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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

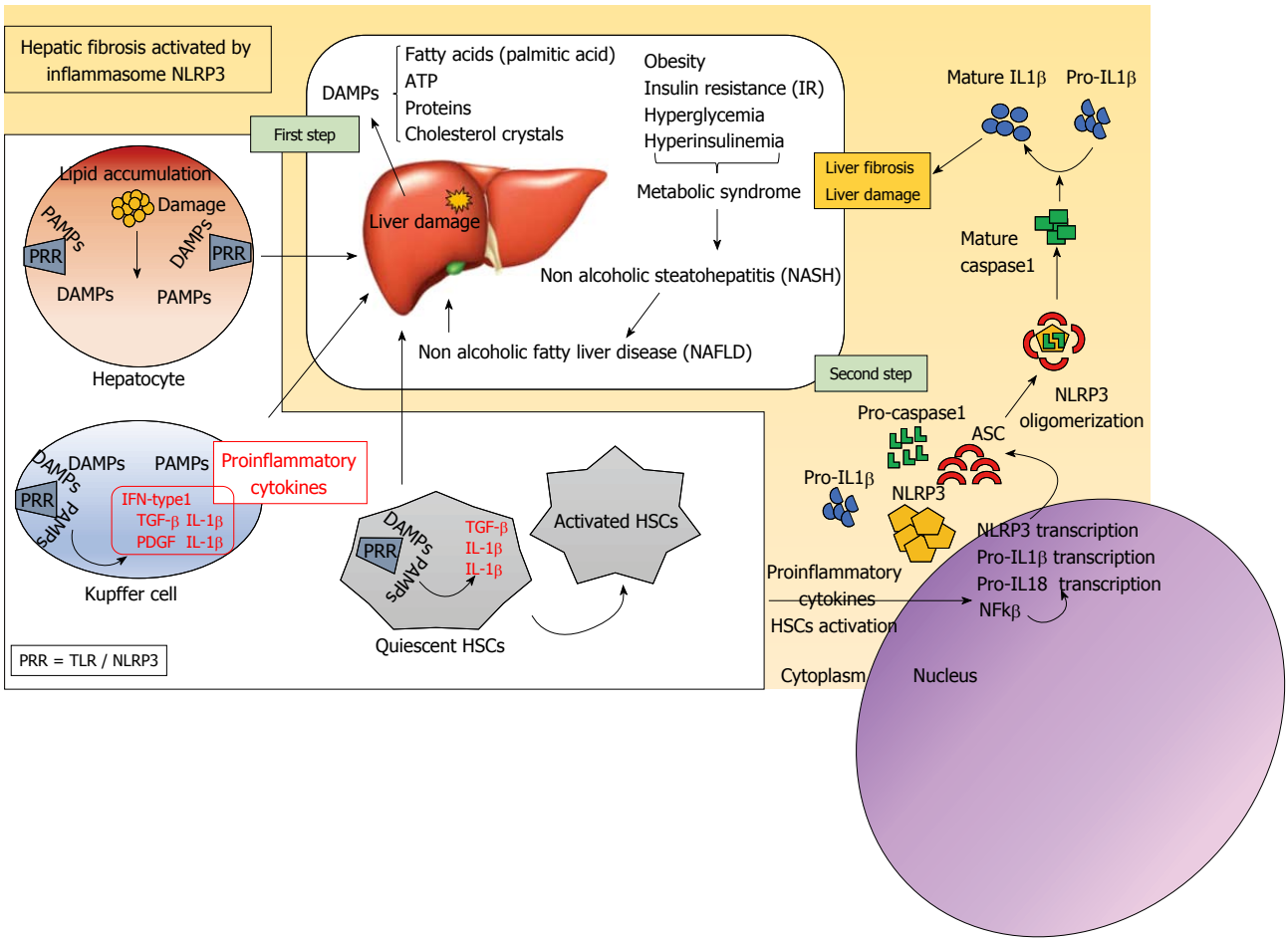

Figure 1 Hepatic fibrosis caused by the activation of the inflammasome-NLRP3 complex. The accumulation of lipids derived from the metabolic syndrome produces a non-alcoholic steatohepatitis (NASH) that progresses to a non-alcoholic fatty liver disease (NAFLD). The accumulated lipids, fatty acids, cholesterol 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.

