence after DAA therapy. The activating receptor natural killer group 2, member D (NKG2D) and its ligands play a crucial role in the immune response to HCC. Reduced NKG2D ligand expression in HCC correlates with early recurrence[43]. NKG2D predicts the early emergence of HCC after interferon-free DAAs[44].

Major histocompatibility complex class I-related chain A (MICA), which is one of the human ligands of NKG2D, has been known to be a key molecule in viral HCC immune surveillance, as the interaction with NKG2D triggers NK cell-mediated cytotoxicity toward the stressed cells[45,46]. Moreover, HCC sheds membrane-bound MICA as soluble MICA and down-regulates the expression of NKG2D on the NK cell surface because escape immune surveillance[47]. Chu *et al*[44] reported that 12% of DAA-treated HCV genotype 1 infected patients developed HCC recurrence or occurrence within 24 wk after EOT. They suggested that a rapid decrease in NKG2D levels at EOT correlated with early HCC emergence in DAA-treated patients. Of interest, this phenomenon was not found in patients treated with the interferon-based regimen. These data may suggest a risk of early HCC after interferon-free DAA treatment, different from interferon-based therapy. Golden-Mason *et al*[48] reported that the frequency of clusters of differentiation (CD) of 56^{bright} immature NKs decreased 2 wk after DAA therapy started and was maintained at SVR12 in HCV genotype 1 infected patients. Moreover, the downregulation of receptors of cytotoxic signaling including TRAIL, NKp30 and NKp46, was observed 12 wk after DAA therapy started and was maintained at SVR12[48]. They suggest that rapid viral clearance induced by DAA therapy normalizes NK cell function and reduces cytotoxic activity.

The other mechanism was explained by the numbers of peripheral $FOXP3^+CD25^+CD4^+$ regulatory T cells[49]. In their report[49], peripheral $CD4^+$ T cells numbers persisted in DAA treatment groups even approximately 51 wk after EOT in HCV genotype 1 infected patients. In HCV genotype 1a/1b patients, DAA therapy reduced the T-cell compartment in the peripheral blood and re-differentiation of the T lymphocyte memory compartment and resulted in a reduction in the expression of the

coinhibitory molecule T cell immunoglobulin and immunoreceptor tyrosine-based inhibition motif domains (TIGITs) in bulk T lymphocytes[50]. They reported that HCV eradication after DAA therapy involves immune reconstitution[50]. These immune reconstitutions may support the successful treatment of oral lichen planus after DAA therapy[51]. Thus, rapid immunological changes, including in NKG2D systems are observed during and after DAA treatment, and they may have several effects on HCC occurrence and recurrence.

HOST GENETIC FACTORS OF HCC OCCURRENCE AND RECURRENCE AFTER SVR BY DAA TREATMENT

In the interferon era, genome-wide association studies (GWAS) identified that several genetic variants in close proximity to interleukin 28B (IL28B; also known as interferon-lambda 3) variants were strongly associated with the response to pegylated-interferon-α plus ribavirin therapy for chronic HCV infected patients[52-54]. Moreover, IL28B variations are independent predictors of the progression of hepatic fibrosis[55,56] and seems to be involved in the hepatocarcinogenesis[57,58]. However, with the induction of interferon-free regimens, the importance of IL28B genetic variants may be diminishing in HCV genotype 1 infected patients[59]. There have been some reports that showed the relation between single nucleotide polymorphisms (SNPs) and hepatocarcinogenesis.

Lange *et al*[60] revealed a SNP in HLA complex P5 (HCP5) rs2244546, which is a upstream of MICA, as a strong predictor of HCV-related HCC. The differentially methylated cytosine-phosphate-guanine (dmCpG) loci were also reported[61]. The dmCpG loci were highly enriched for enhancers, promoters, or CpG islands and the surrounding regions and were hypermethylated in HCC infected with HCV. The dmCpG loci were associated with cellular growth and proliferation although this report has several limitations[61].

Of interest, Matsuura *et al*[62] investigated GWAS data on hepatocarcinogenesis specifically in HCV-infected patients after the eradication of HCV by interferon-based therapy. There was no difference of development of HCC in HCV genotype 1 or 2 infected patients after eradication of HCV. They found a strong association between the SNP rs17047200, located within the intron of the tolloid like 1 gene (TLL1) on chromosome 4, and the development of HCC, and it played a role in hepatic fibrogenesis. It is uncertain whether interferon-free therapy can inhibit TLL1 after the eradication of HCV. Future studies are needed to evaluate this point.

HCV-mediated enhancement of microRNA miR-373 impairs the JAK/STAT signaling pathway[63]. MicroRNAs associated with HCV-related immunopathogenesis which were found to be enriched in exosomes of HCV viremic patients (in particular, miR-122-5p, miR-222-3p, miR-146a, miR-150-5p, miR-30c, miR-378a-3p and miR-20a-5p), were markedly reduced by DAA therapy. Enrichment of immunomodulatory microRNAs in exosomes of HCV patients was correlated with their inhibitory activity on innate immune cell functions[64]. DAAs against HCV may have an impact on extracellular vesicles including microRNAs, leading to immunomodulation. Thus, several host genetic factors and microRNAs are change during and after DAA treatment, which may have several effects on HCC occurrence and recurrence.

CONCLUSION

DAA therapy is more efficacious for HCV eradication with fewer side effects. The use of DAAs does not increase the occurrence or recurrence of HCC according to clinical trials. However, the mechanism that altered the immunological balance because of a rapid decrease of HCV viral load in the short-term after DAA therapy may contribute to early tumor development (Figure 1). Sasaki *et al*[65] demonstrated the changes of complement cascades and neutralizing antibodies after SVR by DAA. Complement-dependent cytotoxic effects[66] and neutralizing antibodies[67] are also important for HCC cells survival. Thus, a longer follow-up period and basic research are required to establish whether there is a risk or advantage of HCC recurrence or occurrence with interferon-free therapy. Moreover, new serum biomarkers that may be altered by DAA therapy should be investigated in the follow-up of HCV-patients with SVR after DAA and interferon-based regimens.

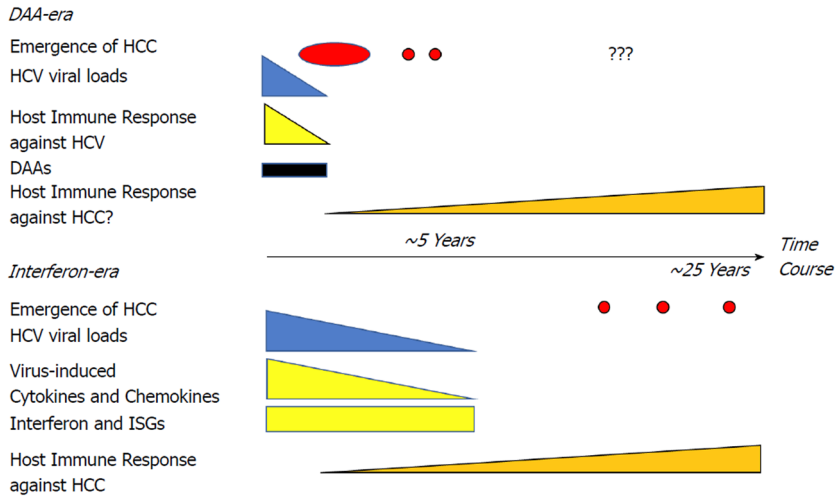

Figure 1 Emergence of hepatocellular carcinoma (HCC) during and after treatment in direct-acting antiviral (DAA) era (upper part) and intreferon era (lower part). It is uncertain whether HCC emerges in patients treated with DAA in more than 5 years after sustained virological response. DAA: direct-acting antiviral; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; ISG: interferon-stimulated gene.

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EDITORIAL

Stem cell transplantation for the treatment of end-stage liver disease

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Abstract

The past two decades have witnessed an explosion of research and clinical application of stem cells, transforming the field of regenerative medicine. Stem cell transplantation has already been performed to treat patients with cancer, liver diseases, and various types of chronic diseases. Indeed, stem cell-based therapies are effective in many diseases, and provide novel insights into the treatment of end-stage liver disease. Several clinical trials have indicated the efficacy profiles of stem cell transplantation in patients with end-stage liver disease, including liver cirrhosis, liver failure, and liver tumors. Animal models of acute liver failure have also provided important insights into the safety, mechanisms, and efficacy of stem cell therapies. Nevertheless, excitement due to this promising field must be tempered with careful and calculated research. In particular, studies on the quality, safety, and efficacy of stem cell transplantation are needed to ensure that qualified products are tested in well-designed clinical trials and approved by governments. Therefore, further investigations are required to effectively balance the safety with the innovation of stem cell transplantation research toward the effective treatment of end-stage liver disease.

Key words: Stem cell transplantation; End-stage liver disease; Clinical treatment; Efficacy; Safety

Core tip: Stem cells have the capacity for multiple rounds of self-renewal and differentiation, and play important roles in numerous biological functions. Treatment of end-stage liver disease *via* stem cell transplantation has emerged as an effective therapeutic alternative in clinical practice. However, caution should be paid to ensuring the safety and efficacy of stem cell transplantation to avoid the use of products that are not rigorously tested that may put patients at risk.

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INTRODUCTION

Due to their capacity for multiple rounds of self-renewal and differentiation, stem cells play roles in numerous biological phenomena including immunomodulation, anti-inflammation, anti-apoptosis regulation, angiogenesis, promotion of tissue repair, and production of growth factors[1-3]. The term "stem cells" represents cells of various origins, including mesenchymal stem cells (MSCs), adipose-derived mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells, hepatic progenitor cells, and hematopoietic stem cells[1,4-8]. However, MSCs are the most common stem cell source for basic and clinical research given the lack of ethical constraints regarding their usage and availability[4,8].

In the past few decades, stem cell transplantation has emerged as a novel and promising therapy for the treatment of patients with cancer, nervous system diseases, eye diseases, orthopedic disorders, diabetes mellitus, and liver diseases. Moreover, advances in stem cell transplantation from basic and translational clinical research have yielded improvements in the survival of patients with benign and malignant hematologic disorders[9] and stem cell transplantation has proven to be an effective therapeutic alternative for central nervous system diseases, including Alzheimer's disease[10]. Moreover, stem cell therapy has been shown to delay or suppress the progression of end-stage liver disease[4,8,11].

TREATMENT OF END-STAGE LIVER DISEASE *VIA* STEM CELL TRANSPLANTATION

To date, there have been numerous clinical studies on stem cell transplantation for the treatment of end-stage liver disease, demonstrating its side effects and efficacy profiles. Furthermore, there were 139 clinical trials registered, including 27 ongoing clinical trials, on the association between stem cell transplantation and liver disease in accordance with the guidelines outlined in ClinicalTrials.gov on July 01, 2018 (http://www.clinicaltrials.gov). Of these, 52 clinical trials were focused on liver cirrhosis (LC), nine on liver failure, and six on liver cancer.

Previous studies indicated that MSC transplantation could constitute an effective treatment for LC. In a multicenter, randomized, open-label, phase 2 trial, autologous bone marrow-derived transplantation of MSCs safely improved liver function and facilitated the quantification of fibrosis following liver biopsy in patients with alcoholic cirrhosis[7]. Another open-label, paired, controlled study from China demonstrated that transplantation of umbilical cord-derived MSCs (UC-MSCs) also improved liver function and reduced ascites in patients with chronic hepatitis B (CHB) and in decompensated LC[1]. MSC transplantation was also shown to improve liver function in LC patients with autoimmune diseases[12]. However, another randomized, controlled phase 2 trial yielded no evidence to support the benefits of granulocyte colony-stimulating factor (G-CSF) administration alone or supplementation of G-CSF with stem-cell transplantation, with no significant dierences in improved liver dysfunction or decreased fibrosis in LC patients after stem cell transplantation[3]. These conflicting results may be associated with differences in LC etiology, an increased frequency of adverse events, and differences in stem cell types used in these studies.

Moreover, studies involving animal models of acute liver failure have shown strong evidence pointing to the success of MSC transplantation in improving liver function, inhibiting hepatocyte apoptosis, and promoting hepatocyte proliferation in animal models of acute liver failure[6], suggesting that MSC transplantation may be used to treat liver failure. In 2012, Shi *et al*[5] performed a case-control study to evaluate the safety and efficacy of UC-MSC transplantation in CHB patients with acute-on-chronic liver failure (ACLF); and found increased survival rates, accompanied by reduced end-stage liver disease scores and enhanced liver function. Another study on MSC transplantation for the treatment of ACLF patients also achieved similar results, in which the treatment increased the 24-wk survival rate, improved liver function, and decreased the incidence of severe infections[11].

Moreover, we recently conducted a systematic review and meta-analysis of MSC transplantation in ACLF patients, which showed that the treatment significantly reduced mortality rates, without increasing the incidence of severe complications[13]. There were also no differences in the incidence of severe complications (*e.g.*, encephalopathy, hepatorenal syndrome, gastrointestinal bleeding) between the standard medical treatment and the MSC treatment group in ACLF patients[5]. Nevertheless, long-term follow-up is needed to confirm the safety of MSC transplantation.

FUTURE PERSPECTIVES

Studies on stem cells and regenerative medicine have received increasing attention in the life sciences in the past 20 years. Stem cells are undifferentiated cells that undergo both self-renewal through symmetric cell division and differentiate into specialized cells, tissues, and organs through asymmetric cell division. Stem cell-based therapies have proven to be effective in many diseases, providing novel insights into the treatment of end-stage liver disease. Indeed, in recent years, numerous studies have reported stem cell-based "cures" for an extraordinary and implausible range of medical conditions.

However, research on the safety and innovation of stem cell transplantation for end-stage liver disease must be well-balanced. Some risky procedures performed without substantial evidence have led to medical accidents, leading to blindness, paralysis, or even death[14].

Moreover, both the administration and the government must be involved in regulations and advisement to ensure the quality, safety, and efficacy of stem cell transplantation. Two finalized tenders regarding guidelines to establish a more stringent policy framework were issued by the Food and Drug Administration (FDA), which included the requirement of sponsors to document a biological license application, request permission from the agency before proceeding to FDA-supervised clinical trials, and obtain agency approval before marketing[15]. To promote rapid yet responsible advancements in the fundamental knowledge and clinical application of stem cells and regenerative medicine, the International Society for Stem Cell Research (ISSCR) has issued three guidelines[2]. The 2016 guidelines revise and extend two prior sets of guidelines (ISSCR, 2006; ISSCR, 2008) and address an integrated set of principles and best practices for ensuring progress in basic, translational, and clinical trials[2]. Overall, safe and effective stem cell transplantation for treating end-stage liver disease will only be achieved from well-designed clinical trials and qualified products approved by the FDA or the government, while avoiding the high risks of unproven cell therapy products.

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MINIREVIEWS

Autoimmune hepatitis: Appraisal of current treatment guidelines

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Abstract

Autoimmune hepatitis affects patients of all ages and gender, across all geographic regions. Although still rare, its incidence and prevalence are increasing. Genetic predisposition conveyed by human leucocyte antigen is a strong risk factor for the disease and may be responsible in part for the wide variation in presentation in different geographic regions. Our understanding of the underlying pathogenic mechanisms is evolving and may lead to development of more targeted immunotherapies. Diagnosis is based on elevated levels of serum aminotransferases, gamma globulins, autoantibodies and characteristic findings on histology. Exclusion of other causes of chronic hepatitis is important. Although undiagnosed disease is associated with poor outcomes, it is readily treatable with timely immunosuppressive therapy in the majority of patients. International guidelines are available to guide management but there exists a disparity in the standard treatment regimens. This minireview aims to review the available guidelines and summarize the key recommendations involved in management of this complex autoimmune disease.

Key words: Autoimmune hepatitis; Treatment; Hypergammaglobulinemia; Autoantibodies; Azathioprine

Core tip: Autoimmune hepatitis, is a rare inflammatory condition of the liver that can affect all ages and gender, across all geographic regions. It has a wide variability in clinical presentation and thus, diagnosis can be challenging. While undiagnosed disease leads to significant morbidity and mortality, timely initiation of treatment leads to favorable outcomes in the majority of cases. Guidelines are available by international societies but there exists a disparity in the standard treatment indications and regimens. In this minireview, we summarize key points from the available literature and guidelines, focusing on appropriate indications and different treatment regimens available.

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INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammatory disease of the liver of unknown cause that can affect children and adults of all ages. The course of the disease can occasionally be fluctuating, but is generally progressive. It is marked by interface hepatitis and lymphoplasmacytic infiltration on histology, serum hypergammaglobulinemia and characteristic circulating autoantibodies. Since it was first described by Waldenström in 1950 as a disease affecting young women characterized by jaundice, high serum gammaglobulins, and amenorrhea causing liver cirrhosis, it has been known by many different labels including "lupoid" hepatitis, but AIH has been accepted as the most appropriate term[1].

Many variant, overlapping forms of AIH exist, particularly with coexisting cholestatic features, primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC). Diagnosis requires exclusion of other causes of chronic hepatitis such as drug induced liver injury (DILI), viral hepatitis, alcoholic hepatitis or non-alcoholic steatohepatitis (NASH) as some of these cases respond to immunosuppressive therapy. A therapeutic response to corticosteroids in AIH was observed in the early 1950s, as well as an early relapse after withdrawal of corticosteroids[2]. By the late 1950s, combined approach with immunomodulators was described and it remains the cornerstone of therapy. Wide heterogeneity of clinical presentation and relatively rare incidence of the disease has limited the advancement in clinical trials. Thus, more than 50 years after its original description, AIH remains a diagnostic and therapeutic challenge.

Guidelines by the American Association for the Study of Liver Diseases (AASLD) (2010) and European Association for the Study of the Liver (EASL) (2015) provide practice guidance on the management of this complex disease[3,4]. The aim of this minireview is to provide an overview of the current treatment guidelines, with an emphasis on appropriate immunosuppressive therapy and difficult to manage cases.

EPIDEMIOLOGY

AIH is a disease that affects all age groups, occurs in all ethnicities and geographic regions but affects the female gender disproportionately. In the United States, women are affected 3.5 times more than men, and 76% of patients in a Swedish study were women[5,6].

Previously considered to be a disease of the young, a recent large Danish nationwide population-based study demonstrated the peak age of incidence at more than 60 years for both men and women. It also showed that both the incidence and prevalence of AIH is rising[7]. Although it is still considered a rare disease, as its prevalence ranges from 16 to 18 cases per 100000 persons in Europe. In Europe and the United States, it accounts for 2% to 3% of the pediatric and 4% to 6% of the adult liver transplantations[3].

The occurrence and clinical course appear to vary according to ethnicity. The disease appears to be more common and more severe in the North American aboriginals compared to the Caucasian population; African-Americans are more likely to present with cirrhosis; patients with Asian or other non-European Caucasoid back ground have poor outcomes. These diverse clinical outcomes between different ethnic groups, within and between countries may reflect differences in genetic predisposition, environmental stimuli as well as complex socioeconomic reasons such as delivery of healthcare[8].

PATHOGENESIS

The model for the pathogenesis of AIH follows the general hypothesis underlying many autoimmune diseases. The disease is thought to arise in a genetically predisposed individual when a potential environmental antigenic trigger sets of a T-

cell mediated immune response directed at liver antigens, leading to a progressive inflammatory process and scarring[9].

Although a definite antigenic trigger has not been found, some of the proposed triggering factors include drugs, toxins and infectious agents. Genetic predisposition to AIH is primarily conveyed by human leucocyte antigen (HLA) haplotype which determines the autoantigen presentation and $CD4^+$ helper T-cell recognition. HLA-DR3 was shown to be strongly associated with the onset of AIH in the Caucasian population. Subsequently characterized as HLA DRB1*0301, it is associated with a younger age of onset and a more severe phenotype. This HLA class II locus determines the shape of the peptide binding groove of the Major histocompatibility complex (MHC) class II complex which presents peptide antigens to the $CD4^+$ T cells. Strong association of DRB1*0301 haplotype with AIH suggests that a specific peptide bound to this complex is recognized by T cells within the liver which then become autoreactive[10].

Various other haplotypes have been found to be associated with AIH in different geographic populations such as DRB1*0401 in Europeans and DRB1*0405 in Japanese[10]. When negative for DRB1*0301, these patients demonstrate a milder form of the disease with older age of onset. Association with varying haplotypes suggests diversity among the peptide antigens triggering the disease but provide a stronger evidence for a T-cell mediated immune reaction driving inflammation and fibrosis.

HLA haplotypes convey the strongest genetic predisposition to AIH. In addition, many other genetic risk factors have been identified, predominantly affecting the immune regulatory function. Particular variant of cytotoxic lymphocyte antigen-4 (CTLA-4)[11], important co-stimulator of T-cells has been associated with AIH. Mutations in the autoimmune regulator (*AIRE*) gene, important for inducing central immune tolerance, leads to a complex autoimmune phenotype, with majority developing AIH.

Knowledge of the underlying genetic predisposition may lead to identification of potential environmental triggers, better understanding of the disease phenotype and development of therapeutic targets in the future, but this as of now appears to be clinically dispensable.

CLINICAL FEATURES

AIH is a heterogenous disease, characterized by a fluctuating course of activity. Therefore, the clinical manifestations are variable. The spectrum of presentation ranges from asymptomatic disease to acute severe hepatitis or debilitating smoldering cirrhosis. Thus, the diagnosis of AIH should be entertained in any patient presenting with signs or symptoms liver disease, whether acute or chronic.

Presentation

Up to a third of adult patients are found to have acute icteric hepatitis[12]. Presentation is similar to acute viral hepatitis and patients may develop non-specific symptoms such as malaise, fatigue, anorexia, nausea, abdominal pain, and arthralgias. Physical exam may be normal or reveal jaundice, hepatomegaly and splenomegaly. Occasionally, patients may have a severe or fulminant presentation with elevated prothrombin time and serum aminotransferase levels in thousands leading to acute liver failure and need for liver transplantation. This presentation is more common in children and relatively rare after 30 years of age.

Many patients with an acute presentation can undergo spontaneous recovery and the initial episode misdiagnosed as a transient illness. Subclinical disease can progress and lead to cirrhosis. Approximately, one third of all adult patients and almost 50% of children already have cirrhosis at the time of diagnosis[13,14]. AIH, can therefore, present for the first time with signs and symptoms of decompensated cirrhosis including ascites, variceal bleeding or hepatic encephalopathy.

Due to the improvement in diagnostic modalities, more than half of all patients diagnosed with AIH have no specific symptoms. They are usually diagnosed upon work up of abnormal liver enzymes detected on routine blood work for other indications. AIH may rarely be diagnosed during pregnancy, or manifest for the first time during post-partum period. Patients with AIH can undergo spontaneous remissions during pregnancy and typically experience flare up in the immediate post-partum period, likely due to immune reconstitution[15].

It is important to keep in mind the concomitant occurrence of other autoimmune diseases with AIH, particularly autoimmune thyroiditis, rheumatoid arthritis, ulcerative colitis, type 1 Diabetes mellitus and celiac disease[16].

Laboratory features

Abnormalities of the liver biochemistry predominantly reflect a hepatocellular pattern with elevated aminotransferases and variable, but usually mild elevation of serum alkaline phosphatase. Any magnitude of serum aminotransferase elevation is possible, and higher elevations are associated with a more severe course and poor outcomes[17].

Generalized elevation of serum gammaglobulins, particularly the IgG fraction is a characteristic feature of AIH. It can be seen in up to 90% of patients with AIH and a diagnosis of AIH should be questioned in patients without hypergammaglobulinemia[18,19].

Autoantibodies

Presence of serum auto-antibodies is a characteristic hallmark of AIH and serological testing is an important part of the diagnostic work up of the disease[1,18]. Antibodies important for the diagnosis of AIH are described in Table 1. Diagnostic value of serological testing also depends on the technique used. Performance parameters for indirect immunofluorescence assays are well defined for diagnosis of AIH and this is the recommended technique for antibody detection[19].

This is performed using rodent tissue or Hep2 cell lines and results are given in titers. It provides the best sensitivity and specificity profile for antinuclear antibody (ANA) and anti-smooth muscle antibody (ASMA). However, it is labor and time intensive, subject to intra-observer variation and requires experienced lab technicians. Therefore, solid phase enzyme immunoassays have gained popularity and are replacing indirect immunofluorescence. These tests are very antigen specific, easy to perform and give rapid results. However, diagnostic parameters for ANA, ASMA detected by enzyme-linked immuno sorbent assay (ELISA) are not well defined and as the recombinant antigens may differ from those detected by indirect immunofluorescence, the results of the two assays should not be equated[20].

Several other antibodies have been evaluated such as antibodies to asialoglycoprotein receptor which are closely associated with histologic activity and may prove useful in defining the treatment endpoint. Antibodies to liver cytosol type 1 can coexist with anti LKM1 in type 2 AIH and are associated with early age of disease onset and severe phenotype. Newer antibodies continue to be characterized to improve the diagnostic accuracy and prognostic value[21].

Histology

Histological confirmation is a prerequisite for diagnosis of AIH[1,18]. It is also useful in guiding therapeutic decisions. Certain characteristic features have been described but none are pathognomonic. Interface hepatitis characterized by inflammation at the parenchymal portal junction is the hallmark feature. Hepatocyte rosette formation, dense plasma cell rich infiltrate and emperiopolesis (active penetration of lymphocytes into hepatocytes) are other common findings. Multi acinar and bridging necrosis is associated with severe disease[22].

DIAGNOSIS AND DIAGNOSTIC CRITERIA

In patients presenting with signs and symptoms of acute or chronic hepatitis, diagnosis of AIH is made on the basis of aforementioned biochemical and serological lab results, and confirmed by liver histology. Difficulties may arise due to the wide variability of presentation, fluctuating disease course, variant forms and presence of co-existing liver diseases. Therefore prior to confirming AIH, it is crucial to exclude other causes of inflammatory hepatitis such as alcoholic or NASH, viral hepatitis and DILI. Other causes of chronic liver disease such Wilson's disease, hemochromatosis and alpha 1 antitrypsin deficiency should be ruled out as well.

Different scoring systems have been proposed to assist in the diagnosis of AIH. In 1999, the International Autoimmune Hepatitis Group (IAIHG) published a comprehensive scoring system which grades every clinical, laboratory and histological feature of AIH, including response to corticosteroid treatment. Initially designed as a research tool for clinical trials, it was useful in clinical practice for patients with few or atypical features of disease. Its complexity and failure to distinguish AIH from cholestatic syndromes limited the clinical utility[1]. In 2008, a simplified scoring system was proposed by the same group for every day clinical practice (Table 2) [18]. It considers the key diagnostic criteria (autoantibodies, degree of hypergammaglobulinemia, liver histology and exclusion of viral hepatitis). It performs well with good sensitivity and specificity (both more than 90%) in diverse populations, and with chronic disease. Its relative ease of use makes it friendly for clinical practice. However, this has not been validated in prospective clinical trials and its utility in acute or fulminant presentations is limited.

Table 1 Serologic markers of autoimmune hepatitis

ANA	Variably expressed with ASMA in type 1 AIH
	Heterogenous antigen profile
	No single staining pattern is pathognomonic for diagnosis of AIH
	Most useful when found with ASMA (diagnostic accuracy 74%)[20]
ASMA	Marker of type 1 AIH along with ANA
	Reacts to several cytoskeletal elements, especially F-actin.
	ELISA against F-actin as the substrate can be used instead of indirect immunofluorescence but may miss the diagnosis in 15% to 20% of cases[20]
Anti-SLA/LP	Only disease specific antibody with specificity of 99% for AIH
	Present in only 15% patients with AIH in the United States
	Known to have a defined antigen, SEPSECS. ELISA is the preferred methodology of testing
	Closely associated with HLA DRB1*03 and Anti-Ro/SSA
	Have prognostic value as it is associated with severe disease, higher risk of relapse and need for lifelong treatment
Anti-LKM1	Serologic marker for type 2 AIH.
	CYP2D6 is the target antigen. Shares homology with hepatitis C virus antigen
	Present mainly in children, worldwide. Rare in adults in the United States (< 4%)
	Associated with HLA DRB*07
Atypical pANCA	Common in type 1 AIH, and absent in type 2 AIH
	Associated with PSC, UC

ANA: Antinuclear antibodies; ASMA: Anti-smooth muscle antibodies; AIH: Autoimmune hepatitis; ELISA: Enzyme linked immunosorbent assay; Anti-SLA/LP: Anti-soluble liver antigen/liver pancreas antibody; SEPSECSA: Sep (phosphoserine) tRNA: Sec (selenocysteine) tRNA synthase; Ro/SSA: Ribonucleoprotein/Sjögren's syndrome A protein; Anti-LKM1: Antibodies to liver kidney microsome type 1; pANCA: Perinuclear antineutrophil cytoplasmic antibodies; PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis.

Scoring systems are not particularly helpful in making the distinction from DILI during acute or hyperacute presentation. Some drugs, such as minocycline and nitrofurantoin, can induce a drug induced autoimmune-like hepatitis and appropriate diagnosis can only be made with passage of time[23]. In severe presentations, steroids should be started and the most likely offending drug should be withdrawn. After normalization of biochemical parameters, steroids should be tapered. *De novo* AIH will typically recur after treatment withdrawal whereas DILI often resolves with the removal of offending agent and does not recur.

TREATMENT

All patients with AIH must be considered candidates for treatment and the timing of therapy rather than the need for therapy is the most important variable to consider. Early studies in 1970s and 1980s showed that untreated patients with moderate to severe AIH, had very poor outcomes, and the 6-month mortality reached as high as 40%. It was also shown that patients treated with immunosuppressive therapy did very well with improvement in biochemical parameters, clinical symptoms and overall mortality[17,24,25].

Liver biopsy should be performed in all patients to make a diagnosis of AIH and before starting treatment. Transjugular liver biopsy may be performed if there is severe coagulopathy. There is general consensus that patients with active AIH [these include patients with aspartate transaminase (AST) > 10 times the upper limit of normal (ULN), AST > 5 × ULN and total IgG > 2 × ULN, or with hepatic activity index > 4/18 on histology] need timely initiation of immunosuppressive therapy. As per the AASLD guidelines, absolute indications for treatment are (1) AST > 10 × ULN; (2) AST > 5 × ULN along serum IgG > 2 × ULN; (3) bridging necrosis or multiacinar necrosis on histology; and (4) incapacitating symptoms such as fatigue and arthralgia[3]. The EASL clinical practice guidelines consensus group recommends treatment for all patients with active AIH[4]. Our recommendation based on review of the literature and clinical guidelines is that all patients with clinical, laboratory or histological features of active liver inflammation should be considered as candidates for treatment as long as they do not have contraindications or risks for significant

Table 2 Simplified diagnostic criteria of the International Autoimmune Hepatitis Group

Parameter	Discriminator	Score
ANA or ASMA	≥ 1:40	+ 1[1]
	≥ 1:80	+ 2[1]
Anti-LKM	≥ 1:40	+ 2[1]
Anti-SLA/LP	Any titer	+ 2[1]
Total IgG	> ULN	+ 1
	> 1.1 × ULN	+ 2
Liver histology	Compatible with AIH	+ 1
	Typical of AIH	+ 2
Absence of viral hepatitis	No	0
	Yes	+ 2

[1]Addition of all points for autoantibodies must be done, maximum 2 points allowed. Definite autoimmune hepatitis (AIH) ≥ 7; Probably ≥ 6. Typical histology for AIH: Each of the following should be present, interface hepatitis, plasma cell infiltrates, emperiopolesis, and hepatic rosette formation. Compatible liver histology: Chronic hepatitis with lymphocytic infiltrations without typical features as above. AIH: Autoimmune hepatitis; ANA: Antinuclear antibody; ASMA: Anti-smooth muscle antibody; Anti-LKM: Anti-liver kidney microsomal antibody; Anti-SLA/LP: Anti-soluble liver antigen/liver pancreas antibody[18].

adverse effects from corticosteroid or azathioprine therapy. Patients with advanced fibrosis and even cirrhosis with active ongoing inflammation on histology should receive therapy as regression of scarring with successful treatment has been reported[17,24,25]. Acute presentation with jaundice as well as subclinical development of fibrosis is more common in childhood, such that more than 50% of the children diagnosed with AIH already have cirrhosis[26]. Therefore, most children with AIH need to be started on treatment. Risks of therapy outweigh any benefits when cirrhosis is already decompensated or there is minimal or no disease activity on histology. Therapy should not be started in such patients.

Benefits of treatment in asymptomatic older individuals without cirrhosis or advanced fibrosis and mild disease activity are unclear. Treatment in such cases must be individualized. The risks of immunosuppression must be weighed against the risk of progression of subclinical disease. Ten-year survival in patients with mild disease without treatment has been reported to range from 67% to 90%[27]. Therefore, the urgency of initiation of treatment is much less in such a patient population. However, AIH can have a fluctuating course with spontaneous remissions and relapses. A significant proportion of asymptomatic patients become symptomatic over time and risk for progression to cirrhosis and HCC is possible. Therefore, the guidelines agree that treatment should be offered to patients with mild disease especially if they do not have contraindications to immunosuppressive therapy. If decision is made to withhold treatment then these patients should be closely monitored with measurement of ALT and total IgG every 3 mo[3,4]. An algorithm for decision making regarding initiation of immunosuppressive therapy is shown in Figure 1.

Induction of remission

The goal of treatment is to obtain complete biochemical and histological resolution of disease. Two treatment regimens are equally effective and are recommended by the AASLD and British Society of Gastroenterology (BSG). First is prednisone monotherapy starting at 60 mg daily, tapered down over 4 wk to 20 mg daily which is then continued until treatment end point. Dose can subsequently be tapered by 5 or 2.5 mg per week to achieve the lowest effective dose of steroids. The BSG, on the other hand, recommends treatment in all cases where the serum aminotransferases are greater than 5 times the ULN, irrespective of the other criteria for treatment[28]. The other is the combination regimen of prednisone starting at 30 mg daily tapered over 4 wk to 10 mg daily and azathioprine 50 mg daily (United States) or 1-2 mg/kg per day (Europe)[3,28].

The evidence for mortality benefit of immunosuppressive therapy with steroids and/or combination with azathioprine was established in a number of controlled trials in the 1960s and 1970s. A sentinel study performed in Mayo clinic in 1972 compared prednisone monotherapy (starting with 60 mg/d, tapered down to 20 mg over four weeks), azathioprine monotherapy (100 mg/d), combination therapy (prednisone starting at 30 mg/d, decreased to 10 mg/d maintenance after 4 wk combined with azathioprine at 50 mg/d) and placebo. There was a significant but similar mortality benefit with prednisone monotherapy and combination therapy

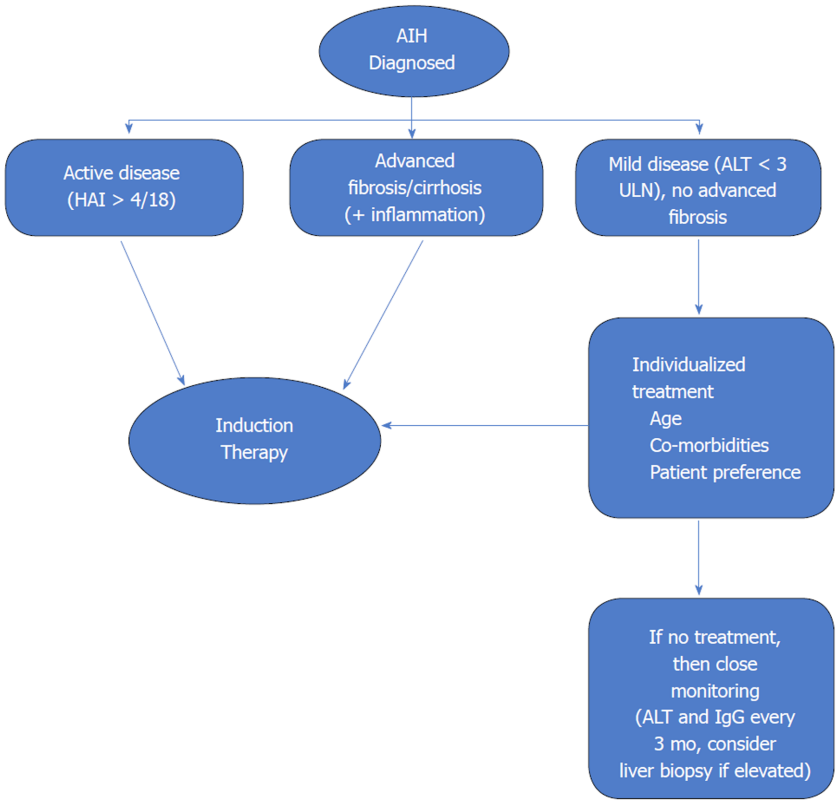

Figure 1 Algorithm for decision making regarding initiation of induction immunosuppressive therapy. Patients with active disease and advanced fibrosis/cirrhosis need initiation of therapy. Patients with mild or asymptomatic disease need an individualized approach. Patients with cirrhosis who have decompensated disease or no inflammatory activity on histology do no benefit from treatment. AIH: Autoimmune hepatitis; ALT: Alanine aminotransferase; ULN: Upper limit of normal; HAI: Hepatic activity index[4].

when compared to placebo (6 % *vs* 7% *vs* 41 %). The combination regimen, however, was associated with fewer side effects (10% *vs* 44%). Seventy-five percent of the patients achieved histological remission, several months after clinical and biochemical remission[17]. This, along with other trials at the time, demonstrated high mortality with azathioprine monotherapy when used for remission induction, likely due to slow onset of action and this was discarded as a valid option.

Thus, the combination regimen was shown to have the best therapeutic profile, and is universally recommended as the first line option[3,4,28].

Upfront combination therapy is especially useful in patients with uncontrolled hypertension, brittle diabetes mellitus, osteoporosis, emotional liability or morbid obesity who are unlikely to tolerate higher doses of steroids well. Similarly, addition of azathioprine may not be appropriate for patients with severe cytopenias, underlying malignancy, pregnancy or established deficiency of thiopurine methyltransferase enzyme (TPMT). Therefore, it is important to individualize regimens based on patient factors and co-morbidities.

Prednisolone is the preferred steroid used in Europe, in contrast to prednisone in the United States, as it does not require intrahepatic conversion to the active form. It also achieves quicker peak plasma concentrations and has greater systemic bioavailability compared to prednisone. Although there does not appear to be a difference in outcomes, it makes sense to use prednisolone in the acute fulminant variant of AIH.

In addition to the classical regimen recommended by the AASLD and BSG, several modifications have been proposed and are being used in clinical practice. A recent questionnaire study by IAIHG evaluated the real-world management of AIH and it suggested wide variations in the initial doses of standard induction therapy and steroid tapering protocols among expert centers[29]. As a general principle, higher the initial steroid dose, faster is the biochemical response with a slightly increased but transient risk of steroid related side effects. Faster induction overall reduces the time to tapering of steroids and thus limiting overall duration of steroid related side effects. In a German cohort, dose of predniso(lo)ne (up to 1 mg/kg per day) in combination with azathioprine lead to more rapid normalization of the

transaminases[30]. A retrospective analysis from Turkey showed a faster biochemical response, less relapses and better survival over 12 mo with starting prednisolone dose of 40 mg and slow taper over 9 weeks in combination with azathioprine in comparison to the standard combination regimen recommended by AASLD[31]. As absence of an early biochemical response is a negative prognostic indicator, strategy to use the most effective dose of steroids for the patient and guide further management based on response is most prudent.

Rate of therapeutic response and dose limiting side effects typically influence the tapering schedule of steroids. Initiation of azathioprine maintenance therapy as soon as possible can help achieve reduction of steroid dose faster. However, in contrast to the AASLD recommendations, and as suggested in the EASL guidelines, it seems reasonable to delay the introduction of azathioprine until early biochemical response is seen from steroid use (usually 2 wk) as that can help clarify diagnostic uncertainties, and differentiate between primary non-response and azathioprine induced hepatotoxicity (although rare, its frequency is increased in advanced liver disease).

It is important to counsel patients about steroid and azathioprine related side-effects. Appropriate adjunctive therapies such as vitamin D and calcium supplementation to prevent bone loss should be given. Vaccination against hepatitis A and B should be completed.

Budesonide, an oral steroid, with a very high first pass metabolism, has been shown in a large, double blind randomized clinical trial to be an effective alternative therapy for AIH[32]. Dose is started at 9 mg daily (3 pills of 3 mg each) in combination with standard azathioprine regimen until remission is induced. A high first pass metabolism leads to less dose limiting side effects when compared to prednisone (28% *vs* 53%). However, this is negated in patients with cirrhosis who may have unpredictable systemic levels due to porto-systemic shunting. Also, the rate of remission induced by budesonide in the study was lower compared to appropriately dosed prednisone[30]. Data regarding the long-term use of budesonide is currently lacking. Therefore, there is a role of budesonide in management of non-cirrhotic patients with AIH who are unable to tolerate systemic side effects of steroids but it is not appropriate for the majority of the patients as a first line agent.

Maintenance therapy

Azathioprine is the drug of choice for maintenance therapy of AIH, as it has been shown to maintain remission effectively in up to 90% patients with fewer side effects compared to low-dose steroid therapy[25,33]. Target dose is usually 1 to 2 mg/kg, but can be titrated up to 2 mg/kg to decrease risk of relapse after steroid withdrawal. To test the tolerance of the drug, it is recommended to start at a lower dose, usually 50 mg daily. Patients should be counseled about the side effects of the drug which include risk of bone marrow suppression, rare risk of malignancy, small risk of drug induced hepatoxicity and pancreatitis. In addition, up to 5% patients demonstrate intolerance to azathioprine manifested by abdominal discomfort, malaise, nausea and fever. Symptoms usually dissipate within 2 to 3 d of stopping the drug. Azathioprine is a category D drug for teratogenic risk, however, all reports from patients treated during pregnancy suggest that it is safe. The risk for maternal and fetal mortality from a disease flare up during pregnancy outweighs any potential harms from the drug, therefore it should be continued at the lowest effective dose to maintain remission during pregnancy.