REFERENCES

1 **Younossi ZM**, Loomba R, Anstee QM, Rinella ME, Bugianesi E, Marchesini G, Neuschwander-Tetri BA, Serfaty L, Negro F, Caldwell SH, Ratziu V, Corey KE, Friedman SL, Abdelmalek MF, Harrison SA, Sanyal AJ, Lavine JE, Mathurin P, Charlton MR, Goodman ZD, Chalasani NP, Kowdley KV, George J, Lindor K. Diagnostic Modalities for Non-alcoholic Fatty Liver Disease (NAFLD), Non-alcoholic Steatohepatitis (NASH) and Associated Fibrosis. *Hepatology* 2017; Epub ahead of print [PMID: 29222917 DOI: 10.1002/hep.29721]

2 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438 [PMID: 7382552]

3 **Chalasani N**, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012; **55**: 2005-2023 [PMID: 22488764 DOI: 10.1002/hep.25762]

4 **Loomba R**, Sanyal AJ. The global NAFLD epidemic. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 686-690 [PMID: 24042449 DOI: 10.1038/nrgastro.2013.171]

5 **Buzzetti E**, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism* 2016; **65**: 1038-1048 [PMID: 26823198 DOI: 10.1016/j.metabol.2015.12.012]

6 **Brunt EM**, Wong VW, Nobili V, Day CP, Sookoian S, Maher JJ, Bugianesi E, Sirlin CB, Neuschwander-Tetri BA, Rinella ME. Nonalcoholic fatty liver disease. *Nat Rev Dis Primers* 2015; **1**: 15080 [PMID: 27188459 DOI: 10.1038/nrdp.2015.80]

7 **Hassan K**, Bhalla V, El Regal ME, A-Kader HH. Nonalcoholic fatty liver disease: a comprehensive review of a growing epidemic. *World J Gastroenterol* 2014; **20**: 12082-12101 [PMID: 25232245 DOI: 10.3748/wjg.v20.i34.12082]

8 **Chisholm JW**, Hong J, Mills SA, Lawn RM. The LXR ligand T0901317 induces severe lipogenesis in the db/db diabetic mouse. *J Lipid Res* 2003; **44**: 2039-2048 [PMID: 12923232]

9 **Begriche K**, Massart J, Robin MA, Bonnet F, Fromenty B. Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. *Hepatology* 2013; **58**: 1497-1507 [PMID: 23299992 DOI: 10.1002/hep.26226]

10 **Diehl AM**, Li ZP, Lin HZ, Yang SQ. Cytokines and the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2005; **54**: 303-306 [PMID: 15647199 DOI: 10.1136/gut.2003.024935]

11 **Walenbergh SM**, Koek GH, Bieghs V, Shiri-Sverdlov R. Non-alcoholic steatohepatitis: the role of oxidized low-density lipoproteins. *J Hepatol* 2013; **58**: 801-810 [PMID: 23183522 DOI: 10.1016/j.jhep.2012.11.014]

12 **Wree A**, McGeough MD, Peña CA, Schlattjan M, Li H, Inzaugarat ME, Messer K, Canbay A, Hoffman HM, Feldstein AE. NLRP3 inflammasome activation is required for fibrosis development in NAFLD. *J Mol Med* (Berl) 2014; **92**: 1069-1082 [PMID: 24861026 DOI: 10.1007/s00109-014-1170-1]

13 **Tilg H**, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. *N Engl J Med* 2000; **343**: 1467-1476 [PMID: 11078773 DOI: 10.1056/NEJM200011163432007]

14 **Martinon F**, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002; **10**: 417-426 [PMID: 12191486 DOI: 10.1016/S1097-2765(02)00599-3]

15 **Ioannou GN**, Haigh WG, Thorning D, Savard C. Hepatic cholesterol crystals and crown-like structures distinguish NASH from simple steatosis. *J Lipid Res* 2013; **54**: 1326-1334 [PMID: 23417738 DOI: 10.1194/jlr.M034876]

16 **Ix JH**, Sharma K. Mechanisms linking obesity, chronic kidney disease, and fatty liver disease: the roles of fetuin-A, adiponectin, and AMPK. *J Am Soc Nephrol* 2010; **21**: 406-412 [PMID: 20150538 DOI: 10.1681/ASN.2009080820]

17 **Miura K**, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. *Hepatology* 2013; **57**: 577-589 [PMID: 22987396 DOI: 10.1002/hep.26081]

18 **Ozaki E**, Campbell M, Doyle SL. Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives. *J Inflamm Res* 2015; **8**: 15-27 [PMID: 25653548 DOI: 10.2147/JIR.S51250]

19 **Takeuchi O**, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**: 805-820 [PMID: 20303872 DOI: 10.1016/j.cell.2010.01.022]

20 **Iracheta-Vellve A**, Petrasek J, Gyongyosi B, Satishchandran A, Lowe P, Kodys K, Catalano D, Calenda CD, Kurt-Jones EA, Fitzgerald KA, Szabo G. Endoplasmic Reticulum Stress-induced Hepatocellular Death Pathways Mediate Liver Injury and Fibrosis via Stimulator of Interferon Genes. *J Biol Chem* 2016; **291**: 26794-26805 [PMID: 27810900 DOI: 10.1074/jbc.M116.736991]

21 **Bian H**, Li F, Wang W, Zhao Q, Gao S, Ma J, Li X, Ren W, Qin C, Qi J. MAPK/p38 regulation of cytoskeleton rearrangement accelerates induction of macrophage activation by TLR4, but not TLR3. *Int J Mol Med* 2017; **40**: 1495-1503 [PMID: 28949380 DOI: 10.3892/ijmm.2017.3143]

22 **Hu W**, Jain A, Gao Y, Dozmorov IM, Mandraju R, Wakeland EK, Pasare C. Differential outcome of TRIF-mediated signaling in TLR4 and TLR3 induced DC maturation. *Proc Natl Acad Sci USA* 2015; **112**: 13994-13999 [PMID: 26508631 DOI: 10.1073/pnas.1510760112]

23 **Kanda T**, Steele R, Ray R, Ray RB. Hepatitis C virus infection induces the beta interferon signaling pathway in immortalized human hepatocytes. *J Virol* 2007; **81**: 12375-12381 [PMID: 17804510 DOI: 10.1128/JVI.01695-07]

24 **Seki E**, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology* 2008; **48**: 322-335 [PMID: 18506843 DOI: 10.1002/hep.22306]

25 **Zhang B**, Xu D, She L, Wang Z, Yang N, Sun R, Zhang Y, Yan C, Wei Q, Aa J, Liu B, Wang G, Xie Y. Silybin inhibits NLRP3 inflammasome assembly through the NAD + /SIRT2 pathway in mice with nonalcoholic fatty liver disease. *FASEB J* 2017 [PMID: 28970254 DOI: 10.1096/fj.201700602R]

26 **Dolunay A**, Senol SP, Temiz-Resitoglu M, Guden DS, Sari AN, Sahan-Firat S, Tunctan B. Inhibition of NLRP3 Inflammasome Prevents LPS-Induced Inflammatory Hyperalgesia in Mice: Contribution of NF-κB, Caspase-1/11, ASC, NOX, and NOS Isoforms. *Inflammation* 2017; **40**: 366-386 [PMID: 27924425 DOI: 10.1007/s10753-016-0483-3]

27 **Csak T**, Velayudham A, Hritz I, Petrasek J, Levin I, Lippai D, Catalano D, Mandrekar P, Dolganiuc A, Kurt-Jones E, Szabo G. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**:

G433-G441 [PMID: 21233280 DOI: 10.1152/ajpgi.00163.2009]
28 **Mridha AR**, Wree A, Robertson AAB, Yeh MM, Johnson CD, Van Rooyen DM, Haczeyni F, Teoh NC, Savard C, Ioannou GN, Masters SL, Schroder K, Cooper MA, Feldstein AE, Farrell GC. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. *J Hepatol* 2017; **66**: 1037-1046 [PMID: 28167322 DOI: 10.1016/j.jhep.2017.01.022]
29 **Bhargava P**, Lee CH. Role and function of macrophages in the metabolic syndrome. *Biochem J* 2012; **442**: 253-262 [PMID: 22329799 DOI: 10.1042/BJ20111708]
30 **Nallagangula KS**, Nagaraj SK, Venkataswamy L, Chandrappa M. Liver fibrosis: a compilation on the biomarkers status and their significance during disease progression. *Future Sci OA* 2017; **4**: FSO250 [PMID: 29255622 DOI: 10.4155/fsoa-2017-0083]
31 **Soehnlein O**, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol* 2010; **10**: 427-439 [PMID: 20498669 DOI: 10.1038/nri2779]
32 **Fagoonee S**, Famulari ES, Silengo L, Camussi G, Altruda F. Prospects for Adult Stem Cells in the Treatment of Liver Diseases. *Stem Cells Dev* 2016; Epub ahead of print [PMID: 27503633 DOI: 10.1089/scd.2016.0144]
33 **Wu X**, Wu X, Ma Y, Shao F, Tan Y, Tan T, Gu L, Zhou Y, Sun B, Sun Y, Wu X, Xu Q. CUG-binding protein 1 regulates HSC activation and liver fibrogenesis. *Nat Commun* 2016; **7**: 13498 [PMID: 27853137 DOI: 10.1038/ncomms13498]
34 **Geerts A**. History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis* 2001; **21**: 311-335 [PMID: 11586463 DOI: 10.1055/s-2001-17550]
35 **Eng FJ**, Friedman SL. Fibrogenesis I. New insights into hepatic stellate cell activation: the simple becomes complex. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G7-G11 [PMID: 10898741 DOI: 10.1152/ajpgi.2000.279.1.G7]
36 **Seki E**, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology* 2015; **61**: 1066-1079 [PMID: 25066777 DOI: 10.1002/hep.27332]
37 **Duarte S**, Baber J, Fujii T, Coito AJ. Matrix metalloproteinases in liver injury, repair and fibrosis. *Matrix Biol* 2015; **44-46**: 147-156 [PMID: 25599939 DOI: 10.1016/j.matbio.2015.01.004]
38 **Henao-Mejia J**, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 2012; **482**: 179-185 [PMID: 22297845 DOI: 10.1038/nature10809]
39 **Pierantonelli I**, Rychlicki C, Agostinelli L, Giordano DM, Gaggini M, Fraumene C, Saponaro C, Manghina V, Sartini L, Mingarelli E, Pinto C, Buzzigoli E, Trozzi L, Giordano A, Marzioni M, De Minicis S, Uzzau S, Cinti S, Gastalderi A, Svegliati-Baroni G. Lack of NLRP3-inflammasome leads to gut-liver axis derangement, gut dysbiosis and a worsened phenotype in a mouse model of NAFLD. *Sci Rep* 2017; **7**: 12200 [PMID: 28939830 DOI: 10.1038/s41598-017-11744-6]