TPMT is one of the enzymes that is involved in azathioprine metabolism. Patients with genetically determined TPMT deficiency (present in up to 2% of the general population) may be at a higher risk for severe bone marrow suppression. EASL guidelines recommend that when available, serum TPMT testing be performed prior to initiation of azathioprine in patients with AIH. However, not all patients with low levels of TPMT develop bone marrow toxicity and screening for blood TPMT activity has not reduced the frequency of azathioprine related side effects compared with unscreened patients with AIH[34,35]. Therefore, a more pragmatic approach appears to be the initiation of the drug at low dose (50 mg daily) and close monitoring of complete blood count (CBC) (every 2 wk) in the first few months of therapy.

In the first three months of therapy, monitoring of blood counts is done every 2 wk after which it can be spread out. Dose of predniso(lo)ne is tapered in parallel down to 10 mg daily until normalization of transaminases and IgG occurs. Subsequently, steroids can be tapered slowly (in steps of 2.5 to 5 mg daily) every 4 to 12 wk. Transaminases should be closely monitored during this time to detect reactivation of the disease which can be controlled by a transient increase in the steroid dose. Rarely, azathioprine related hepatotoxicity can occur. Usually, IgG levels remain normal in this case and can help differentiate with insufficient treatment response, insufficient dose or lack of compliance. There may be a role of checking thiopurine metabolites (6

thioguanine, 6 methylmercaptopurine) as low levels may indicate lack of compliance. Liver biopsy can help when differentiation between reactivation of AIH or azathioprine related hepatoxicity remains unclear.

Patients who are intolerant to azathioprine, have several alternative modalities for maintenance. 6-mercaptopurine, the active metabolite of azathioprine, can be used in up to 50% of the patients intolerant to azathioprine. However, this should not be tried in patients have had pancreatitis, hepatotoxicity or severe bone marrow suppression secondary to azathioprine.

Mycophenolate mofetil (MMF) has been established as an effective second line agent for AIH[35,36]. It appears to be more useful for patients who suffer from azathioprine intolerance rather than treatment failure with azathioprine. It is tolerated very well and at a target dose of 2 g/d, it can maintain a stable remission rate around 70%[37]. It has been reported to have teratogenic properties and should be prescribed to women of childbearing age with due precaution. Lastly, for patients with mild disease and good tolerance to steroids, chronic low dose predniso(lo)ne at 10 mg daily or less is a viable option to maintain remission.

With good compliance and standard treatment regimens, majority of the patients achieve and maintain sustained remission. 10-year life expectancy for patients with and without cirrhosis and AIH is 89% and 90% respectively, in tertiary referral centers. Overall 10-year survival rate of 93% approaches that of age matched cohorts of the general population[38].

Treatment withdrawal

Most patients with AIH will need lifelong maintenance therapy. This is because only 20% of patients with AIH can maintain a sustained remission after withdrawal of all immunosuppressive therapy[39]. However, this does not preclude a consideration of trial of treatment withdrawal in appropriate candidates. Treatment should be continued for at least 2 years after complete biochemical remission (normal transaminases and normal total IgG) has been achieved. This is because histological resolution lags behind biochemical remission. Patients with persistent mild elevation of transaminases and/or IgG, or intermittent flares during maintenance therapy are likely to experience disease relapse, and treatment withdrawal should not be attempted in them. A recent study showed that patients with ALT levels less than half the ULN and IgG levels in the lower range of normal (< 12 g/L) were much more likely to maintain sustained remission of medications[40]. Liver biopsy can be helpful in excluding a trial of withdrawal as mild ongoing inflammation [hepatic activity index (HAI) > 3] is a strong predictor of relapse. However, a normal liver biopsy is a poor predictor of the probability of relapse. When a decision is made to attempt treatment withdrawal, azathioprine is slowly decreased with careful monitoring of transaminases. Patients who have been successfully weaned off medical therapy should be monitored at regular intervals as up to 50% suffer a relapse within 6 mo, most within 2 years but relapses decades after remission have also been described. Treatment with the initial induction regimen usually helps to get the disease under control. Patients who undergo repeated relapse have higher incidence of cirrhosis, death from liver failure, higher rate of drug induced side effects and overall adverse outcomes[41]. Therefore, lifelong maintenance therapy is needed in patients who suffer a relapse.

Difficult to treat patients: Incomplete and non-responders

Most patients respond well to standard immunosuppressive regimen and at least 10% to 15% appear to be refractory. This can be due to non-compliance, partial response or true non-response.

As biochemical response to immunosuppressive regimen is the norm, non-response to treatment (lack of more than 25% reduction in transaminases after two weeks) should lead to re-evaluation of the diagnosis. Alternative etiologies such as Wilson's disease, DILI, NASH should be definitively ruled out. Occasionally variant forms with overlapping features of PBC, PSC preclude full normalization of enzymes.

Compliance with treatment regimen should be ascertained. Measurement of 6 thioguanine (6TGN) levels can be helpful in this regard. A level > 220 pmol per 8×10^8 red blood cells has been shown to be associated with remission in AIH patients[42]. Lack of detectable serum levels would indicate lack of compliance.

Patients who present with a severe acute hepatitis are more likely to fail standard therapy. Limited data is available on management of such patients. Overall mortality is high (19% to 45%) and liver transplant (LT) evaluation should be initiated. A trial of high dose intravenous corticosteroids (> 1 mg/kg) should be given however a definite futility threshold is not defined. Generally, failure to improve model for end-stage liver disease (MELD)-Na, or serum bilirubin within 7 d of initiation of therapy should lead to alternative treatment strategies including LT.

Some patients with AIH fail to achieve full clinical, laboratory and histologic remission after 3-year standard therapy and are said to have incomplete response. Attempt should be made to optimize dosing of the standard regimen (increasing azathioprine to 2 mg/kg per day with addition of 5 to 10 mg of predniso(lo)ne). If complete response remains elusive, then the goal of therapy is to maintain lowest possible biochemical activity while minimizing side effects. Serum transaminases less than 3 times the ULN are acceptable and azathioprine monotherapy at 2 mg/kg per day is usually reasonable.

Fortunately, true non-responders to standard regimens rare (< 5%) and alternative immunosuppressive agents are needed for these patients. Data for use of these agents in AIH is limited and based on small, mainly retrospective case series as no randomized control trial has been conducted. Largest experience is available for calcineurin inhibitors (CNI) cyclosporine and tacrolimus, primarily as salvage therapy with very effective biochemical response (> 90% for both)[43,44]. These drugs are associated with significant long-term side effects including risk of infections, hypertension, renal dysfunction and diabetes mellitus, and once initiated, they need to be continued permanently.

Recently, role of anti-tumor necrosis factor (TNF) monoclonal antibody, infliximab has been shown to have a positive response in difficult to treat patients with AIH[45]. In addition to inducing stable remission, it was shown to have a beneficial effect on liver histology. Its efficacy is supported pathophysiologically by presence of increased TNF secretion and TNF-positive T cells in the liver of patients with AIH. It should be noted, however, that anti-TNF biologics can themselves induce an AIH-like drug induced syndrome.

LT

AIH and its complications account for up to 5% of all liver transplants, typically for acute fulminant presentations or for advanced decompensated cirrhosis. LT for AIH is an effective intervention, with 10-year patient survival of approximately 75%[3]. Both recurrent and "*de novo*" AIH can occur post liver transplantation, and are treated similarly, using a combination of glucocorticoids and azathioprine. Anti-rejection medications including CNI have not been shown to prevent nor effectively treat recurrent AIH. For refractory cases, switching azathioprine to MMF or changing the calcineurin inhibitor has been recommended[3].

CONCLUSION

Management of AIH, since its initial description, has seen tremendous growth (Figure 2). Most patients can expect near normal life expectancy and a reasonable quality of life. However, there still exists wide disparity in delivery of care and patient outcomes. Our understanding of the underlying pathogenetic mechanisms, although advanced over the years, is still limited and far away from having significant clinical implications. Rarity and heterogeneity of the disease, excellent response to standard treatment regimens besides economic factors guiding the pharmaceutical industry has limited the development of more specific therapies. Further research into the pathogenesis of the disease may lead to development of more definitive serological diagnostic tests as well as targeted immunotherapies addressing the underlying inflammatory mechanisms in the future.

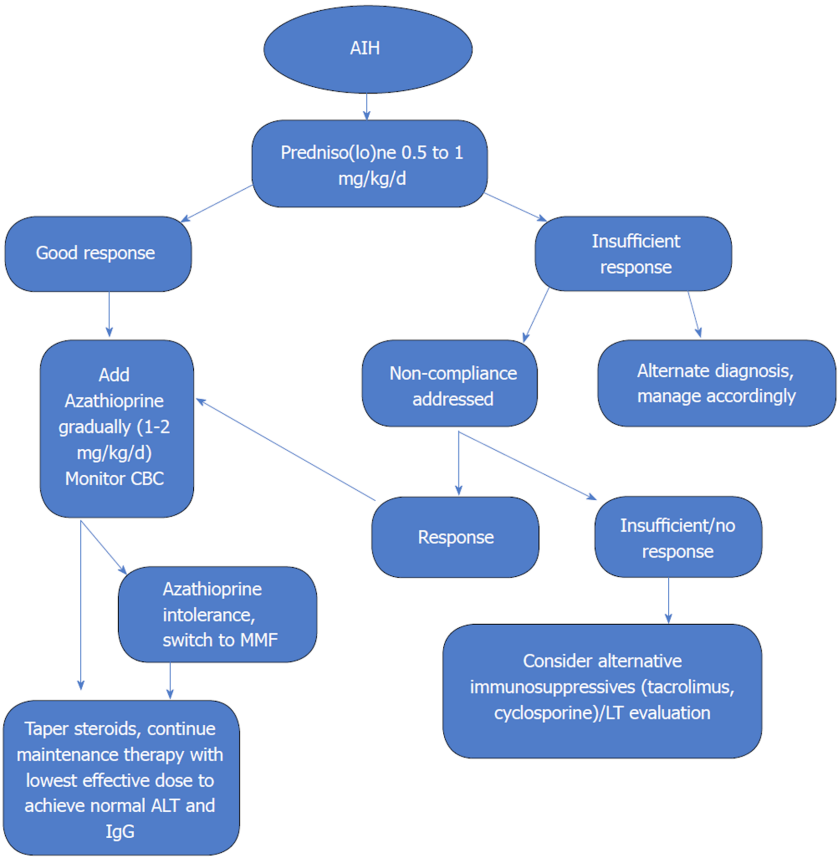

Figure 2 Treatment strategy in autoimmune hepatitis. Treatment includes induction and maintenance therapy to achieve biochemical remission. Induction is achieved by steroids and after a positive response (more than 25% reduction in serum aminotransferases after two weeks) is seen, azathioprine is introduced to achieve long term remission. Timely and appropriate maintenance therapy with azathioprine allows for steroid withdrawal. AIH: Autoimmune hepatitis; ALT: Alanine aminotransferase; CBC: Complete blood count; MMF: Mycophenolate mofetil; LT: Liver transplant[4].

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ORIGINAL ARTICLE

Basic Study

Body mass index and its effects on liver fat content in overweight and obese young adults by proton magnetic resonance spectroscopy technique

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Abstract

AIM

To assess the association between liver fat content (LFC) and weight status in young adults using proton magnetic resonance spectroscopy (^{1}H MRS) technique.

METHODS

Seventy-eight healthy young adults, between 19-30 years of age participated in this study. This group was then separated into a control of 39 subjects and an overweight/obese group (OW/OB group) consisting of 39 subjects. Blood biochemical quantity and ^{1}H MRS was performed for LFC assessment.

RESULTS

LFC was found to be almost three times higher in OW/OB group when compared to the control group. A 48.7% incidence of non-alcoholic fatty liver disease in the OW/OB group was found. Blood biochemical measurements showed statistically higher low-density lipoproteins and triglyceride, lower high-density lipoproteins, and increased glycosylated hemoglobin and fasting glucose in the OW/OB group. Body mass index was a significant independent predictor for LFC after adjusting for age and sex (multiple linear regression; $\beta = 0.459$, $P < 0.001$).

CONCLUSION

Due to the prevalence of high LFC in the OW/OB group, it can be proposed that weight gain and obesity are sensitive indicators of high hepatic fat content.

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Core tip: Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases. The prevalence of NAFLD in young adults is a growing public health concern. Interestingly, the liver fat content (LFC) of an overweight/obese group was approximately three times higher than the control group. This result suggests that obesity can increase LFC and is a risk factor for higher NAFLD in overweight and obese young adults. This current study also demonstrated the importance of Body Mass Index as a tool for risk prevention and control of NAFLD and metabolic syndromes.

Pasanta D, Tungjai M, Chancharunee S, Sajomsang W, Kothan S. Body mass index and its effects on liver fat content in overweight and obese young adults by proton magnetic resonance spectroscopy technique. *World J Hepatol* 2018; 10(12): 924-933
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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases and is increasing at an alarming rate. Previous studies have reported a positive correlation between BMI and lipid accumulation in the liver, which leads to a higher risk of NAFLD, cirrhosis[1,2], and dyslipidemia[3].

Due to modern lifestyles and diet, there has been a persistent increase in the number of NAFLD patients. This increase occurred at the same time that there were also increases in the number of people considered to be obese all over the world[4,5]. NAFLD in young adults is a topic that has received slight recognition, yet this age group is the most likely to gain weight and develop obesity from diet and lifestyle as they are transitioning into adulthood[6]. The prevalence of NAFLD in young adults has increased almost 2.5 times over 30 years with half of morbidly obese young adults having NAFLD[7]. However, despite the growing public health concern about obesity and NAFLD in young adults, necessary information addressing the effects of obesity and NAFLD pathogenesis in this age group is lacking, and there is an urgent need for better consideration of its effects and mechanisms[8,9]. Proton magnetic resonance spectroscopy (^{1}H MRS) is a well-established non-invasive technique for liver metabolite assessment and is known for its high accuracy for determining liver fat quantification when compared to biopsy[10,11]. As far as we know, there's no study to date that has investigated the effects of obesity on liver fat content (LFC) by ^{1}H MRS in healthy young adults.

The aim of this present study is to assess the association between LFC by ^{1}H MRS technique, blood serum biochemical measures of total cholesterol (Cho), low-density lipoproteins (LDL), high-density lipoproteins (HDL), fasting plasma glucose (FG), glycosylated hemoglobin (HbA1c), and being overweight/obese (OW/OB) as a young adult.

MATERIALS AND METHODS

Study population

The subjects of this current study were 78 healthy young adults between 19-30 years of age. Subjects were randomly chosen from a young adult population residing in Chiang Mai, Thailand through recruitment efforts using posters, or were personally invited to join the study. The control group was comprised of 39 subjects who had engaged in moderate physical activity, and who had a body mass index (BMI) in the normal range according to the World Health Organization (15.8-24.9 kg/m^2)[12]. The OW/OB group was comprised of 39 subjects who had a BMI that was in the overweight and obese range (> 25 kg/m^2)[12]. Exclusion criteria for both groups was diagnoses with a chronic disease or liver injury in any form, alcohol consumption of more than 150 g/wk, hyperglycemia (FBS > 140 mg/dL), hypertriglyceridemia (TG >

300 mg/dL), hepatotoxic medication usage, athletes, contraindication for magnetic resonance imaging (MRI), and poor ^{1}H MRS resolution. Subjects were given a questionnaire about health and lifestyle in order to include or exclude subjects for the study. Eating and exercise habits, occupation, and personal and family medical history were also provided. The Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (AMSEC-61EX-016) approved all procedures.

LFC assessment by 1H MRS

Liver metabolite spectra were obtained by ^{1}H MRS technique on MRI 1.5 T (Achieva, Philips Medical Systems, Best, The Netherlands) using sense cardiac coil. T2-weighted turbo spin echo (TSE) transverse (TR/TE = 871/80 ms) and coronal T2-weighted (TR/TE = 829/80 ms) images were applied for localization. PRESS sequence with TR = 2000 ms, TE = 43 ms, number of signal averages = 96. Voxel size of 10 × 10 × 10 mm^3 was carefully placed in right lobe of the liver (Couinaud lobe segment V-VIII), carefully avoiding any large vessels and bile duct. The liver metabolite signals without water suppression were obtained and analyzed for metabolized quantification by AMARES algorithm available on jMRUI software[13-15]. Spectrum fitting and quantification was done for water peak (4.72 ppm), and major lipid spectrum peaks (CH_3= 0.9 ppm, CH_2= 1.3 ppm, 2.1 ppm) with prior knowledge and Gaussian line shape was then applied[16]. Signal intensity correction was done for T2 relaxation using linear least-square equation with previous determination for T2 of water and fat. LFC was calculated by a validated method described elsewhere[17,18]. NAFLD was determined as LFC > 5.56%[18].

Blood examination

Blood collection of subjects was done by The Associated Medical Science Clinical Service Center, Chiang Mai University. Ten milliliters of intravenous blood was drawn from antecubital veins and was biochemically analyzed using a fully automated analyzer (Architect ci8200, Abbott Diagnostic). The test focused on Cho, HDL, VLDL, TG, FG, and HbA1c. Subjects were told to fast for 10-12 h prior to blood examination. Later, LDL concentration was calculated from novel adjustable LDL estimation equations[19,20].

Dyslipidemia was described as an abnormality of Cho levels in plasma including increased Tri and LDL, and decreased HDL. The National Cholesterol Education Project (NCEP) Adult Treatment Panel (ATP) III has defined dyslipidemia as Cho ≥ 200 mg/dL, Tri ≥ 150 mg/dL, LDL ≥ 130 mg/dL, and HDL ≤ 40 mg/dL[21]. Normal FG ranges should be between 70-100 mg/dL, and FG between 100-125 mg/dL is considered prediabetes. Normal HbA1c levels should be less than 6%[22].

Anthropometry

The same examiner measured every subject. Subjects wore only an examination cloth. Height and bodyweight were measured to the nearest 0.5 cm and 0.1 kg respectively. Waist circumference (WC) and hip circumference (HC) was acquired while instructed to breathe out mildly. Both measurements were done using non-elastic tape. WC was measured at the midpoint of the lower margin of the rib and the top of the iliac crest. HC was measured at the widest section of the buttocks. Waist-to-hip ratio (W/H ratio) was calculated from WC divided by HC.

Statistical analysis

Statistical analysis was performed using SPSS statistical software version 17.0. Normal distribution results are expressed as mean ± SD. The Kolmogorov-Smirnov test and the Shapiro-Wilk test were performed to determine data normality. Comparison of LFC and blood biochemical examination between groups was then further compared with an unpaired samples *t*-test. Relationship between groups was done with Pearson correlation. Multiple stepwise linear regression analysis was used to verify the relationships between LFC and independent of significant corelate variables. Results with P value < 0.05 were considered statistically significant.

RESULTS

A total of 78 healthy subjects in the young adult age group (19-30 years old) participated in this study. The control group of 39 subjects and the OW/OB group of 39 subjects had an average BMI of 20.9 ± 0.3 and 31.3 ± 0.5 kg/m^2, respectively. The characteristics of LFC, anthropometric, and biochemical data of all subjects are shown in Table 1.

Seventy-eight spectra were obtained and were analyzed for LFC. The corrected

Table 1 Characteristic and biochemical analysis of 78 subjects in the control and overweight/obese groups

	Control group	OW/OB group	*P*-value	Correlation with LFC	
				r	*P* value
n	39	39	-	-	-
Gender (male/female)	12/27	24/15	-	-	-
LFC (%)	2.7 ± 0.2	8.1 ± 1.0	< 0.001[b]	-	-
Age	22.3 ± 1.6	22.1 ± 0.3	0.662	-0.058	0.611
BMI (kg/m^2)	20.9 ± 0.3	31.3 ± 0.5	< 0.001[b]	0.531	< 0.001[b]
WC (cm)	74.6 ± 1.4	112.6 ± 7.4	< 0.001[b]	0.259	0.022[a]
HC (cm)	90.7 ± 1.3	122.5 ± 7.5	< 0.001[b]	0.212	0.062
W/H ratio	0.8 ± 0.0	0.9 ± 0.0	< 0.001[b]	0.388	< 0.001[b]
FG (mg/dL)	83.1 ± 1.1	89.9 ± 1.1	< 0.001[b]	0.144	0.21
Cho (mg/dL)	187.3 ± 6.8	200.7 ± 6.1	0.147	0.093	0.419
Tri (mg/dL)	77.8 ± 5.2	117.1 ± 8.8	< 0.001[b]	0.223	0.05
HDL (mg/dL)	59.3 ± 2.5	47.7 ± 1.4	< 0.001[b]	-0.185	0.105
LDL (mg/dL)	111.1 ± 5.6	130.1 ± 5.1	0.014[a]	0.133	0.246
HbA1c (%)	5.1 ± 0.1	5.5 ± 0.1	< 0.001[b]	0.345	0.002[a]

Data expressed as mean ± SD.
[a]$P < 0.05$;
[b]$P < 0.001$. OW/OB: Overweight/obese; LFC: Liver fat content; BMI: Body mass index; WC: Waist circumference; HC: Hip circumference; W/H ratio: Waist-to-hip ratio; FG: Fasting plasma glucose; Cho: Cholesterol; Tri: Triglyceride; HDL: High-density lipoproteins; LDL: Low-density lipoproteins; HbA1c: Glycosylated hemoglobin.

value of liver fat by weight was calculated by a method validated by Longo *et al*[17] and Szczepaniak *et al*[18]. Representative spectrum from the right lobe of the liver was shown in Figure 1.

As expected, LFC, anthropometric, and biochemical results were significantly different between the two groups with the exception of age and Cho. The OW/OB group reported statistically higher BMI, LFC, WC, HC, FG, Tri, LDL, HbA1c, and statistically lower HDL. Cho also was found to be increased in the OW/OB group, but this tendency was not statistically significant. The prevalence of dyslipidemia in the OW/OB group (69.2%) was higher than in the control group (48.7%). There were no subjects in the control group who exceeded the normal FG and HbA1c ranges.

Interestingly, the LFC of the OW/OB group was approximately three times higher than the control group. Additionally, 19 subjects (48.7%) in the OW/OB group had LFC > 5.56%, which is considered to be a cut off point for NAFLD according to a previous large cohort ^{1}H MRS LFC study[18]. Furthermore, dyslipidemia was present in 47.4% of participants in the OW/OB group, and abnormal HbA1c was found in 10.5% of OW/OB subjects.

The data in this study was normally distributed. Pearson correlation analysis was conducted as preliminary analysis for possible predictor variable for LFC and is presented in Table 1. Various statistically significant correlations of LFC and variables were found, with moderate correlation occurring with BMI and mild correlation with W/H ratio, HbA1c, and waist circumference. Among the blood biochemical results, HbA1c showed the highest correlation with LFC followed by Tri. The Pearson correlations and data distribution by sex in both groups is shown in Figure 2. This indicates that the overall data between male and woman in each group is distributed in the same way.

The correlation was then compared between HbA1c and FG to determine the indicator for diabetes. Even if a low positive correlation was found in FG, it is not statistically significant, while the HbA1c showed a statistically significant positive correlation with LFC. The correlation of diabetes (HbA1c and FG) markers is compared in Figure 3.

A multiple linear regression was used to predict the LFC from significantly correlated blood biochemical marker (HbA1c, Tri) and anthropography marker (BMI, W/H ratio). Standardized coefficient and correlations are presented in Table 2. BMI and HbA1c were found to be significant positive independent predictor for LFC after adjusting for age and sex. However, only BMI remained statistically significant as an

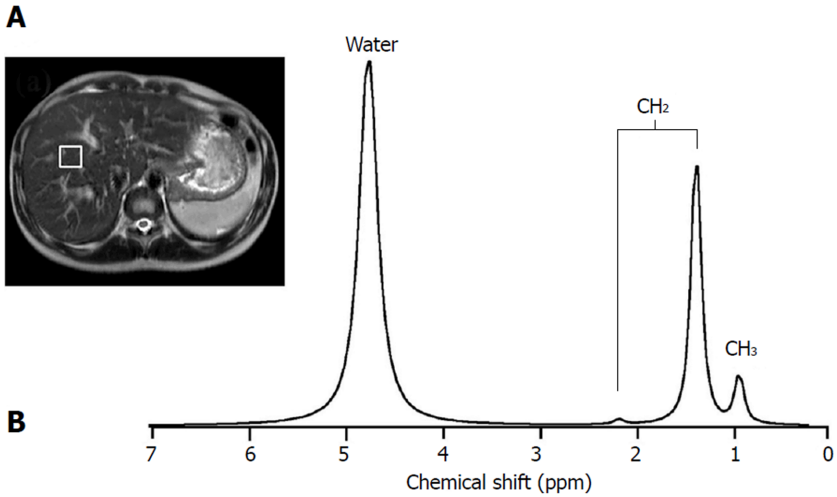

Figure 1 Proton magnetic resonance spectroscopy technique was used for liver fat assessment. Water peak was shown occurring at 4.72 ppm, peaks in fat for CH_3 occurred at 0.9 ppm, and CH_2 peaked at 1.3 ppm and 2.1 ppm. A: Magnetic resonance imaging axial image of abdomen show voxel localization in right lobe liver for liver fat content quantification. B: Representative fitted proton magnetic resonance spectroscopy spectrum of right lobe liver.

independent predictor for LFC after HbA1C adjusting for age, sex, and BMI.

DISCUSSION

In recent years, the prevalence of NAFLD in young adults has been increasing at an alarming rate that parallels with the global epidemic of weight gain and obesity. The prevalence of obesity in young adult is double that of younger ages[8]. Various studies have stated the close relationships between obesity, dyslipidemia, insulin resistance, and NAFLD[23,24]. MRI has proven to be a powerful imaging tool for liver cirrhosis diagnosis[25], and is known for its ability to non-invasively and accurately quantify liver fat using the ^{1}H MRS technique, which is suitable for longitudinal follow-ups when compared to liver biopsy. Liver biopsies are the gold standard, but are an invasive method.

The results of this study confirmed once again the association between BMI and LFC, the higher risk of dyslipidemia, the probability of insulin resistance, and the prospect of metabolic disease in young adults.

The highlight of this study is that LFC in the OW/OB group is higher when compared to the control group, even if both groups were revealed to be healthy. This prevalent rate is consistent with earlier findings where 57.4% of NAFLD subjects in young adults also had high BMI[7,26]. This tendency should also be considered with a higher prevalence of dyslipidemia, prediabetes, and hyperglycemia among subjects with LFC > 5.56%. In accordance with the present results, previous studies have demonstrated that the risk for dyslipidemia starts to increase progressively with a BMI over 21 kg/m^2 and LDL and Tri levels are used to evaluate the risk for coronary artery disease[27].

A new important finding is that the biochemical and anthropographic markers associated with LFC are significantly different between the OW/OB group and the control group. Among the blood lipid markers, Tri and LDL were found to be statistically higher, and HDL was found to be statistically lower when compared to the control group. However, no significant differences were found for Cho, even though the Cho in the OW/OB subjects had increased slightly with almost half of the control group having dyslipidemia. This could be explained by the fact that the two characteristics of the subjects in this age group were that they were exposed to high caloric, low fiber "ready-to-eat" foods, consumed sugary beverages, and had low physical activity. It can be expected that these effects can change the Cho levels in blood[6,28].

The Pearson correlation analysis showed moderate correlation of BMI and LFC and mild correlation with W/H ratio and WC. The association of BMI and LFC was additionally confirmed by multilinearity regression analysis as a significant independent variable after being adjusted for age, sex, and other anthropometric variables. This outcome is dissimilar with previous studies that proposed that W/H ratio can be used as a tool to predict the risks of liver cirrhosis and NAFLD in place of

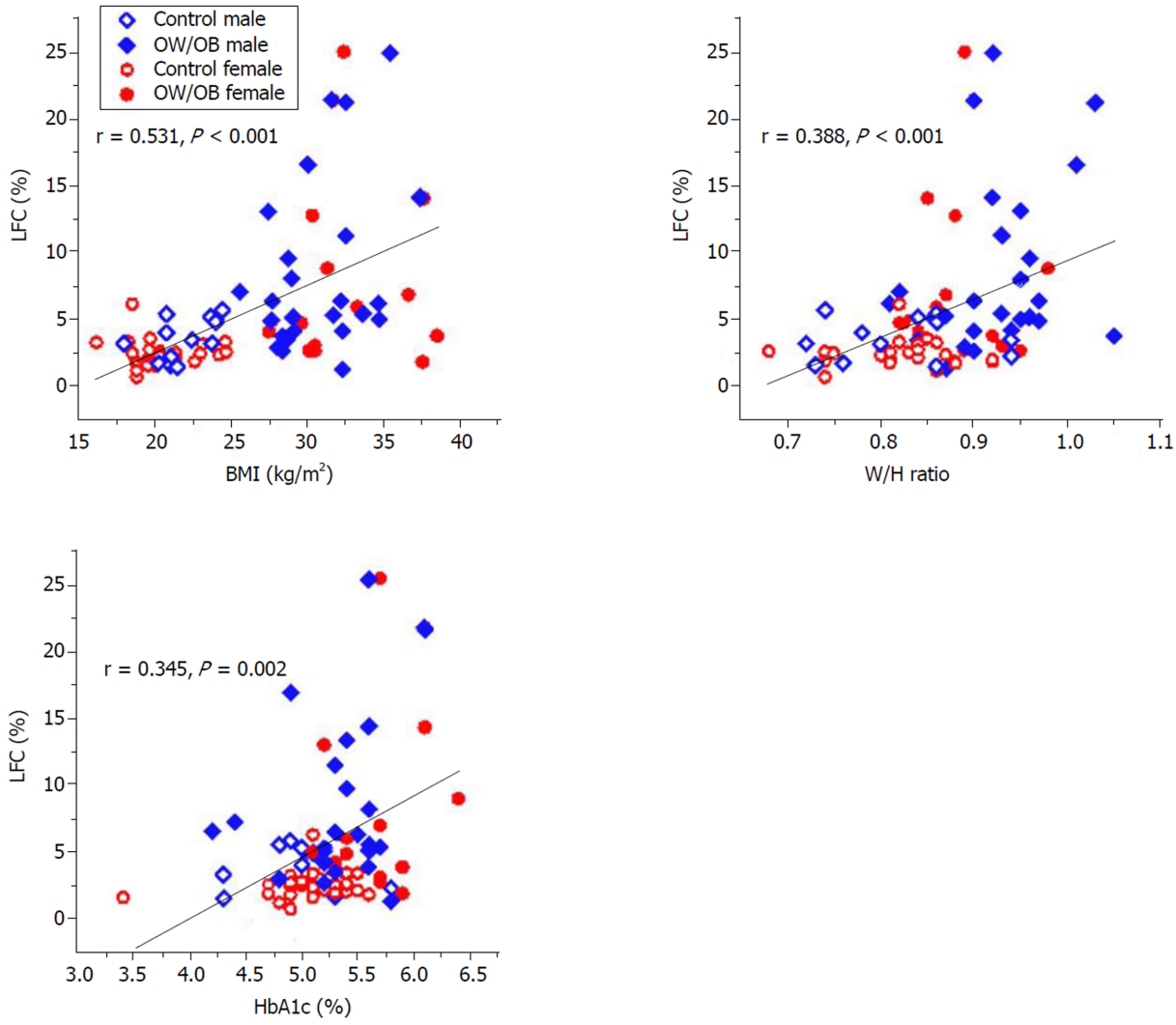

Figure 2 Pearson correlation coefficient (r) and data distribution by sex in each group between body mass index, waist-to-hip ratio, glycosylated hemoglobin, and liver fat content as measured by proton magnetic resonance spectroscopy. BMI: Body mass index; HbA1c: Glycosylated hemoglobin; LFC: Liver fat content; W/H ratio: Waist-to-hip ratio.

BMI[29,30]. A possible explanation is the difference in fat accumulation mechanisms and that weight gain is the main pathogenic mechanism of liver fat accumulation in this age group as was previously proposed by Van Wagner *et al*[31].

HbA1c and FG are also found to be statistically different between the two groups with a slightly positive correlation with LFC. However, only HbA1c is a statistically significant independent variable for LFC after adjusting for age and sex. This result may suggest that HbA1c is a better tool for reflecting the NAFLD effects on insulin resistance than the FG. This assumption is reflected in other research done on the association between HbA1c and NALFD in non-diabetic subjects[32] and on the association of prediabetes characteristics independent of total body fat in obese adolescents with high liver fat assessment by MRI[23]. Elevated HbA1c further confirms the high risk of cardiovascular disease and insulin resistance in overweight and obese young adults.

This study has a few limitations such as the high prevalence of dyslipidemia in the control group that may be caused by the sample characteristics. This group being mostly comprised of young adults engaged in academic studies and whose exercise levels were determined by a questionnaire. There may have been a potential for over reporting by the subjects. A second limitation is that LDL was calculated by an adjustable ratio equation and was not measured directly by biochemical assessment.

To our knowledge, this is the first study on the topic of non-invasive assessment of LFC by ^{1}H MRS technique in healthy young adults without any complications or earlier diagnoses of chronic disease. The high prevalence of NAFLD (LFC > 5.56%) contributed to the impact of silent chronic disease in young adults that had become obese. Although the current study is based on a small sample of subjects, the findings have drawn together various interesting subjects on the effects of BMI, how it

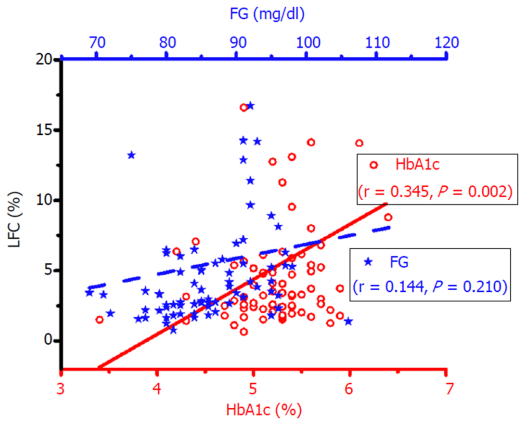

Figure 3 Pearson correlation between glycosylated hemoglobin (circle in red, lower x axis), fasting plasma glucose (star in blue, upper X axis), and liver fat content as measured by proton magnetic resonance spectroscopy. BMI: Body mass index; FG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; LFC: Liver fat content.

contributes to LFC, and how it is a high risk factor of metabolic syndrome in young adults. Previous studies on young adults after a 39 year follow-up has shown that an obese young adult who remained obese throughout their adult life increased their risk of developing severe liver disease[26]. This study suggested a role of BMI in increasing LFC and as a factor in higher NAFLD risk for overweight and obese young adults. The importance of weight control as the primary risk prevention and control of NAFLD and many metabolic syndromes has been proposed. However, this study may reveal the importance in raising awareness for early prevention before NAFLD transitions into chronic liver disease later in adulthood. Future studies on this topic are therefore recommended as young adults are at a high risk for developing severe liver disease. Further, implications of these findings may be forthcoming in future research using longitudinal studies with larger groups of subjects.

In conclusion, it is proposed that the prevalence of high LFC in the OW/OB group can be the result of weight gain and obesity, and may be a leading pathogenic mechanism of liver fat accumulation in young adults. This current study demonstrated the importance of BMI as a tool for the prevention and control of NAFLD and metabolic syndrome in young adults.

Table 2 Multiple linear regression analysis showing relationship of blood biochemical marker and anthropometry marker with LFC as the dependent variable

	Model 1			Model 2			Model 3		
	R^2	β(SE)	*P*	R^2	β(SE)	*P*	R^2	β(SE)	*P*
HbA1c	0.135	0.306	0.002	0.174	0.339	0.004	0.298	0.120	0.327
		(1.273)			(1.283)			(1.379)	
Tri		0.131	0.247		0.065	0.590		-0.029	0.590
		(0.012)			(0.013)			(0.012)	
BMI	0.295	0.463	< 0.001[a]	0.299	0.459	< 0.001[a]			
		(0.109)			(0.111)				
WC		-0.026	0.824		-0.034	0.774			
		(0.016)			(0.017)				
W/H ratio		0.145	0.247		0.136	0.288			
		(8.768)			(9.018)				

Model 1 is unadjusted model. Model 2 is Model 1 adjusted for sex and age. Model 3 is Model 2 adjust for body mass index. Statistical significance: [a]$P < 0.001$. β: Standardized coefficient; SE: Estimated error; R^2: Correction coefficient; LFC: Liver fat content; HbA1c: Glycosylated hemoglobin; Tri: Triglyceride; BMI: Body mass index; WC: Waist circumference; W/H ratio: Waist-to-hip ratio.

ARTICLE HIGHLIGHTS

Research background

In recent years, the prevalence of non-alcoholic fatty liver disease (NAFLD) in young adults has been increasing at an alarming rate that parallels the global epidemic of weight gain and obesity. NAFLD in young adults is a topic that has received little recognition, yet this age group is the most likely to gain weight and develop obesity from their diet and lifestyle as they are transitioning into adulthood. However, despite the growing public health concern about obesity and NAFLD in young adults, necessary information addressing the effects of obesity and NAFLD pathogenesis in this age group is lacking.

Research motivation

NAFLD is a chronic liver disease that is one of the most common health problems among young adults. We aim to identify the effects of obesity on liver fat content (LFC) and health in this age group. This information is crucial for primary prevention and a better understanding of NAFLD pathogenesis in young adults.

Research objectives

The aim of this present study is to assess the association between LFC by proton magnetic resonance spectroscopy (^{1}H MRS) technique. Using biochemical tests, the total cholesterol (Cho), low-density lipoproteins (LDL), high-density lipoproteins (HDL), fasting plasma glucose (FG), glycosylated hemoglobin (HbA1c), and being overweight/obese (OW/OB) will be determined.

Research methods

A total of 78 healthy subjects in the young adult age group (19-30 years old) participated in this study. A control group was made up of 39 healthy subjects, and the experimental group was made up of 39 overweight or obese (OW/OB) subjects. We performed the liver fat assessment by ^{1}H MRS technique on MRI 1.5 T that was calculated into LFC. Intravenous blood was drawn for biochemical analysis. The test focused on Cho, HDL, VLDL, TG, FG, and HbA1c. The waist circumference (WC) and hip circumference (HC) of each subject was measured, and the waist-to-hip ratio (W/H ratio) was calculated.

Research results

LFC from the OW/OB group (8.1% ± 1.0%) was found to be statistically higher when compared to the control group (2.7% ± 0.2%) ($P < 0.001$). Additionally, 48.7% of subjects in the OW/OB group had LFC > 5.56%, which is considered to be a cut off point for NAFLD. The OW/OB group reported statistically higher BMI, LFC, WC, HC, FG, Tri, LDL, HbA1c, and statistically lower HDL. Cho was increased in the OW/OB group compared to the control group, but was not statistically significant. The association of BMI and LFC was additionally confirmed by multilinearity regression analysis as a significant independent variable after being adjusted for age and sex ($P < 0.001$). These findings indicated that BMI is a sensitive marker for LFC in young adults.

Research conclusions

It is proposed that the prevalence of high LFC in the OW/OB group can be the result of weight gain and obesity, and may be a leading pathogenic mechanism of liver fat accumulation in

young adults. Moreover, high BMI is a risk factor for metabolic syndrome in young adults. This current study demonstrated the importance of weight control as a tool for the prevention and control of NAFLD and metabolic syndrome in young adults.

Research perspectives

Further study on this topic may require larger groups of subjects, and should also investigate the alteration of LFC and BMI throughout the adult years as a longitudinal study.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Non-invasive prediction of non-alcoholic steatohepatitis in Japanese patients with morbid obesity by artificial intelligence using rule extraction technology

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Institutional review board statement: This study was approved by institutional review boards of Yotsuya Medical Cube and Gunma University.

Informed consent statement: For this study using artificial intelligence using rule extraction technology, opt-out was obtained.

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Abstract

AIM

To construct a non-invasive prediction algorithm for predicting non-alcoholic steatohepatitis (NASH), we investigated Japanese morbidly obese patients using artificial intelligence with rule extraction technology.

METHODS

Consecutive patients who required bariatric surgery underwent a liver biopsy during the operation. Standard clinical, anthropometric, biochemical measurements were used as parameters to predict NASH and were analyzed using rule extraction technology. One hundred and two patients, including 79 NASH and 23 non-NASH patients were analyzed in order to create the prediction

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model, another cohort with 77 patients including 65 NASH and 12 non-NASH patients were analyzed to validate the algorithm.

RESULTS
Alanine aminotransferase, C-reactive protein, homeostasis model assessment insulin resistance, albumin were extracted as predictors of NASH using a recursive-rule extraction algorithm. When we adopted the extracted rules for the validation cohort using a highly accurate rule extraction algorithm, the predictive accuracy was 79.2%. The positive predictive value, negative predictive value, sensitivity and specificity were 88.9%, 35.7%, 86.2% and 41.7%, respectively.

CONCLUSION
We successfully generated a useful model for predicting NASH in Japanese morbidly obese patients based on their biochemical profile using a rule extraction algorithm.

Key words: Non-alcoholic steatohepatitis; Artificial intelligence; Rule extraction; Morbid obesity; Liver biopsy; Non-invasive prediction

Core tip: The prevalence of non-alcoholic steatohepatitis (NASH) in Japanese morbidly obese patients is extremely high, and early intervention should be undertaken. However, it is difficult to perform a percutaneous liver biopsy or elastography in routine medical care, especially in morbidly obese patients. We therefore attempted to construct a non-invasive prediction algorithm in order to predict NASH in Japanese morbidly obese patients using artificial intelligence with rule extraction technology. Although further studies with larger numbers of patients are needed to confirm the results, this algorithm may be useful for non-invasively predicting NASH in morbidly obese Japanese patients in the clinical setting.

Uehara D, Hayashi Y, Seki Y, Kakizaki S, Horiguchi N, Tojima H, Yamazaki Y, Sato K, Yasuda K, Yamada M, Uraoka T, Kasama K. Non-invasive prediction of non-alcoholic steatohepatitis in Japanese patients with morbid obesity by artificial intelligence using rule extraction technology. *World J Hepatol* 2018; 10(12): 934-943
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INTRODUCTIO

Non-alcoholic fatty liver disease (NAFLD) includes non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver (NAFL); a pathological diagnosis is the gold standard for the diagnosis of NASH[1,2]. The prevalence of NAFLD is reported to be 29.7% and is increasing in Japan[3]. The number of patients with morbid obesity is also increasing worldwide, and this increase is a major social problem[4]. Although the prevalence of morbid obesity is low in Japan (0.25%-0.3%) compared with to the United States[5,6], it is also increasing in Japan.