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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)

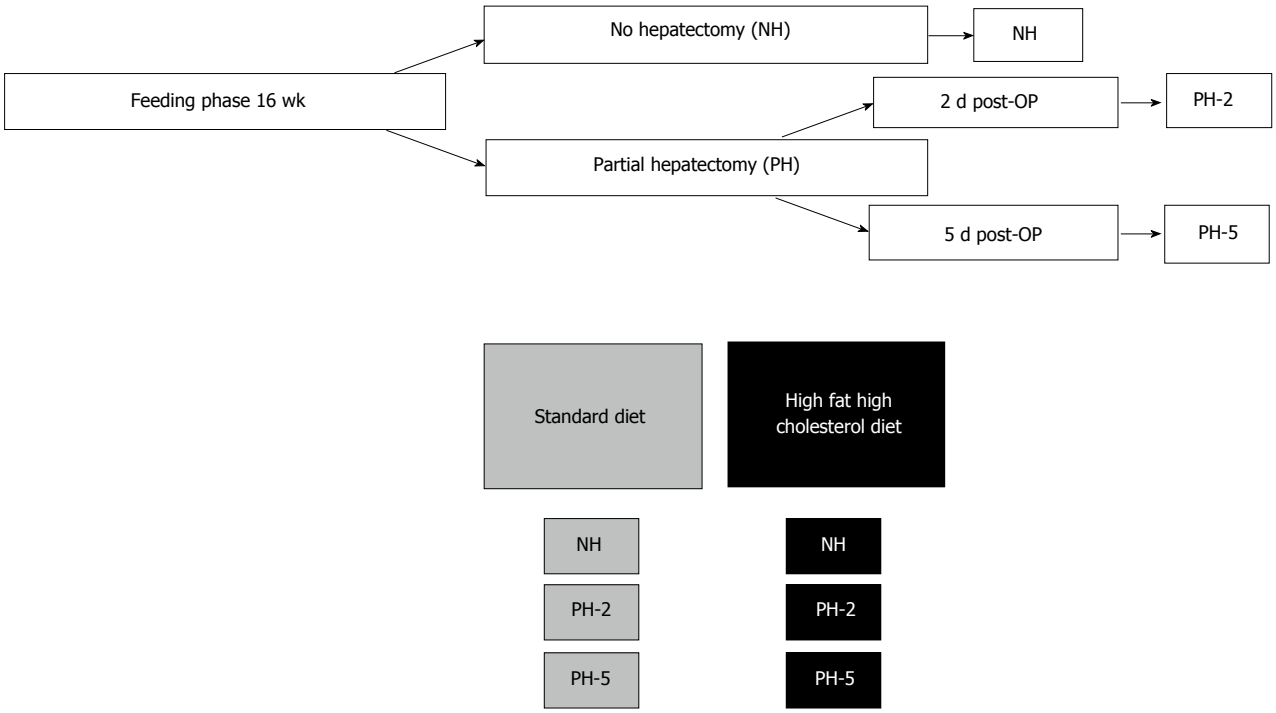

Figure 1 Overview of the groups, 9 animals in each group. PH-2: Partial hepatectomy and 2 d; PH-5: Partial hepatectomy and 5 d.