Morbid obesity is frequently complicated by metabolic diseases such as type 2 diabetes mellitus, hypertension, hyperlipidemia, and ischemic heart disease. The prevalence of NASH in patients with morbid obesity is also high. We previously reported that the prevalence of NASH in Japanese morbidly obese patients was extremely high (77.5%)[7]. Among non-morbidly-obese patients, the survival rate of NASH patients is significantly lower than in the general population[8]. As a result, an early diagnosis and early intervention are important for NASH patients[7].

A percutaneous liver biopsy is usually performed to diagnose liver diseases, including NASH[9]. Since a percutaneous liver biopsy requires the insertion of a long needle through the subcutaneous tissues to reach the liver, this method is difficult to perform in obese patients with a thick layer of subcutaneous fat. In addition, a percutaneous liver biopsy carries a risk of complications and sampling error in patients with a thick layer of subcutaneous fat. Elastography is non-invasive and sometimes used to estimate the liver stiffness or the extent of fibrosis in patients with

liver disease, including NASH[9]. Since elastography is also performed percutaneously, a thick layer of subcutaneous fat is again an obstacle for the accurate measurement and sometimes leads to a misdiagnosis. Magnetic resonance (MR) elastography is useful for predicting fibrosis and NASH[10]. However, it is also difficult to perform in morbidly obese patients due to limitations in the size or the ability of MR equipment to accommodate the patient. Given the above limitations, a safer, non-invasive, convenient diagnostic method for predicting NASH in morbidly obese patients is needed.

Predictive calculation formulas, such as the NAFIC score, fibrosis-4 index, and NAFLD fibrosis score are usually used to diagnose fibrosis and NASH[11-16]. These formulas are usually constructed using the data from patients whose body weight is within the general range and are not specialized for patients with morbid obesity. The HAIR [hypertension, alanine aminotransferase (ALT), insulin resistance] score[16] and BARD score[17] [includes body mass index (BMI), aspartate aminotransferase (AST)/ALT ratio and presence of diabetes mellitus] were constructed using data of morbidly obese patients from Australia[16] and the United States[17], respectively. However, there are racial differences between Asian people (including Japanese) and Caucasians[7,18,19]. When the BMI is similar, the degree of liver dysfunction and prevalence of NASH is worse in Asian patients than in Caucasians[7,18,19].

Recently, there has been remarkable progress in artificial intelligence, and we are now able to generate highly accurate and interpretable predictive models[20,21]. Rule extraction is a technique that attempts to find a compromise between requirements by building a simple rule set that mimics how well-performing complex predictive models (black-box, *i.e.*, not understandable) make their decisions for physicians and clinicians. We proposed continuous recursive-rule extraction (Re-RX) with J48graft as a promising algorithm for rule extraction[22]; this was based on the Re-RX algorithm[23] to simultaneously enhance the accuracy and interpretability of the extracted classification rules.

In the present study, we extracted new classification rules to predict NASH based on the biochemical profile of Japanese morbidly obese patients using Continuous Re-RX with J48graft. This predictive algorithm, which was named the Japanese algorithm of Morbid Obesity for NASH Prediction (JOMO algorithm), may be useful for predicting NASH in Japanese or Asian morbidly obese patients in the clinical setting.

MATERIALS AND METHODS

Study design

To create the prediction algorithm, we used the data of the cohort of Japanese morbidly obese patients who participated in our previous study[7]. The patient characteristics are shown in Supplemental Table 1 and Table 2 (modified from reference 7). One hundred and two patients, including 79 NASH and 23 non-NASH patients were analyzed using rule extraction technology. The main purpose of this study was to generate a highly accurate and interpretable predictive model for NASH in Japanese morbidly obese patients using an accuracy-priority rule extraction algorithm.

To validate the algorithm, another cohort of 77 patients was collected from October 2012 to September 2014. This cohort included 65 NASH and 12 non-NASH patients. Consecutive obese patients undergoing bariatric surgery for the management of morbid obesity at a single center (Weight Loss and Metabolic Surgery Center, Yotsuya Medical Cube, Tokyo, Japan) underwent a liver biopsy during the operation[7]. The precise methods for liver biopsies and sampling have been described in previous study[7]. NASH was histologically defined as steatosis with at least two of the following[24]: (1) Lobular necro-inflammatory foci; (2) Ballooning degeneration of hepatocytes with or without Mallory bodies; and (3) Perisinusoidal fibrosis. The indications for bariatric surgery followed the criteria established in 2005 by the Asia–Pacific Bariatric Surgery Group consensus meeting[18] as follows: Asian patients with a BMI of > 37 kg/m^2 or > 32 kg/m^2 (with diabetes or 2 other obesity-related comorbidities) and failure to show any improvement with nonsurgical treatment.

The standard clinical, anthropometric (weight, height, waist and hip circumferences) and biochemical measurements, including AST, ALT, γ-glutamyl transpeptidase (γ-GTP), cholinesterase (ChE), uric acid (UA), albumin (Alb), C-reactive protein (CRP), Fe (iron), fasting blood insulin, fasting plasma glucose (FPG), hemoglobin (Hb) A1c, triglyceride, and low density lipoprotein-cholesterol (LDL-cho) levels, were obtained before bariatric surgery. All blood samples were collected after overnight fasting. The homeostasis model assessment insulin resistance (HOMA-IR) index was used as an index of insulin resistance and was calculated by the following

Table 1 The positive predictive value, negative predictive value, sensitivity, specificity and predictive accuracy rate of each category

	Positive predictive value	Negative predictive value	Sensitivity	Specificity	Predictive accuracy rate
HAIR score	92.6%	20.0%	38.5%	83.3%	45.5%
BARD score	78.0%	8.3%	49.2%	25.0%	45.5%
JOMO algorithm	88.9%	35.7%	86.2%	41.7%	79.2%

formula: glucose (mg/dL) × insulin (μU/mL) /405. Computed tomography was performed, and the areas of subcutaneous and visceral fat were calculated. The diagnosis of type 2 diabetes mellitus was based on the Japanese Diabetes Society criteria[25]. Hypertension was diagnosed if the patient had a history of hypertension and was on antihypertensive medication or if the patient had a resting recumbent blood pressure of ≥ 140/90 mmHg on two repeated occasions.

Written informed consent was obtained from every patient, and the study was approved by the institutional review boards (IRB) of Yotsuya Medical Cube (YMC-2009-3). The study was performed in accordance with the principles of the Declaration of Helsinki. For this study using artificial intelligence using rule extraction technology, new IRB statements (Gunma University-2017-004) and opt-out were obtained.

Continuous Re-RX with J48graft

We recently proposed a new accuracy-priority rule extraction algorithm using Re-RX with J48graft[26] and continuous attributes (Continuous Re-RX with J48graft[23,27]). In order to extract more accurate and concise classification rules, we proposed replacing the conventional Re-RX algorithm, which uses C4.5 as a decision tree[28], with Re-RX with J48graft[23,29]. The conventional pruning used in J48 both complements and contrasts with that used in J48graft. The performance of the Re-RX algorithm is thought to be greatly affected by the decision tree.

In contrast, Continuous Re-RX[29] is a rule extraction algorithm that aims to achieve very high accuracy rather than concision. We found that the combination of Continuous Re-RX with J48graft and Continuous Re-RX simultaneously enhanced the accuracy and interpretability[27] and was well suited to generating a considerably better predictive model in this cohort. The age, Sex, BMI, waist-hip ratio, visceral fat area, AST, ALT, γ-GTP, ChE, UA, Alb, CRP, Fe, platelets, LDL-cho, TG, FPG, HbA1c, HOMA-IR, type 2 diabetes mellitus, and hypertension were used as parameters.

Statistical analysis

All data are shown as the mean ± SD. Differences between the groups were analyzed by Fisher's exact probability test and Mann-Whitney *U* tests when a significant difference was obtained by the Kruskal-Wallis test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Patient characteristics

We classified the data of the pathological findings and the results of a blood test obtained from a routine medical examination in the NASH and Non-NASH groups and analyzed the data using Continuous Re-RX with J48graft. The algorithm demonstrated the following rules for classification: R1: ALT ≤ 19 then non-NASH; R2: ALT > 19; CRP ≤ 0.15, HOMA-IR ≤ 6.37, Alb ≤ 4.15 then NASH; R3: ALT > 19, CRP ≤ 0.15, HOMA-IR ≤ 6.37, Alb > 4.15 then Non-NASH; R4: ALT > 19, CRP ≤ 0.15, HOMA-IR > 6.37 then NASH; R5: ALT > 19, CRP > 0.15 then NASH (Figure 1). The patients classified in R1 and R3 were predicted to be non-NASH, while NASH was predicted in the patients who were classified into R2, R4 and R5. This algorithm was based on 10 runs of 5 cross validation (CV)[30].

We validated the algorithm in the other cohort of 77 patients. To validate the performance of the extracted rules in classifying NASH patients, we confirmed the rate of agreement of the results. The 77 cases were classified using the prediction formula as follows: R1, *n* = 6; R2, *n* = 3; R3, *n* = 8; R4, *n* = 1; and R5, *n* = 59 (Figure 2). Fourteen cases were classified as non-NASH (R1 + R3) by the algorithm, and 5 cases were classified as non-NASH according to the results of the histopathological examinations. Sixty-three cases were classified into the NASH group (R2 + R4 +R5), while NASH was histopathologically diagnosed in 56 cases (Figure 2). Figure 3A shows the predictive value using our algorithm. The positive predictive value,

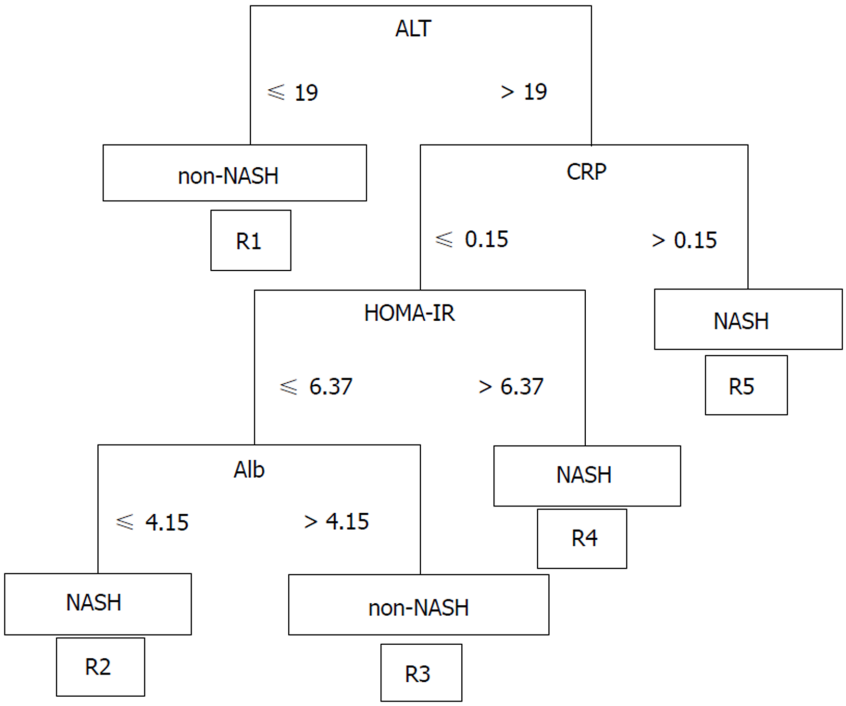

Figure 1 Prediction formula created using a highly accurate rule extraction algorithm. NASH: Non-alcoholic steatohepatitis; ALT: Alanine aminotransferase; CRP: C-reactive protein; Alb: Albumin; HOMA-IR: homeostasis model assessment insulin resistance.

negative predictive value, sensitivity and specificity were 88.9%, 35.7%, 86.2% and 41.7%, respectively (Table 1). The predictive accuracy rate was 79.2%.

Figure 3B shows the predictive value of the conventional predictive method using the HAIR score[16]. The HAIR score was constructed based on data from Australian patients with morbid obesity. The positive predictive value, negative predictive value, sensitivity and specificity were 92.6%, 20.0%, 38.5%, and 83.3%, respectively (Table 1). The predictive accuracy rate was 45.5%.

Figure 3C shows the prediction values using BARD score[17]. The BARD score was constructed based on a large cohort of patients from the United States with a median BMI of 33 kg/m^2. The positive predictive value, negative predictive value, sensitivity and specificity were 78.0%, 8.3%, 49.2%, and 25.0%, respectively. The predictive accuracy rate was 45.5% (Table 1).

The Receiver Operating Characteristic (ROC) curve of our algorithm for all patients is shown in Figure 4. The area of ROC was 0.772.

Our newly developed predictive algorithm showed high predictive accuracy in Japanese obese patients. We named our new predictive algorithm the JOMO algorithm.

DISCUSSION

It is difficult to perform a liver biopsy in morbidly obese patients during the course of a typical consultation. We therefore generated a new predictive model, the JOMO algorithm, to predict NASH in Japanese morbidly obese patients using Continuous Re-RX with J48graft. This algorithm showed substantially better results than conventional predictive formulae. We used 2 cohorts of 102 and 77 Japanese morbidly obese patients to generate and to validate the algorithm, respectively. Although a larger number of cases would enable us to improve the accuracy of the classification and interpretability, it is difficult to collect liver biopsy data from Japanese morbidly obese patients. These cohorts of 102 and 77 patients may be the largest cohorts of Japanese morbidly obese patients to have undergone a liver biopsy. For this reason, we adopted an artificial intelligence approach using Continuous Re-RX with J48graft. Continuous Re-RX with J48graft provided a more accurate predictive accuracy without the need for a validation cohort. Since we generated a predictive model by artificial intelligence using rule extraction technology, we were able to extract more accurate and interpretable classification rules from a limited cohort.

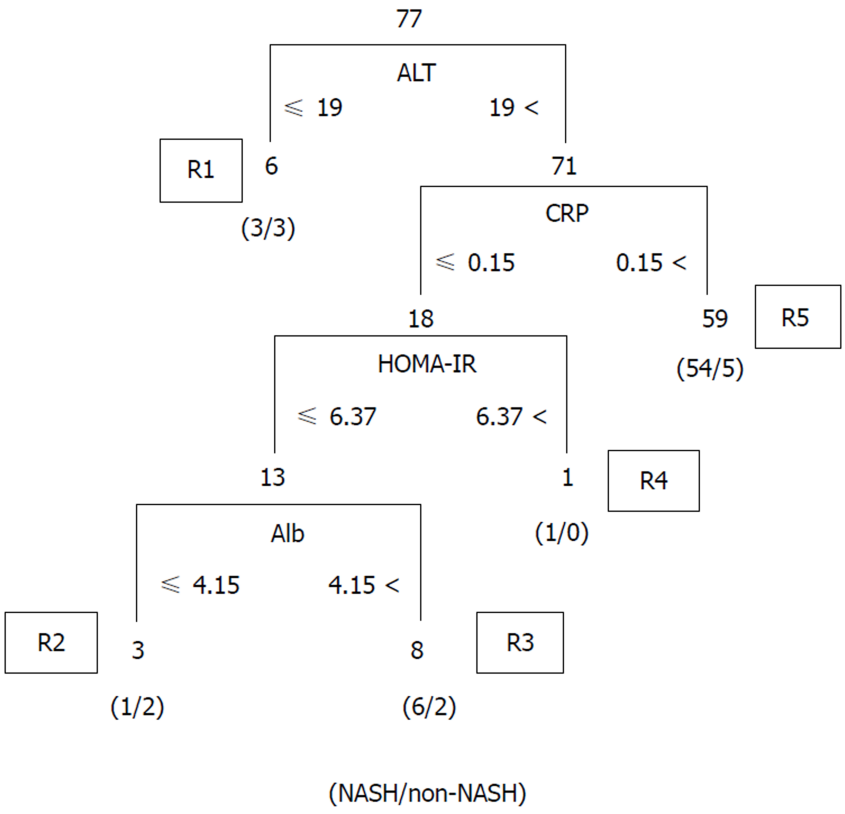

Figure 2 Predictive accuracy of the new developed JOMO algorithm in Japanese morbidly obese patients. NASH: Non-alcoholic steatohepatitis; ALT: Alanine aminotransferase; CRP: C-reactive protein; Alb: Albumin; HOMA-IR: Homeostasis model assessment insulin resistance.

In this study, we performed *k*-fold cross-validation when crafting this algorithm[30,31]. Regarding the experimental settings and data splitting technique, *k*-fold CV[30] was applied, *i.e.*, the original database was partitioned into *k*-subsets/folds of equal sizes where each subset had to be trained and tested. Accordingly, the final output was predicted by taking the averages of all partitions/folds that had been tested. Therefore, we applied 10 runs of 5-fold CV which was averaged to obtain the robust classification accuracy. Although the application of rule extraction technology is new in the medical field, strategies using Continuous Re-RX with J48graft, *i.e.*, rule-based classifiers, have already been established in the field of artificial intelligence[23,27]. Our algorithm using Continuous Re-RX with J48graft can therefore be adapted to different cohorts without a loss of generality.

In our previous study[7], a multivariate analysis for predicting NASH indicated that the waist-hip ratio, ALT level, fasting plasma glucose level, and HOMA-IR were independent factors associated with NASH. However, the JOMO algorithm using Continuous Re-RX with J48graft selected the ALT level, HOMA-IR, CRP level, and Alb level as predictive values. The ALT level and HOMA-IR were both selected in our previous multivariate analysis as well as in this JOMO algorithm. However, the waist-hip ratio and fasting plasma glucose level were not chosen by the JOMO algorithm. On using the predictive factors determined in the previous multivariate analysis, the predictive accuracy rates of the waist-hip ratio, fasting plasma glucose level, ALT level and HOMA-IR were found to be 54.1%, 35.1%, 77.9%, and 79.2%, respectively. The predictive values of the JOMO algorithm were thus considerably better than those of the multivariate analysis. Furthermore, since the attributes selected by artificial intelligence in this study are more concise, they are easily checked by blood sampling. This is an advantage with the JOMO algorithm and fulfills the purpose of this study.

CRP was selected as a predictive parameter in this JOMO algorithm. The predictive value of CRP in the diagnosis of NASH remains controversial at present. Anty *et al*[32] reported the CRP levels were not predictive of the diagnosis of NASH in severely obese patients because the liver as well as the adipose tissue can produce CRP. However, Yoneda *et al*[33] demonstrated that high-sensitivity CRP was useful for making a prediction in cases of NASH compared with in cases of simple nonprogressive steatosis. They also suggested that high-sensitivity CRP might be a

A JOMO algorithm

Number of cases		Pathological diagnosis	
		NASH	non-NASH
Prediction model	NASH	56	7
	non-NASH	9	5

B HAIR score

Number of cases		Pathological diagnosis	
		NASH	non-NASH
Prediction model	NASH	25	2
	non-NASH	40	10

C BARD score

Number of cases		Pathological diagnosis	
		NASH	non-NASH
Prediction model	NASH	32	9
	non-NASH	33	3

Figure 3 A cross table comparing the results of the prediction model and the pathological diagnosis. A: JOMO algorithm; B: HAIR score; C: BARD score. NASH: Non-alcoholic steatohepatitis.

clinical feature that indicates the severity of hepatic fibrosis in cases of NASH[33]. It is of note that there was no statistically significant difference in the CRP levels between the NASH and non-NASH as groups in our cohort (Supplemental Table 2), and thus the introduction of CRP into the predictive model demonstrated the power and usefulness of artificial intelligence.

The level of CRP can be affected by many factors. However, the cohort used in this study was relatively homogenous: All patients were candidates for bariatric surgery, and blood samples were collected from all patients who were roughly in the same condition before surgery. As such, our cohort may be protected against miscellaneous factors that influence the CRP level. The prevalence of NASH patients was high, and the number of non-NASH patients was low in the cohort used in this study. This may have influenced the accuracy of our predictive algorithm. Therefore, further evaluation of the role of CRP in this algorithm will be needed before it should be applied to other cohorts. It the future, we plan to improve this algorithm further using a larger number of NASH and non-NASH patients.

In this study, we generated a new predictive model that used Continuous Re-RX with J48graft to predict NASH in Japanese morbidly obese patients. This algorithm enables the prediction of NASH using parameters available from blood tests in routine medical examinations. Although further studies with larger numbers of patients are needed to confirm the results, this algorithm may be useful for non-invasively predicting NASH in morbidly obese Japanese patients in the clinical setting.

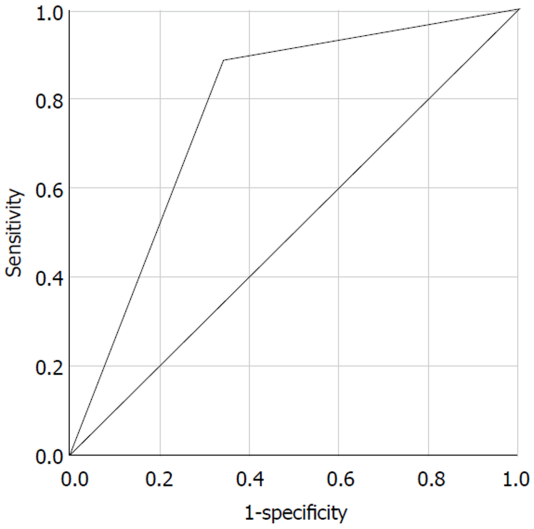

Figure 4 Receiver operating characteristic curve of the JOMO algorithm.

ARTICLE HIGHLIGHTS

Research background

Pathological diagnosis is the gold standard for the diagnosis of non-alcoholic steatohepatitis (NASH). However, it is difficult to perform a percutaneous liver biopsy in routine medical care, especially in morbidly obese patients. Predictive calculation formulas are usually used to diagnose fibrosis and NASH.

Research motivation

Recently, there has been remarkable progress in artificial intelligence. We therefore attempted to construct a non-invasive prediction algorithm in order to predict NASH using artificial intelligence with rule extraction technology.

Research objectives

Morbidly obese Japanese patients who required bariatric surgery underwent a liver biopsy during the operation. Standard clinical, anthropometric, biochemical measurements were used as parameters for making prediction model.

Research methods

One hundred and two patients, including 79 NASH and 23 non-NASH patients were analyzed in order to create the prediction model, another cohort with 77 patients including 65 NASH and 12 non-NASH patients were analyzed to validate the algorithm. We used Continuous recursive-rule extraction with J48graft for rule extraction to predict NASH.

Research results

Alanine aminotransferase, C-reactive protein, homeostasis model assessment insulin resistance, albumin were extracted as predictors of NASH. When we adopted the extracted rules for the validation cohort, the predictive accuracy was 79.2%. The positive predictive value, negative predictive value, sensitivity and specificity were 88.9%, 35.7%, 86.2% and 41.7%, respectively.

Research conclusions

We successfully generated a useful model for predicting NASH in Japanese morbidly obese patients based on their biochemical profile using a rule extraction algorithm.

Research perspectives

Although further studies with larger numbers of patients are needed to confirm the results, this algorithm may be useful for non-invasively predicting NASH in morbidly obese Japanese patients in the clinical setting.

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ORIGINAL ARTICLE

Retrospective Study

Impact of sepsis and non-communicable diseases on prognostic models to predict the outcome of hospitalized chronic liver disease patients

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Author contributions: Abid S contributed to the study idea and design; Qazi Arisar FA, Shaikh PA, Awan S, and Abid S contributed to acquisition, analysis, or interpretation of data; Qazi Arisar FA wrote the initial draft; Abid S and Shaikh PA made critical revision for important intellectual content; all authors approved the final version of the manuscript and are accountable for all aspects of the work.

Institutional review board statement: This study was reviewed and granted exemption by the Ethical Review Committee of the Aga Khan University Hospital, Karachi.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained through a retrospective review of charts.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

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Abstract

AIM

To evaluate the impact of sepsis and non-communicable diseases (NCDs) on the outcome of decompensated chronic liver disease (CLD) patients.

METHODS

In this cross-sectional study, medical records of patients with CLD admitted to the Gastroenterology unit at the Aga Khan University Hospital were reviewed. Patients older than 18 years with decompensation of CLD (*i.e.*, jaundice, ascites, encephalopathy, and/or upper gastrointestinal bleed) as the primary reason for admission were included, while those who were admitted for reasons other than decompensation of CLD were excluded. Each patient was followed for 6 wk after index admission to assess mortality, prolonged hospital stay (> 5 d), and early readmission (within 7 d).

RESULTS

A total of 399 patients were enrolled. The mean age was 54.3 ± 11.7 years and 64.6% (n = 258) were male. Six-week mortality was 13% (n = 52). Prolonged hospital stay and readmission were present in 18% (n = 72) and 7% (n = 28) of patients, respectively. NCDs were found in 47.4% (n = 189) of patients. Acute kidney injury, sepsis, and non-ST elevation myocardial infarction were found in 41% (n = 165), 17.5% (n = 70), and 1.75% (n = 7) of patients, respectively. Upon multivariate analysis, acute kidney injury, non-ST elevation myocardial infarction, sepsis, and coagulopathy were found to be statistically significant predictors of mortality. While chronic kidney disease (CKD), low albumin, and high Model for End-Stage Liver Disease (MELD)-Na score were found to be statistically significant predictors of morbidity. Addition of sepsis in conventional

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MELD score predicted mortality even better than MELD-Na (area under receiver operating characteristic: 0.735 *vs* 0.686; $P < 0.001$). Among NCDs, CKD was found to increase morbidity independently.

CONCLUSION
Addition of sepsis improved the predictability of MELD score as a prognostic marker for mortality in patients with CLD. Presence of CKD increases the morbidity of patients with CLD.

Key words: Chronic liver disease; Mortality; Morbidity; Prognostic factors; Non-communicable diseases; Sepsis

Core tip: Chronic liver disease is one of the leading causes of mortality. Child-Pugh and Model for End-Stage Liver Disease scores have been designed to predict the outcome in cirrhotic patients. Infection and renal insufficiency can worsen the outcome in cirrhotic patients. Myocardial infarction, sepsis,, and coagulopathy are associated with poor outcomes in patients with cirrhosis. The addition of sepsis can improve the predictability of the Model for End-Stage Liver Disease score as a prognostic marker for mortality in hospitalized patients with liver cirrhosis. Presence of chronic kidney disease increased the morbidity of cirrhotic patients. There is no direct impact of non-communicable disease over mortality in hospitalized patients with liver cirrhosis.

Qazi Arisar FA, Abid S, Shaikh PA, Awan S. Impact of sepsis and non-communicable diseases on prognostic models to predict the outcome of hospitalized chronic liver disease patients. *World J Hepatol* 2018; 10(12): 944-955
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INTRODUCTION

The massive global burden of chronic liver disease (CLD) has been well documented[1], with more than one million deaths per year worldwide[2] making it the 14th leading cause of death globally[3]. Many prognostic models have been developed over the years to help classify the severity of liver disease and direct the aggressiveness of medical care. The Child-Pugh Turcotte (CTP) Score and the Model for End-Stage Liver Disease (MELD) score are two of the most commonly used scoring systems worldwide[4-6].

Child and Turcotte proposed the CTP score initially using nutritional status, the presence of ascites, hepatic encephalopathy, total bilirubin, and albumin as parameters to determine mortality risk in patients undergoing portosystemic shunt surgery. Later, Pugh *et al*[4] modified it to its current version by replacing nutritional status with prothrombin time or international normalized ratio (INR), making it the most widely used scoring system for estimation of prognosis in CLD patients. However, the subjectivity of variables (ascites, encephalopathy) as well as inter-laboratory variability limited the accuracy of the CTP score, with the waitlist mortality for liver transplantation continuing to rise[7].

MELD score was introduced primarily to determine the survival of patients undergoing transjugular intrahepatic portosystemic shunt placement[5]. MELD score incorporates total bilirubin, creatinine, and INR. It has not only become the mainstay for prioritizing patients for liver transplant, but also for predicting mortality in non-transplant surgical procedures, alcoholic hepatitis, and acute variceal hemorrhage[6,8]. However, there are still several comorbidities such as hepatocellular carcinoma, hepatopulmonary syndrome, and portopulmonary hypertension (HTN) that can affect the prognosis of CLD patients and that are not taken into account by the MELD score[9,10].

Several studies have been done on the MELD score with proposals made for revision of the scoring system to include other factors to improve the predictive accuracy of the score. Some of these include modified CTP, MELD-Na, Reweighted MELD and the Refit MELD, which have been shown to be superior to the current CTP

and MELD scores[11-14]. Such studies have led several leading researchers to investigate other variables and scoring systems to better predict mortality in patients hospitalized for decompensated CLD.

Acute kidney injury (AKI) is a devastating complication that is frequently progressive and independently associated with mortality in a stage-dependent fashion in CLD patients[15]. Bacterial infections are common in liver disease, especially in decompensated patients. Infections increase the mortality four-fold in patients with end-stage liver disease[16]. Inflammation stemming from infections plays a key role in the outcome of cirrhosis. The presence of systemic inflammatory response syndrome with or without infection is a major predictor of prognosis in CLD patients[17].

There is an alarming rise in the prevalence of non-communicable diseases (NCDs) worldwide. Diabetes, HTN, cardiovascular diseases, chronic obstructive pulmonary diseases, and cancers have emerged as leading causes of mortality globally[18]. In a recent cohort of the Asian population, diabetes was found to impact mortality in cirrhotic patients[19]. However, the effect of other NCDs on the outcomes of liver disease has not been elucidated.

Charlson *et al*[20] proposed a comorbidity index to predict all-cause mortality on the basis of a number of comorbid conditions. Seventeen different diseases, each allocated a score from one to six, are incorporated in the Charlson comorbidity index. The total sum gives the total burden of comorbidities in that patient[20]. Since the 1980s, the score has been extensively used to estimate mortality in a different subset of cohorts including liver disease patients[21,22]. However, the severity of liver disease was not incorporated in those studies.

The aim of this study is to evaluate the impact of NCDs on the outcome of patients admitted for decompensated CLD. We also aimed to construct a model over and above the existing scoring system.

MATERIALS AND METHODS

Study design and settings

This study employed a cross-sectional design. Medical records of CLD patients who were admitted to the Gastroenterology unit at the Aga Khan University Hospital in Karachi, Pakistan were reviewed. Patients older than 18 years with decompensation of CLD (*i.e*. jaundice, ascites, encephalopathy, and/or upper gastrointestinal (GI) bleed) as the primary reason for admission were included. Those admitted for reasons other than decompensation of CLD were excluded. Each patient was followed for 6 wk after index admission to assess mortality and morbidity. The study was conducted after approval from the institutional ethical review committee.

Variables analyzed

(1) Demographics: age, gender, weight, duration of documented CLD, smoking, and current alcohol use; (2) Clinical presentation: blood pressure, heart rate, temperature, respiratory rate, Glasgow coma scale; (3) CLD complications/decompensation: presence of jaundice, ascites, encephalopathy, esophageal varices, GI bleeding, hepatorenal syndrome, spontaneous bacterial peritonitis, and hepatopulmonary syndrome; (4) NCDs: Diabetes mellitus, HTN, chronic obstructive lung disease, ischemic heart disease, congestive heart failure, peripheral vascular disease, chronic kidney disease (CKD), cerebrovascular disease (prior strokes), acquired immunodeficiency syndrome, cancer, dementia, connective tissue diseases, and peptic ulcer disease; (5) Laboratory markers: complete blood count, electrolytes, liver function tests, serum albumin, creatinine, prothrombin time, C-reactive protein; (6) Scoring Systems: Child-Pugh Score, MELD score, MELD-Na score, and Charlson comorbidity index. While calculating Charlson comorbidity index, patients with Child A disease were given a score of 1 (mild), while those with Child B and C disease were given a score of 3 (moderate to severe) as per standard score; and (7) Primary outcome: To assess mortality within 6 wk of admission and morbidity defined as either prolonged hospital stay > 5 d (120 h) or readmission within 7 d of the index admission.

Operational definitions

Sepsis: Sepsis was defined as the presence of any source of infection along with at least two of the following[23]: (1) Temperature > 38 °C (100.4 °F) or < 36 °C (96.8 °F); (2) Heart rate > 90 bpm; (3) Respiratory rate > 20 or $PaCO_2$ < 32 mmHg; and (4) Total leukocyte count (TLC) > 12000/mm^3, < 4000/mm^3, or > 10% bands.

AKI: AKI was defined as per kidney disease: Improving Global Outcomes Acute Kidney Injury Work Group, and revised consensus recommendations of the

International Club of Ascites[24], *i.e.* increase in serum creatinine ≥ 0.3 mg/dL within 48 h; or a percentage increase serum creatinine ≥ 50% from the baseline which is known, or presumed, to have occurred within the prior 7 d.

Statistical analysis

The sample size was calculated using Open Epi for proportion, using mortality rate due to infection, and AKI between 36%-38%[16]. Taking into account the 95% confidence interval (CI), 80% power, and an odds ratio (OR) of 1.5, the final sample size was approximately 384 CLD patients.

Data were analyzed using Statistical Package for Social Sciences version 20. The frequency for all variables was calculated. Data were expressed as a mean and standard deviation for normally distributed continuous variables. The significance of association was calculated using the Student *t*-test. Categorical variables were recorded in their absolute value and analyzed using the chi-squared test. Univariate analysis was used to identify parameters associated with mortality and morbidity. Multiple logistic regressions were done in order to identify independent predictors of poor outcome in these patients. These factors were incorporated in the existing MELD score. Receiver operating characteristic curves of MELD, MELD-Na, and the new score were made. Significance tests and CIs were assessed through the nonparametric bootstrap. The area under the curve was compared between the three scores. Fisher exact test was used to determine the association of NCDs with predictors of mortality. All *P* values were two-sided and a *P* value of < 0.05 was considered statistically significant.

RESULTS

Demographics

Records of 399 patients admitted primarily due to decompensation of liver disease were reviewed. Mean age was 54.3 ± 11.7 years and 64.6% (*n* = 258) of patients were male. Hepatitis C was found to be the leading cause of CLD. The length of hospital stay of more than five days was 18%, while readmission rate within 1 wk was 7%. Six-week mortality was observed in 13% (*n* = 52) of patients. Table 1 describes the demographic details of patients.

NCDs were present in 47.4% (*n* = 189) of patients. AKI, sepsis, and non-ST elevation myocardial infarction (NSTEMI) were present in 41% (*n* = 165), 17.5% (*n* = 70), and 1.75% (*n* = 7) of patients, respectively.

Factors predicting mortality

Upon univariate analysis, hypotension, tachycardia, tachypnea, hypoxia, NSTEMI, sepsis, renal insufficiency, encephalopathy, pneumonia, anemia, leukocytosis coagulopathy, high CTP and MELD scores, and frequent admissions in the last 1 to 3 mo were found to be associated with 6 wk mortality.

Upon multivariate analysis, sepsis (OR = 6.50; 95%CI: 3.007-14.06; *P* < 0.001), AKI (OR = 2.69; 95%CI: 1.17-6.20; *P* = 0.02), and INR (OR = 1.75; 95%CI: 1.14-2.69; *P* < 0.001) were significant independent predictors of mortality (Table 2). Figure 1 shows a flow diagram of 6 wk mortality for patients with advanced cirrhosis and concomitant sepsis based on the logistic regression model. Sepsis, AKI, and INR were used in a hierarchical pattern based on the OR.

Factors predicting morbidity

Upon multivariate analysis, CKD, low albumin, and high MELD-Na scores were found to be independent factors in predicting morbidity (Table 3).

Factors predicting both mortality and morbidity

Upon multivariate analysis, CKD, high TLC, and high Child class were found to affect both mortality and morbidity (Table 4).

Addition of sepsis in MELD score

Once the value of sepsis was determined as a significant independent predictor of mortality based on multivariate analysis, we tried to formulate a new score by adding a factor of 6.5 into existing MELD scores. This factor was derived from the odds ratio of sepsis for mortality upon multivariate analysis. The new score labeled as MELD-sep was found to predict mortality better than MELD and MELD-Na as depicted by the area under receiver operating characteristic of 0.735 for MELD-sep in contrast with 0.686 for MELD-Na and 0.671 for MELD score (Figure 2).

Impact of NCDs

Table 1 Baseline demographic details

Variables		*n* (%) or mean ± SD
Age (yr)		54.56 ± 11.74
Gender	Male	258 (64.6)
	Female	136 (34.4)
Etiology (viral)	Hepatitis C	260 (65.1)
	Hepatitis B	31 (7.7)
	Hepatitis B + Hepatitis D	12 (3)
	Hepatitis B + Hepatitis C	6 (1.5)
	Non-B, Non-C	61 (15.2)
	(Unknown etiology)	
	Alcohol	20 (5)
	Autoimmune hepatitis	8 (2)
	Hemochromatosis	1 (0.25)
Duration of chronic liver disease (yr)		4.25 ± 3.71
NCDs	Diabetes	148 (37)
	Hypertension	96 (24)
	Chronic kidney disease	30 (7.5)
	Ischemic heart disease	23 (5.7)
	Chronic obstructive pulmonary disease	13 (3.2)
Infections on admission	Sepsis	70 (17.5)
	Lower respiratory tract infection	24 (6)
	Urinary tract infection	30 (7.5)
Non-ST Elevation myocardial infarction		7 (1.75)
Stroke		3 (0.75)
Decompensation on admission	Ascites	300 (75.1)
	Presence of esophageal/gastric varices	235 (58.8)
	Portosystemic encephalopathy	170 (42.6)
	Acute kidney injury	165 (41.3)
	Upper GI bleed	118 (29.5)
	Hepatocellular carcinoma	98 (24.5)
	Hepatorenal syndrome	86 (21.5)
	Spontaneous bacterial peritonitis	76 (19)
	Hepato-hydrothorax	12 (3)
Investigations	Hemoglobin (g/dL)	9.81 ± 2.17
	Total leukocyte count (× 109/L)	9.87 ± 6.17
	Platelets (× 109/L)	121.29 ± 108.12
	Prothrombin time (s)	17.37 ± 7.87
	International normalizing ratio	1.63 ± 0.65
	Creatinine (mg/dL)	1.65 ± 1.37
	Sodium (mmol/L)	132.3 ± 7.62
	Potassium (mmol/L)	4.17 ± 0.88
	pH	7.37 ± 0.12
	Bicarbonate (mmol/L)	20.13 ± 4.79
	Total bilirubin (mg/dL)	5.47 ± 7.57
	Alanine transaminase (IU/L)	78.7 ± 128.21
	Gama glutamyl transferase (IU/L)	119.26 ± 159.87
	Alkaline phosphatase (IU/L)	178.35 ± 133.8
	Albumin (g/dL)	2.59 ± 0.58
Child class	A	39 (9.8)
	B	142 (35.6)
	C	218 (54.6)
Prognostic scores	MELD score	18.0 ± 8.55

	MELD-Na	21.73 ± 8.31
	Charlson index	4.21 ± 1.63
	Charlson age adjusted score	5.33 ± 2.20
Outcomes	Mortality	52 (13)
	Prolong stay	72 (18)
	Readmission	28 (7)

NCDs: non-communicable diseases; GI: gastrointestinal; MELD: Model for End-Stage Liver Disease.

NCDs were found to be associated with an increased readmission rate (28.6% without NCDs *vs* 71.4% with NCDs; $P = 0.03$). However, there was no effect on length of stay (length of stay > 5 d in 51.4% without NCDs *vs* 48.6% with NCDs; $P = 0.45$). Upon multivariate analysis, among all NCDs, only CKD was directly related with increased morbidity (OR = 3.18; 95%CI: 1.30-7.82; $P = 0.01$). Moreover, the presence of NCDs was not found to be an independent predictor of mortality in our series (mortality rate of 12.4% without NCDs *vs* 13.9% with NCDs; $P = 0.65$). Similarly, the presence of multiple comorbid conditions did not appear to impact the mortality directly (mortality rate of 12.2% without NCDs *vs* 14.1% with 2 or fewer NCDs *vs* 15.6% with 3 or more NCDS; $P = 0.97$). However, the presence of NCDs was directly related with NSTEMI, which was a major predictor of mortality in our study (Table 5). Similarly, Charlson comorbidity index was used to calculate the burden of NCDs in our patients, and this index did not appear to predict the outcome in cirrhotic patients.

DISCUSSION

Decompensated liver disease is a state of organ failure related to multi-organ consequences such as encephalopathy, renal insufficiency, volume overload, GI bleeding, infections, and frailty[25-28]. In this study of hospitalized decompensated cirrhotic patients, we evaluated the impact of sepsis and NCDs on patient outcome.

Traditionally, CTP and MELD scores have been used to predict the mortality in cirrhotic patients. However, the group of patients used to create and validate the MELD score was devoid of acute reversible complications such as sepsis[29]. Infections significantly increase the mortality in end-stage liver disease with some studies reporting a 30% death rate in 1 mo[16]. Prevention and treatment of sepsis were shown to reduce mortality in patients with cirrhosis and AKI[17]. AKI is a common and overwhelming complication in patients with end-stage liver disease. Belcher *et al*[15] also showed AKI to be associated with high mortality and complications in hospitalized patients with cirrhosis. Our study found that AKI, myocardial infarction, sepsis, and coagulopathy on admission were associated with high mortality in cirrhotic patients.

Interestingly, the MELD and Charlson comorbidity index scores were not associated with high mortality in our analysis. However, high Child class appeared to affect both mortality and morbidity. Based on our observations, we propose that the addition of sepsis as a factor in the MELD score gives a better prediction of mortality as compared to conventional MELD and MELD-Na scores in CLD patients admitted with acute decompensation. We also found that CKD, low albumin, and high MELD-Na scores were able to predict morbidity.

Chirapongsathorn *et al*[30] and Shu *et al*[31] related longer hospital stays with a high rate of 30-d readmission while Masadeh *et al*[32] found the opposite relationship. In our analysis, prolonged hospital stay was associated with subsequent early readmission upon univariate analysis but did not stand out as an independent factor upon multivariate analysis.