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-

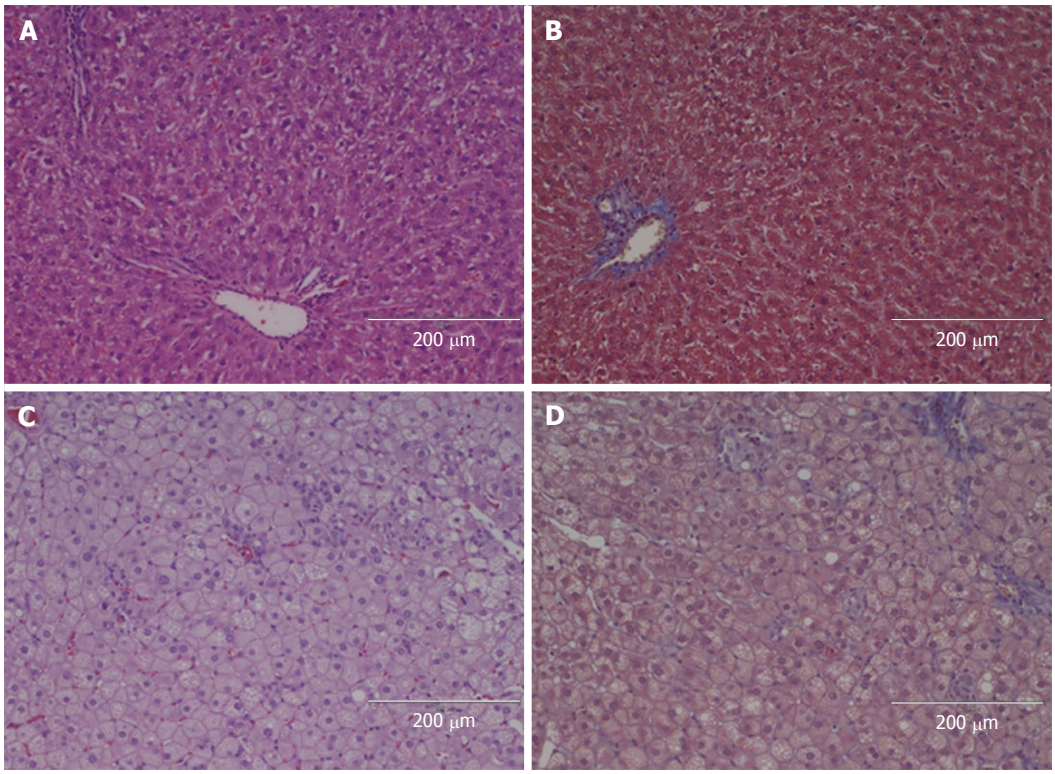

Figure 2 Examples of liver histology. A and C shows Hematoxylin and Eosin; B and D shows Masson Trichome; A and B are from an animal fed the standard diet and euthanised without hepatectomy; C and D are from an animal fed the high fat high cholesterol diet and euthanised without hepatectomy.

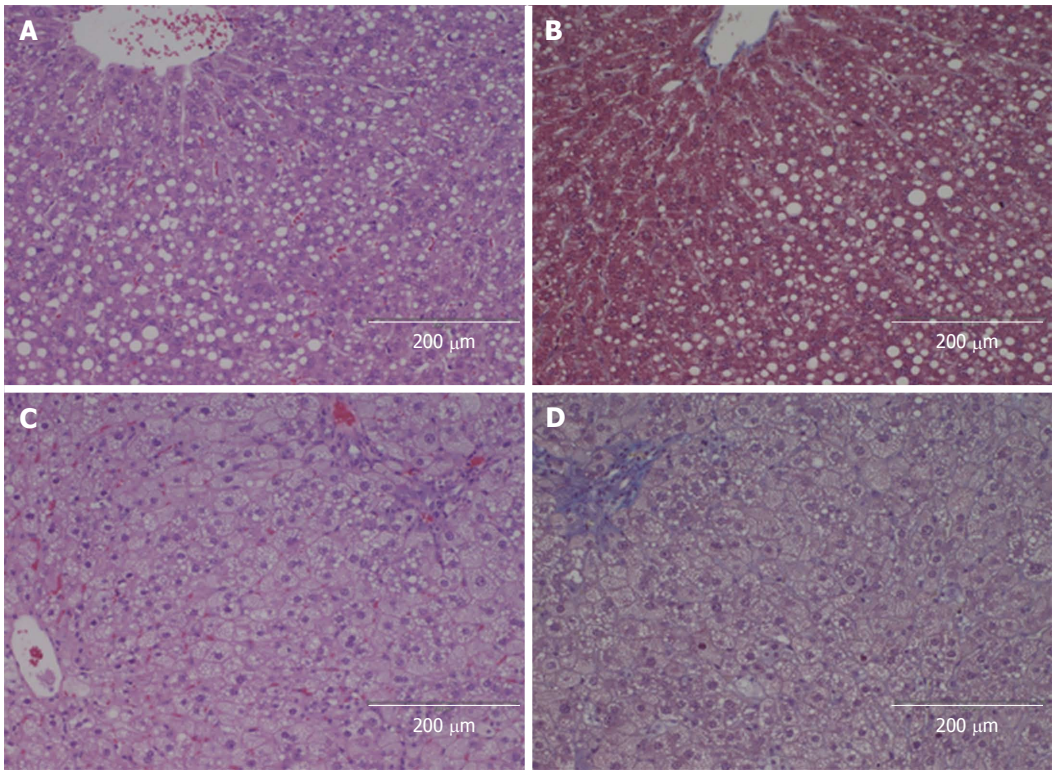

Figure 3 Examples of liver histology. A and C shows Hematoxylin and Eosin; B and D shows Masson Trichome; A and B are from an animal fed the standard diet and euthanised two days after hepatectomy; C and D are from an animal fed the high fat high cholesterol diet and euthanised two days after hepatectomy.

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

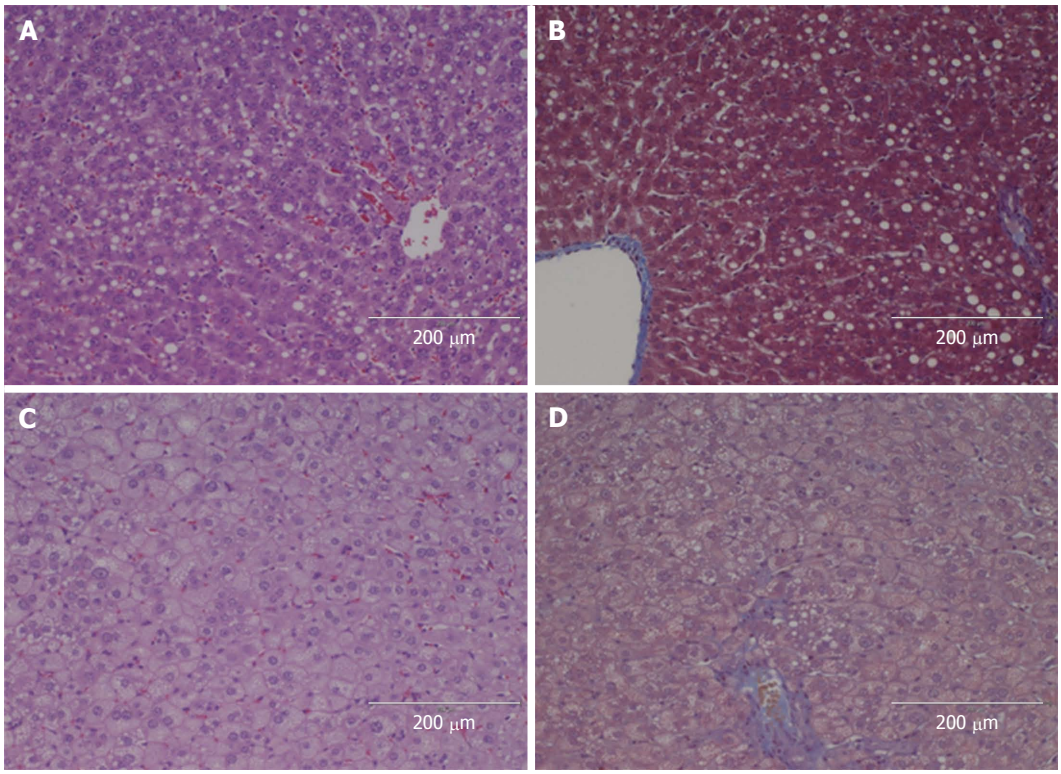

Figure 4 Examples of liver histology. A and C shows Hematoxylin and Eosin; B and D shows Masson Trichome; A and B are from an animal fed the standard diet and euthanised five days after hepatectomy; C and D are from an animal fed the high fat high cholesterol diet and euthanised five days after hepatectomy.