The prognostic value of chronic NCDs in cirrhotic patients has not been extensively studied. Recently, diabetes has been associated with high mortality[19,33,34], higher readmission rate[35], and a major factor in determining liver-related outcomes in cirrhotic patients[36]. We were unable to relate diabetes directly to mortality and morbidity in our series. However, among NCDs, CKD was found to directly predict morbidity in our series. Moreover, the presence of NCDs was associated with NSTEMI, which was one of the major predictors of mortality in our cohort. This observation indicates NCDs as an important factor that could influence the outcome of CLD patients independent of well-known prognostic variables incorporated in Child-Pugh and MELD scores for advanced liver disease patients.

Despite previous studies supporting the use of the Charlson comorbidity index for prediction of poor outcome in CLD patients[21,22], our study did not find a direct

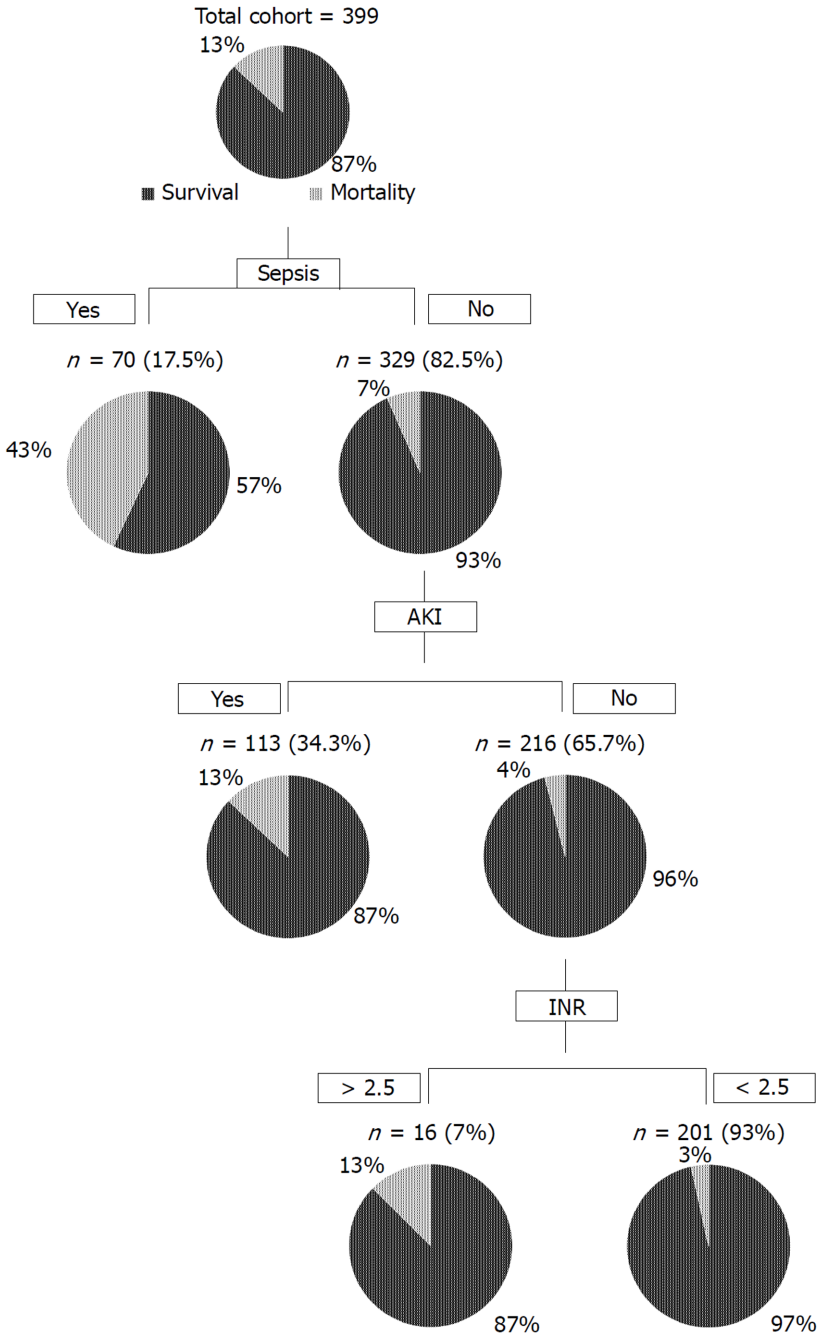

Figure 1 Flow diagram of 6 wk mortality for patients with advanced cirrhosis and concomitant sepsis based on the logistic regression model. AKI: Acute kidney injury; INR: International normalized ratio.

relationship between Charlson comorbidity index and morbidity upon multivariate analysis. This could be due to the difference between patient characteristics. We selectively enrolled patients who were admitted due to decompensated liver disease while previous studies included patients who were labeled as cirrhotic in their database regardless of compensation status[21,22]. Moreover, the follow-up period was longer in the Danish cohort[22].

The present study takes into account readmissions at our center. The possibility of readmissions at other centers was not accounted for. Other limitations include the cross-sectional design, shorter follow-up, and single center focus. Further large-scale multicenter studies with a longer follow-up would be helpful in strengthening the impact of NCDs and sepsis on the determination of outcome in hospitalized cirrhotic patients.

In conclusion, the addition of sepsis improves the predictability of the MELD score as a prognostic marker for mortality in patients with decompensated CLD. Presence

Table 2 Multivariate analysis of factors predicting 6 wk mortality in chronic liver disease patients

		OR (95%CI)	*P* value
NSTEMI	No	1	
	Yes	16.03 (2.01-127.46)	0.009
Sepsis	No	1	
	Yes	6.50 (3.01-14.06)	< 0.001
AKI	No	1	
	Yes	2.69 (1.17-6.20)	0.02
INR		1.75 (1.14-2.69)	< 0.001

NSTEMI: non-ST-elevation myocardial infarction; AKI: acute kidney injury; INR: international normalized ratio; OR: odds ratio; CI: confidence interval.

of CKD increases morbidity of patients with CLD.

Table 3 Multivariate analysis of factors predicting morbidity

		OR (95%CI)	*P* value
CKD	No	1	
	Yes	3.18 (1.30-7.82)	0.01
MELD-Na		1.05 (1.01-1.08)	0.005
Albumin		0.55 (0.32-0.92)	0.02

Prolonged hospital stay > 5 d (120 h) or readmission within 7 d of the index admission; CKD: chronic kidney disease; MELD: Model for End-Stage Liver Disease; OR: odds ratio; CI: confidence interval.

Table 4 Multivariate analysis of factors predicting both mortality and morbidity

		OR (95%CI)	*P* value
CKD	No	1	
	Yes	3.12 (1.21-8.06)	0.01
TLC		1.08 (1.03-1.12)	< 0.001
Child Class		3.57 (2.20-5.79)	< 0.001

CKD: chronic kidney disease; TLC: total leukocyte count; OR: odds ratio; CI: confidence interval.

Table 5 The relationship between predictor of mortality and non-communicable diseases, *n* (%)

	NCDs		*P* value
	Yes	No	
	210 (52.6)	189 (47.3)	
NSTEMI	7 (3.3)	0	0.01
Sepsis	31 (14.8)	39 (20.6)	0.14
AKI	92 (44.7)	76 (40.2)	0.41

NSTEMI: non-ST-elevation myocardial infarction; INR: international normalized ratio; AKI: acute kidney injury; NCDs: non-communicable diseases.

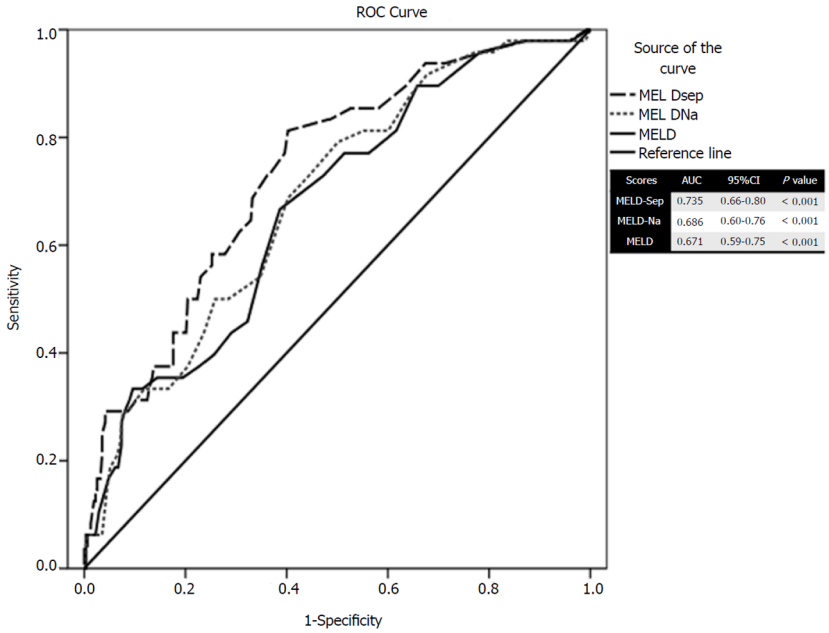

Figure 2 Receiver operating characteristic curve shows 6 wk mortality prediction of MELD-Sep *vs* MELD and MELD-Na. MELD: Model for End-Stage Liver Disease.

ARTICLE HIGHLIGHTS

Research background

Patients with decompensated chronic liver disease (CLD) are at high risk of complications. Various scores have been used to classify the severity of liver disease and to predict mortality. Recently, diabetes was found to impact mortality in cirrhotic patients. However, the impact of other comorbidities on mortality and morbidity has not been studied. Moreover, the impact of sepsis on available predictability scores has not been determined.

Research motivation

Given the limitations with the use of Child-Pugh and Model for End-Stage Liver Disease (MELD) scores, we wanted to come up with a new score to predict mortality and morbidity.

Research objective

The objective for this study included determination of sepsis, non-communicable diseases (NCDs), and acute kidney injury (AKI) in patients admitted with decompensated liver disease, along with their impact of NCDs on mortality and morbidity parameters. We also wanted to evaluate whether the addition of any other variable makes MELD a better tool as a prognostic marker.

Research methods

We performed a retrospective analysis of medical records of patients with CLD admitted at the Aga Khan University Hospital. All adult patients with decompensation of CLD (*i.e.*, jaundice, ascites, encephalopathy, and/or upper gastrointestinal (GI) bleed) as the primary reason for admission were included. Multivariate analysis was performed to assess predictors of 6 wk mortality, prolonged hospital stay (> 5 d), and early readmission (within 7 d).

Research results

Six-week mortality rate was 13%. Prolonged hospital stay and readmission rates were 18% and 7%, respectively. NCDs were present in 47.4% of patients. AKI, sepsis, and NSTEMI were present in 41%, 17.5%, and 1.75% patients, respectively. Factors associated with mortality included AKI, NSTEMI, sepsis, and coagulopathy. The factors found responsible for morbidity included chronic kidney disease (CKD), low albumin, and high MELD-Na score. By adding sepsis to the conventional MELD score, the predictability of mortality increased significantly. CKD was found to impact morbidity independently.

Research conclusion

This study highlighted multiple factors associated with early mortality, readmission, and prolonged hospital stay. This study also determined the significance of the addition of sepsis in the MELD score to improve its predictability as a prognostic marker for mortality in patients with decompensated CLD. Presence of CKD increased morbidity of patients with CLD.

Research perspective

We need to amend factors linked to mortality, readmission, and prolonged stay not only to control mortality and morbidity, but also to minimize the cost burden by patients.

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ORIGINAL ARTICLE

Observational Study

One in five hepatocellular carcinoma patients in the United States are Hispanic while less than 40% were eligible for liver transplantation

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Author contributions: Robinson A and Wong RJ contributed to the study concept and design, acquisition of data, and drafting of the manuscript; Wong RJ contributed to statistical analysis and study supervision; all authors contributed to analysis and interpretation of data, and critical revision of the manuscript for important intellectual content.

Institutional review board statement: The study was reviewed and determined to be exempt by the Alameda Health System Institutional Review Board because human subjects were not involved, as per United States Department of Health and Human Services guidelines, and the SEER database is publicly available without individually identifiable private information.

Informed consent statement: Given the observational study design and large registry-based cohort, this study was granted IRB exemption by Alameda Health System Institutional Review Board because human subjects were not involved, as per United States Department of Health and Human Services guidelines, and the SEER database is publicly available without individually identifiable private information. Thus informed consent was not required and not possible.

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Abstract

AIM

To evaluate trends and disparities in hepatocellular carcinoma (HCC) outcomes among Hispanic patients in the United States with a focus on tumor stage at diagnosis.

METHODS

We retrospectively evaluated all Hispanic adults (age > 20) with HCC diagnosed from 2004 to 2014 using United States Surveillance, Epidemiology, and End Results (SEER) cancer registry data. Tumor stage was assessed by SEER-specific staging systems and whether HCC was within Milan criteria at diagnosis. Multivariate logistic regression models evaluated for predictors of HCC within Milan criteria at diagnosis.

RESULTS

Overall, Hispanics accounted for 19.8% of all HCC (73.3% men, 60.9% had Medicare or commercial insurance, 33.5% Medicaid, and 5.6% uninsured). Thirty-eight percent of Hispanic HCC patients were within Milan criteria at diagnosis. With latter time periods, significantly more patients were diagnosed with HCC within Milan criteria, and in 2013-2014, 42.6% had HCC within Milan criteria. On multivariate regression, Hispanic males (OR *vs* females: 0.76, 95%CI: 0.68-0.83, $P < 0.001$), Hispanics > 65 years (OR *vs* age < 50: 0.67, 95%CI: 0.58-0.79, $P < 0.001$), and uninsured patients (OR *vs* Medicare/commercial: 0.49, 95%CI: 0.40-0.59, $P < 0.001$) were significantly less likely to have HCC within Milan criteria at diagnosis.

CONCLUSION

While one in five HCC patients in the United States are of Hispanic ethnicity, only 38% were within Milan criteria at time of diagnosis, and thus over 60% were

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ineligible for liver transplantation, one of the primary curative options for HCC patients. Improved efforts at HCC screening and surveillance are needed among this group to improve early detection.

Key words: Liver cancer; Surveillance; Epidemiology; End results; Milan criteria; Hepatocellular carcinoma

Core tip: The Hispanic population represents a major contributor to the hepatocellular carcinoma (HCC) burden. However, our current study demonstrates that over 60% of Hispanic HCC patients were ineligible for liver transplantation given the extent of disease severity. This advanced cancer stage at diagnosis likely reflects suboptimal implementation of early and timely HCC screening and surveillance in high-risk populations. These findings emphasize the need to be more vigilant about HCC screening and surveillance, especially among the Hispanic population.

Robinson A, Ohri A, Liu B, Bhuket T, Wong RJ. One in five hepatocellular carcinoma patients in the United States are Hispanic while less than 40% were eligible for liver transplantation. *World J Hepatol* 2018; 10(12): 956-965
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INTRODUCTION

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide[1-3]. The number of deaths per year from HCC is nearly identical to the worldwide incidence, highlighting the incredibly high case fatality rate of this aggressive disease[3]. While the highest prevalence of HCC is among populations in Asia and Africa, the incidence of HCC has been increasing in the United States, due to the growing number of patients with nonalcoholic steatohepatitis (NASH) and chronic hepatitis C virus (HCV) who are at risk for HCC[1,4-8]. However, the rising HCC incidence is not uniform across all groups, and an increasingly disproportionate rise in HCC incidence among minority groups, and in particular Hispanics, have been reported[9-13]. A recent study by White *et al*[7] reported a 4.5% annual increase in overall HCC incidence in the United States from 2000 to 2009. However, race/ethnicity-specific differences were observed with significantly greater increases in HCC incidence among Hispanics, such that beginning in 2012, HCC incidence among Hispanics surpassed that of Asians in the United States. While the exact etiology underlying this epidemiology is unclear, these trends likely reflect the successful impact of HBV vaccination in Asians and the emergence of nonalcoholic fatty liver disease (NAFLD) in the Hispanic population. Because the Hispanic population carries a disproportionate burden of NAFLD, understanding trends in HCC among the Hispanic population in the United States may provide insight on NAFLD-HCC trends[11,14-19]. The aim of the current study is to evaluate overall trends and disparities in HCC outcomes among Hispanic HCC patients in the United States with a focus on HCC tumor stage at presentation.

MATERIALS AND METHODS

The National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) population based cancer registry was reviewed to identify all Hispanic adults (age 20 years and older) with HCC from 2004 to 2014 in the United States[20]. The 2004-2014 SEER data includes data from 18 regions in the United States (San Francisco-Oakland, Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, Atlanta, San Jose-Monterey, Los Angeles, Alaska Natives, Rural Georgia, California excluding San Francisco/San Jose/Los Angeles, Kentucky, Louisiana, New Jersey, and Greater Georgia) and represents approximately 28% of the United States population.

The International Classification of Disease for Oncology, Third Edition was used by the SEER database to define HCC[21]. HCC tumor stage was evaluated using SEER

historic summary staging, which is unique to SEER and is not used particularly for prognosis, but to describe the extent of disease[21]. Tumors confined to only one lobe of the liver with or without vascular invasion were defined as localized. Tumors involving more than one lobe through contiguous growth of a single lesion, extension to adjacent structures (gallbladder, diaphragm, or extrahepatic bile ducts), or spread to regional lymph nodes were defined as regional. Metastatic disease or extension of cancer to distant lymph nodes or nearby organs such as the stomach, pleura, or pancreas was defined as distant. In addition to SEER HCC staging, we also evaluated tumor characteristics including size and number of tumors to determine whether a patient's tumor met Milan criteria (single lesion less than 5 cm or no more than 3 lesions each less than 3 cm) with no extrahepatic or vascular involvement. We specifically chose to evaluate whether or not a patient's tumor met Milan criteria, as this affects eligibility for liver transplantation, which is one of the major curative treatment options for HCC[22].

Insurance status was evaluated using SEER classifications, which included three categories: Medicare or commercial insurance, Medicaid, and uninsured/no insurance. Medicare or commercial insurance includes patients with private insurance (fee-for-service, managed care, HMO, PPO, Tricare) or Medicare (administered through a managed care plan, Medicare with private supplement, or Medicare with supplement, NOS and Military). Medicaid includes patients with Medicaid (including administered through managed care plans, Medicare with Medicaid eligibility, and Indian/Public Health Service). Uninsured is defined as those who were either not insured or self-pay at time of HCC diagnosis. Age-specific comparisons utilized three categories of age at time of HCC diagnosis (age < 50 years, age 50 - 64 years, and age > 65 years).

Statistical analysis

Demographic characteristics of the study cohort were presented as proportions and frequencies. Overall proportion of adult Hispanic patients with localized HCC or HCC within Milan criteria were stratified by sex, age at time of diagnosis, insurance status, and year of diagnosis, and compared across groups using $\chi 2$ test. Predictors of HCC tumor stage at presentation and HCC treatment received were evaluated using multivariate logistic regression models. Forward stepwise regression methods were used with variables demonstrating significant associations in the univariate model (P < 0.10) or those with biologic significance determined a priori (*e.g.*, age and sex) included in the final model. Statistical significance was met with a 2-tailed P-value < 0.05. All statistical analyses were performed with Stata version 14 (Stata Corp, College Station, TX). The study was reviewed and determined to be exempt by the Alameda Health System Institutional Review Board because human subjects were not involved, as per United States Department of Health and Human Services guidelines, and the SEER database is publicly available without individually identifiable private information.

RESULTS

Hispanics accounted for 19.8% of all HCC among United States adults in the 2004-2014 SEER registry, with the majority of subjects with HCC being men (n = 9856, 73.3%). Among both Hispanic men and women with HCC, 60.9% had Medicare or commercial insurance at time of HCC diagnosis, 33.5% had Medicaid, and 5.6% were uninsured (Table 1). The proportion of the HCC cohort represented by Hispanic patients increased from 17.6% in 2004-2006 to 20.1% in 2013-2014.

When evaluating HCC tumor stage at diagnosis, 38.0% of Hispanic HCC patients were within Milan criteria, and 54.0% of Hispanic HCC patients had localized HCC (Table 2). Compared to female HCC patients, male HCC patients had significantly lower rates of localized HCC (52.8% *vs* 57.6%, P < 0.001) and significantly lower rates of HCC within Milan criteria (37.4% *vs* 39.7%, P = 0.01). Compared to HCC patients age < 50 years at time of diagnosis, higher rates of localized HCC and HCC within Milan criteria were observed in those age 50-64, but HCC patients age > 65 years had lower rates of localized HCC or HCC within Milan criteria (Table 2). Compared to Medicare or commercially insured patients, Hispanic HCC patients with Medicaid or no insurance had significantly lower rates of localized HCC or HCC within Milan criteria.

When stratified by year of HCC diagnosis, Hispanic HCC patients in the latter time periods had significantly higher rates of localized HCC at time of diagnosis, and in 2013-2014, 56.9% of Hispanic HCC patients had localized tumor stage. Similarly, the proportion of Hispanic patients with HCC within Milan criteria also increased with

Table 1 Characteristics of the study cohort

	Hispanics		Asians		Non-Hispanic White	
	Percent (%)	Frequency (*n*)	Percent (%)	Frequency (*n*)	Percent (%)	Frequency (*n*)
Sex						
Female	26.7	3584	29.5	3273	24.9	9273
Male	73.3	9856	70.6	7840	75.1	27940
Age						
< 50 yr	10.7	1440	10.7	1191	6.1	2268
50-64 yr	47.1	6325	36.4	4045	45.6	16973
> 65 yr	42.2	5675	52.9	5877	48.3	17972
Insurance						
Medicare or Commercial	60.9	5969	66.3	5177	80.2	21265
Medicaid	33.5	3280	29.5	2299	16.1	4257
Uninsured	5.6	549	4.3	335	3.7	983
Year of diagnosis						
2004-2006	17.6	2722	17.6	2713	51.8	8009
2007-2008	17.4	2115	16.3	1985	52.5	6376
2009-2010	18.9	2550	15.4	2084	51.3	6927
2011-2012	20.1	2870	13.9	2133	51.9	7690
2013-2014	20.1	3183	13.9	2198	51.7	8211
HCC SEER summary stage						
Localized	54	6091	51.8	2250	52.6	8644
Regional	29.5	3324	31	1345	31.2	5129
Distant	16.5	1858	15	747	16.2	2652
HCC within Milan criteria						
No	62	8337	59.1	2832	55.4	10530
Yes	38	5103	40.9	1958	44.6	8465

HCC: Hepatocellular carcinoma; SEER: Surveillance, Epidemiology, and End Results.

time, and in 2013-2014, 42.6% of Hispanic HCC patients were within Milan criteria (Table 3). When stratified by sex, age, and insurance status, similar trends were seen, such that HCC patients in latter time periods had the highest rates of more localized stage of disease.

On multivariate regression, in comparison to Medicare/commercially insured Hispanic HCC patients, uninsured patients were significantly less likely to have HCC within Milan criteria at time of diagnosis (OR 0.49, 95%CI: 0.40-0.59, $P < 0.001$), whereas no significant difference was observed in Medicaid HCC patients (Figure 1). Compared to 2004-2006, Hispanic HCC patients in both the 2011-2012 and 2013-2014 cohorts were significantly more likely to have HCC within Milan criteria at diagnosis (2011-2012: OR 1.17, 95%CI: 1.04-1.32, $P = 0.007$; 2013-2014: OR 1.30, 95%CI: 1.16-1.45, $P < 0.001$). Compared to patients age < 50 years at the time of diagnosis, Hispanic HCC patients age 65 years and older were significantly less likely to have HCC within Milan criteria (OR 0.67, 95%CI: 0.58-0.79, $P < 0.001$), whereas patients age 50-64 years old were significantly more likely to have HCC within Milan criteria (OR 1.33, 95%CI: 1.15-1.54, $P < 0.001$). Males were less likely than females to be within Milan criteria (OR 0.76, 95%CI: 0.68-0.83, $P < 0.001$) (Figure 1).

Similar trends were observed when evaluating predictors of SEER localized stage of HCC at diagnosis. Uninsured Hispanic HCC patients and those with Medicaid were significantly less likely to have localized disease compared to those patients with Medicare or commercial insurance (Uninsured: OR 0.43, 95%CI: 0.33-0.55, $P < 0.001$, Medicaid: OR 0.85, 95%CI: 0.74-0.97, $P = 0.018$). Patients diagnosed with HCC during 2013-2014 were more likely to have localized disease as compared to those diagnosed from 2004-2006 (OR: 1.28, 95%CI: 1.08-1.52, $P = 0.004$), however there was no significant difference when comparing 2011-2012 or 2007-2008 to 2004-2006. Males were less significantly less likely to have localized disease compared to females (OR 0.79, 95%CI: 0.68-0.91, $P = 0.001$) (Figure 2).

Table 2 Proportion of Hispanic hepatocellular carcinoma patients with localized hepatocellular carcinoma or hepatocellular carcinoma within Milan criteria at time of diagnosis

	Localized HCC at Time of diagnosis			HCC within Milan criteria at time of diagnosis		
	Percent (%)	Frequency (*n*)	*P*-value	Percent (%)	Frequency (*n*)	*P*-value
Total	54	6091		38	5103	
Sex						
Female	57.6	1680	< 0.001	39.7	1422	0.01
Male	52.8	4411		37.4	3681	
Age						
< 50 yr	51.7	630	< 0.01	36.8	530	< 0.001
50-64 yr	55.5	3030		44.6	2820	
> 65 yr	52.9	2431		30.9	2753	
Insurance						
Medicare or Commercial	56.2	3004	< 0.001	42.7	2547	< 0.001
Medicaid	53.8	1607		42.9	1407	
Uninsured	39.4	191		29.9	164	
Year of Diagnosis						
2004-2006	52.8	1195	< 0.001	33.4	908	< 0.001
2007-2008	31.7	553		33.8	714	
2009-2010	52.7	1368		37.3	951	
2011-2012	56.3	1368		40.9	1173	
2013-2014	56.9	1525		42.6	1357	

HCC: Hepatocellular carcinoma.

DISCUSSION

Using United States population-based cancer registry data, the current study evaluated disparities in HCC tumor stage at diagnosis with a focus on Hispanics with HCC. Overall, the proportion of HCC in the Hispanic population increased during the study period such that over 20% of HCC patients in the United States during 2013-2014 were of Hispanic ethnicity. Despite this huge disease burden among the Hispanic population, less than half of Hispanic HCC patients were within Milan criteria at the time of diagnosis in the most recent time period studied. Furthermore, significant disparities among this group were observed, with a disproportionately higher risk of advanced tumor stage at presentation among Hispanic men, older Hispanic patients, and uninsured Hispanic patients.

Nearly one in five HCC patients in the United States are of Hispanic ethnicity with Hispanic patients showing the greatest increase in the incidence of HCC from 2004-2006 to 2013-2014[20]. These observations are consistent with previous SEER registry based studies evaluating trends in HCC incidence. For example, El-Serag *et al*[23] reported significantly greater age-adjusted HCC incidence rates in Hispanic patients in comparison to non-Hispanic white and black populations between 1992 and 2002. This increasing HCC incidence among the Hispanic population in the United States has continued to rise such that HCC incidence among Hispanics surpassed HCC incidence among Asians in 2012 to become the ethnic group with the greatest incidence of HCC in the United States and representing over 20% of all HCC among adults in the United States[7]. While our current study is limited by the ability to evaluate the liver disease etiology contributing to HCC, the Hispanic population in the United States carries a disproportionate burden of NAFLD[14-17,24-28]. Furthermore, during this same period where Hispanics have become the ethnic group with the most rapidly rising incidence of HCC, it is also important to note that prevalence of NASH-related HCC has also risen in parallel[8,18,26,29-31]. Two recent studies using the SEER-Medicare database and the United Network for Organ Sharing registry demonstrate the continued rising prevalence of NAFLD-related HCC in the United States[8,18].

While the growing burden of HCC among the Hispanic population is concerning, it is even more alarming that the Hispanic population faces significant disparities in access to treatment options for HCC, which contributes to significantly lower survival[32-35]. A recent study by Ha *et al*[34] using the SEER database found that Hispanic patients with HCC had significantly lower rates of curative HCC treatment

Table 3 Trends in the proportion of Hispanic hepatocellular carcinoma patients with localized hepatocellular carcinoma and hepatocellular carcinoma within Milan criteria over time

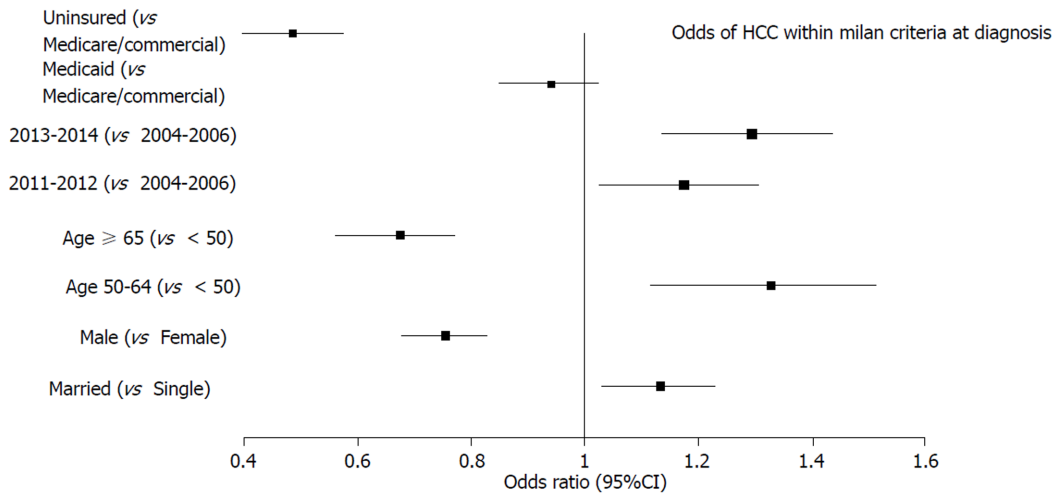
Localized HCC	Overall		Female		Male		Age < 50		Age 50-64		Age 65 and over		Uninsured		Medicaid		Medicare/Commercial	
	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
2004-2006	52.8	1195	58	342	51	853	48.7	165	54.1	544	52.9	486	-	-	-	-	-	-
2007-2008	31.7	553	55.5	236	48.1	635	46	115	50.9	424	50.1	332	33.9	38	49.3	281	52.1	531
2009-2010	52.7	1368	55.3	588	51.8	844	51.5	121	55.4	589	49.6	422	36	40	52.6	397	54.4	675
2011-2012	56.3	1368	58	363	55.7	1005	60.2	121	57.5	706	54.1	541	39.6	53	56.4	456	58	835
2013-2014	56.9	1525	59.7	451	55.7	1074	56	108	57.6	767	56.1	650	46.9	60	55.5	473	58.4	963
P-Value		< 0.001		< 0.03		<0.001		< 0.02		< 0.02		< 0.01		0.17		0.14		< 0.001
HCC Within Milan	**Overall**		**Female**		**Male**		**Age < 50**		**Age 50-64**		**Age 65 and Over**		**Uninsured**		**Medicaid**		**Medicare/Commercial**	
	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*
2004-2006	33.4	908	34.6	255	34.3	653	32.9	141	39.9	462	26.4	305	-	-	-	-	-	-
2007-2008	33.8	714	32.8	179	35.5	535	34.1	105	40.6	397	25.2	212	27.5	36	37.8	237	37.6	428
2009-2010	37.3	951	40.6	260	35.3	691	36.2	97	45.5	559	28.2	295	30.3	37	40.1	337	40.4	558
2011-2012	40.9	1173	41.1	310	40.2	863	40.8	92	47.7	676	33.1	405	30.7	47	47.5	420	42.4	687
2013-2014	42.6	1357	46.1	418	36.8	939	41.2	95	47.1	726	30.9	536	30.8	44	44.2	413	47.8	874
P-Value		< 0.001		< 0.001		< 0.001		0.3		< 0.001		< 0.001		0.92		< 0.001		< 0.001

HCC: Hepatocellular carcinoma.

when compared to non-Hispanic whites. Hispanics were also significantly less likely to receive liver transplantation even after correcting for stage of disease at time of diagnosis. Our current analysis of the most recent SEER database continues to highlight these disparities among the Hispanic HCC population. Despite improvements in earlier tumor stage of diagnosis over time, even in the most recent 2013-2014 period only 42.6% of Hispanic HCC patients were within Milan criteria at the time of diagnosis, which means that nearly 60% of patients were not eligible for liver transplantation, one of the main curative options for HCC patients.

A greater frequency of HCC was observed among male patients within our study, which is consistent with prior data showing HCC rates in males are two to four times as high as rates in females[35,36]. This sex-specific trend in HCC risk is also observed among NAFLD HCC populations. Corey *et al*[36] evaluated at risk factors for HCC in 94 patients with NAFLD with HCC and 150 patients with NAFLD without HCC. The strongest association with risk of HCC was being male, with males having a four-fold higher risk of developing HCC compared to females. The majority of Hispanic HCC patients in our study were men, and they had more advanced HCC at diagnosis compared to females. Similarly Yang *et al*[37] observed significantly better outcomes in females with HCC. In their study of primarily non-Hispanic Caucasian patients, the distribution of tumor burden showed a higher incidence of single lesions among women and a higher incidence of vascular invasion among men.

Our study utilized a large population-based cancer registry, which represents a large proportion of the United States population. The SEER registry allowed for a large sample size and comprehensive analysis of HCC epidemiology and outcomes. Despite this, the current study has several limitations that should be acknowledged. SEER does not include etiology of HCC (*e.g.*, chronic hepatitis B virus, chronic HCV, alcoholic liver disease, or NAFLD), which may have affected rates of disease progression or rates of timely HCC screening and surveillance. Along the same lines, liver disease-specific treatment data (*e.g.*, antiviral therapies) were not available for inclusion in the analysis. While our study observed more advanced tumor stage among older patients, this observation may be confounded by other factors. Older patients may have had more significant co-morbidities (hypertension, diabetes mellitus, or cardiac disease) that may have affected referral for HCC screening and surveillance. However, these additional co-morbidities and risk factors such as alcohol use, obesity, and concurrent diabetes mellitus were not available in the database for inclusion in our analyses. While our study also investigated disparities related to insurance status, we acknowledge that insurance status is only one key factor that is tightly linked to other demographic and socioeconomic factors in a complex manner. Furthermore, in the SEER database, Medicare and commercially

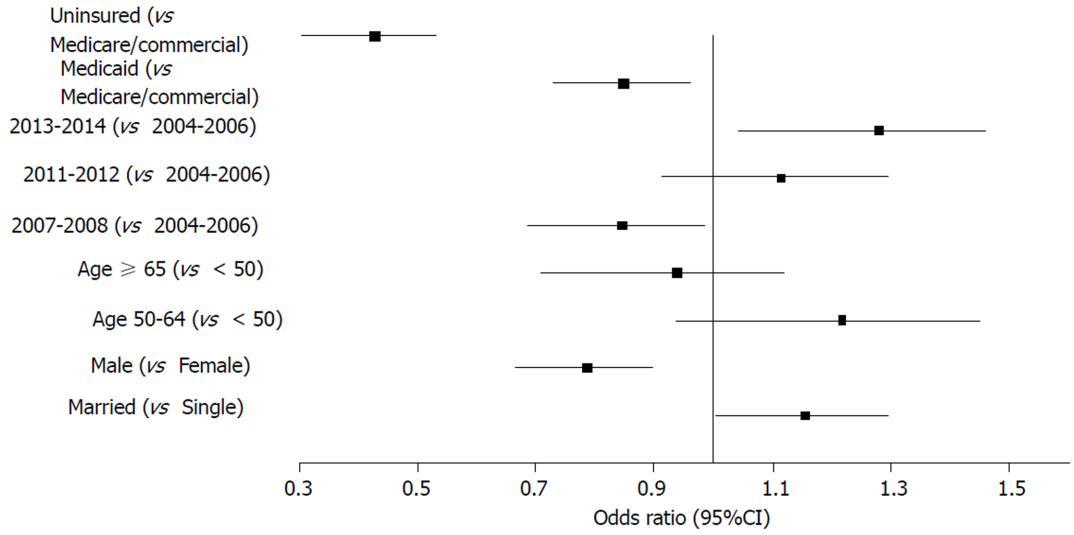


	Odds ratio	95%CI	*P*-Value
Uninsured (*vs* Medicare/commercial)	0.49	0.40-0.59	< 0.001
Medicaid (*vs* Medicare/commercial)	0.94	0.86-1.03	0.203
2013-2014 (*vs* 2004-2006)	1.30	1.16-1.45	< 0.001
2011-2012 (*vs* 2004-2006)	1.17	1.04-1.32	0.007
Age ⩾ 65 (*vs* < 50)	0.67	0.58-0.79	< 0.001
Age 50-64 (*vs* < 50)	1.33	1.15-1.54	< 0.001
Male (*vs* Female)	0.76	0.68-0.83	< 0.001
Marital status (Married *vs* Single)	1.13	1.04-1.24	0.005

Figure 1 Multivariate logistic regression model evaluating predictors of hepatocellular carcinoma within Milan criteria at diagnosis. These data specifically evaluate the independent risk of having hepatocellular carcinoma (HCC) within Milan criteria at diagnosis when evaluated by insurance status at time of diagnosis, the year of HCC diagnosis, the age at time of HCC diagnosis, sex, and marital status. An odds ratio of > 1.00 indicates increased likelihood of having HCC within Milan criteria at time of diagnosis.

insured patients are combined into one category, and demographic and clinical differences between those with Medicare and commercial insurance may affect HCC stage at diagnosis. However, access to care, including HCC screening and surveillance that would affect tumor stage and HCC treatment are expected to be similar between those with Medicare and commercial insurance in the United States. While our study found significant disparities in HCC stage among uninsured patients, these differences may reflect difficulty accessing healthcare due to complex socioeconomic needs. However, these data were not available to assess further in the database. Another factor not readily available for analysis is concurrent substance use and high-risk behavior, which may have affected timely access and adherence to HCC surveillance. Lastly, surveillance data were not available to evaluate how the differences in successful screening and surveillance rates contributed to the observed disparities in HCC stage at diagnosis. Despite these limitations, our study provides valuable information regarding a vulnerable subset of the United States population.

Understanding HCC trends provides valuable data to guide clinicians and policy makers to identify high-risk groups as well as those that suffer disparities towards which resources can be targeted to improve HCC screening and surveillance for early detection and treatment. Our current study focused specifically on Hispanic HCC patients, a group that has the highest incidence of HCC in the United States since 2012 and represents 20% of all adults with HCC. Among this group less than 40% had HCC within Milan criteria at the time of diagnosis, and thus over 60% were ineligible for liver transplantation, one of the major curative options for HCC patients. Part of this problem revolves around effective HCC screening and surveillance, and despite established guidelines, overall HCC screening rates remain low[38-40]. In one United States population-based study, less than 20% of patients with cirrhosis who developed HCC received regular surveillance, and only 29% of patients who had a diagnosis of HCC had undergone annual surveillance in the three years before receiving the diagnosis[41]. Tavakoli *et al*[42] observed that less than half of cirrhotic patients received first time HCC screening among safety-net populations with cirrhosis, with the largest disparity occurring among patients with NASH cirrhosis. Improved efforts at HCC screening and surveillance are needed, particularly among Hispanic patients, a growing population with a high prevalence of NAFLD.



	Odds ratio	95%CI	*P*-Value
Uninsured (*vs* Medicare/commercial)	0.43	0.33-0.55	< 0.001
Medicaid (*vs* Medicare/commercial)	0.85	0.74-0.97	0.018
2013-2014 (*vs* 2004-2006)	1.28	1.08-1.52	0.004
2011-2012 (*vs* 2004-2006)	1.12	0.94-1.32	0.208
2007-2008 (*vs* 2004-2006)	0.85	0.71-1.01	0.072
Age 65+ (*vs* < 50)	0.94	0.76-1.17	0.573
Age 50-64 (*vs* < 50)	1.22	0.99-1.50	0.063
Male (*vs* Female)	0.79	0.68-0.91	0.001
Married (*vs* Single)	1.16	1.02-1.31	0.025

Figure 2 Multivariate logistic regression model evaluating predictors of localized stage hepatocellular carcinoma at diagnosis. These data specifically evaluate the independent risk of having localized cancer stage hepatocellular carcinoma (HCC) at diagnosis compared to advanced cancer stage when evaluated by insurance status at time of diagnosis, the year of HCC diagnosis, the age at time of HCC diagnosis, sex, and marital status. An odds ratio of > 1.00 indicates increased likelihood of having localized cancer stage HCC at time of diagnosis.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is a leading cause of morbidity and mortality worldwide. The Hispanic population represents a major contributor of HCC prevalence, particularly in the United States. Understanding HCC epidemiology and disparities in HCC outcomes among this cohort will help guide interventions to improve HCC care.

Research motivation

Given the significant burden of HCC among the Hispanic population, understanding HCC epidemiology and outcomes among this group is critical. Data gathered from studying HCC epidemiology can help identify potential areas where quality improvement programs can be developed and implemented to improve management of HCC.

Research objectives

The main objective of this study was to evaluate disparities in cancer stage at diagnosis among Hispanic HCC patients.

Research methods

The current study utilized a large United States national-based cancer registry. We utilized a retrospective observational cohort study design to evaluate HCC epidemiology and outcomes among Hispanic adults diagnosed with HCC from 2004 to 2014.

Research results

We identified that over 60% of Hispanic HCC patients were diagnosed with advanced cancer stage that was beyond eligibility for liver transplantation. This highlights an important disparity and may reflect suboptimal utilization of timely HCC screening and surveillance among this population.

Research conclusions

These findings may suggest that the Hispanic population at risk for HCC may experience suboptimal receipt of or delays in timely HCC screening and surveillance. These study findings further add to the existing literature highlighting the poor adherence to HCC screening and surveillance among at-risk populations. Specifically, our study identified a high-risk group in the Hispanic population, which is particularly concerning given the higher risk of nonalcoholic fatty liver disease in this population, a disease that is increasing in prevalence.

Research perspectives

Our study findings emphasize the importance of timely and consistent implementation of HCC screening and surveillance that will translate into improved early HCC detection. This will ultimately improve treatment options for curative intent and thus improve long-term survival outcomes among HCC patients.

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