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

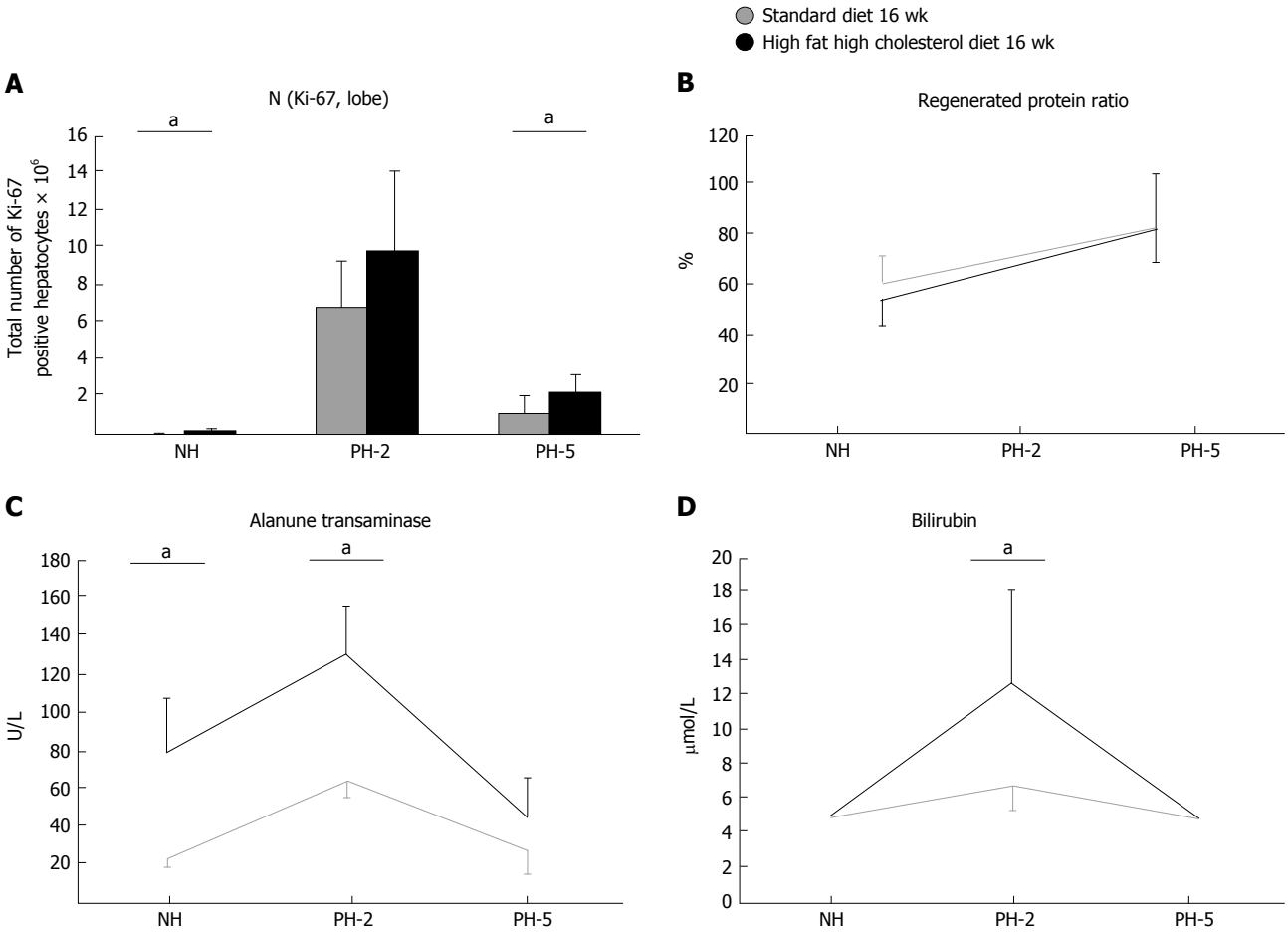

Figure 5 Total number of Ki-67 positive hepatocytes (A); regenerated protein ratio (B); Alanine transaminase (C) and Bilirubin (D). Means and standard derivation displayed. $^{a}P < 0.05$ compared to respective standard diet group. NH: No hepatectomy; PH-2: Partiel hepatectomy and 2 d; PH-5: Partiel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

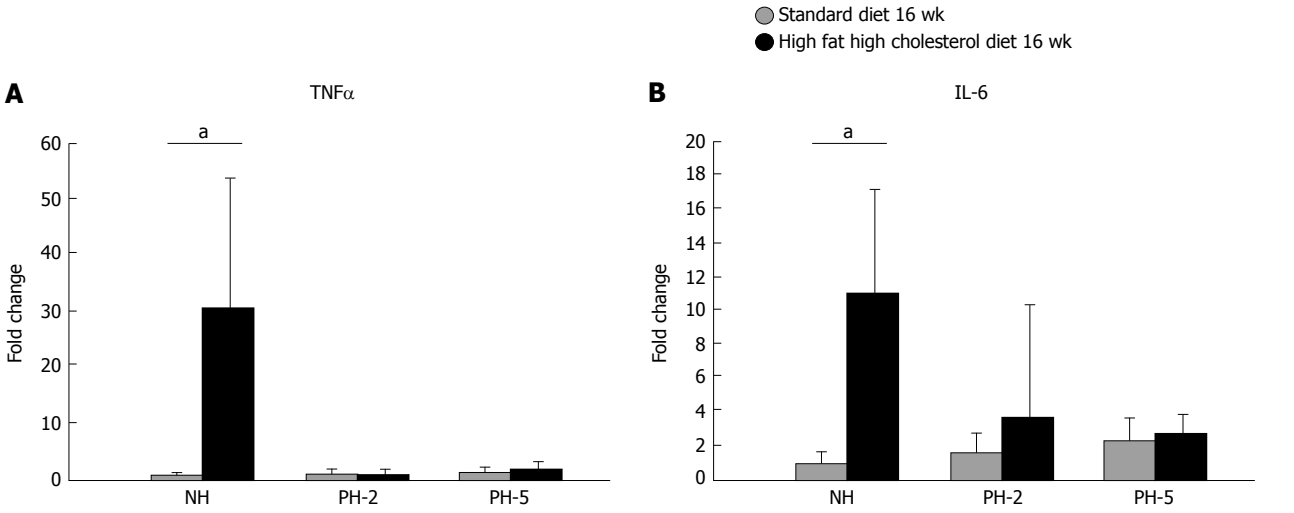

Figure 6 Relative mRNA expression. A: Tumor necrosis factor α (TNFα); B: Interleukin-6 (IL-6) standardized to glyceraldehyde 3-phosphate dehydrogenase. Means and standard derivation displayed. Statistical analysis made using the ANOVA test, $^{a}P < 0.05$ compared to respective standard diet group. NH: 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-

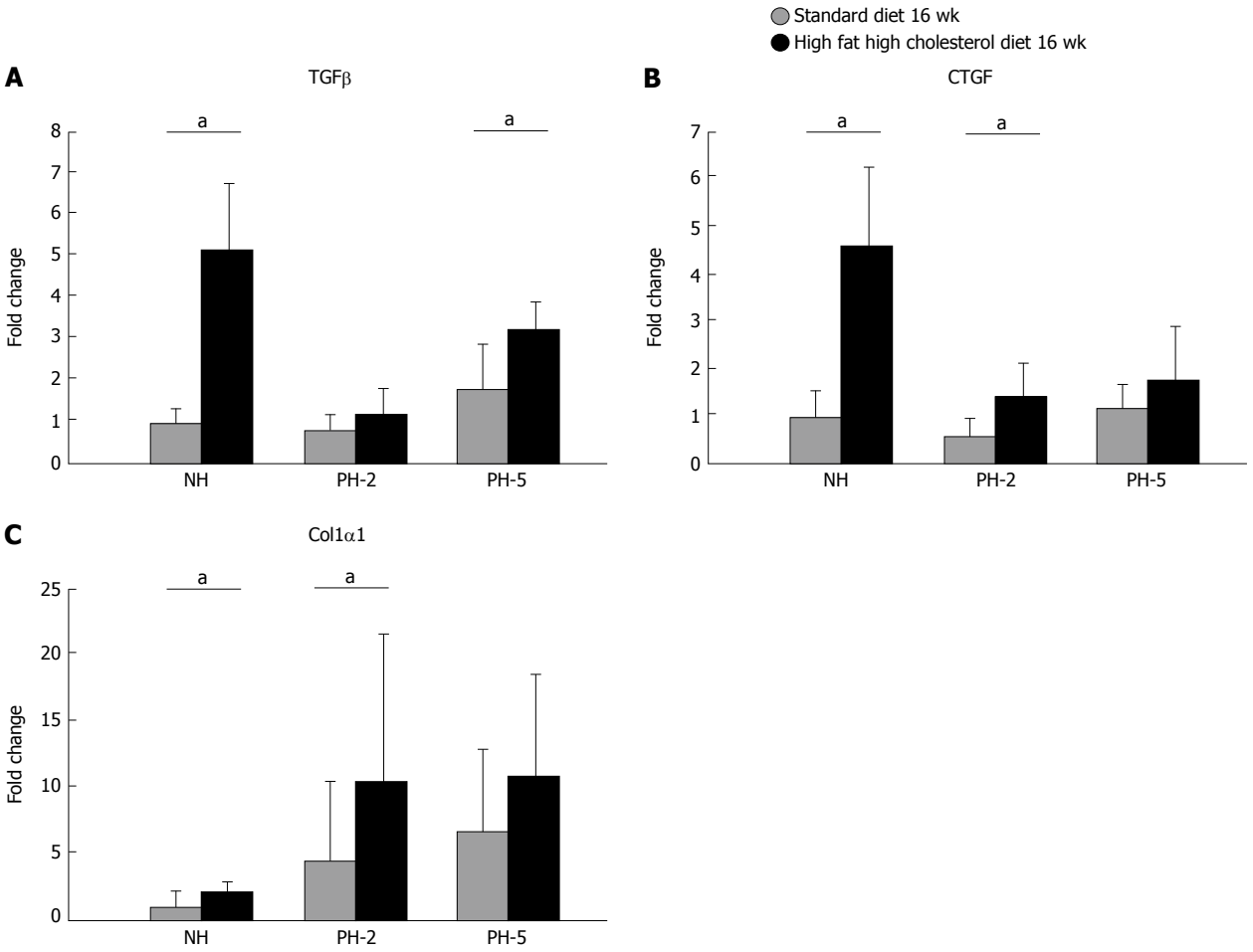

Figure 7 Relative mRNA expression. A: Transforming Growth Factor β (TGFβ); B: Connective Tissue Growth Factor (CTGF); and C: Collagen 1α1 standardized (Col1α1) to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Means and standard derivation displayed. Statistical analysis made using the ANOVA test, $^{a}P < 0.05$ 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

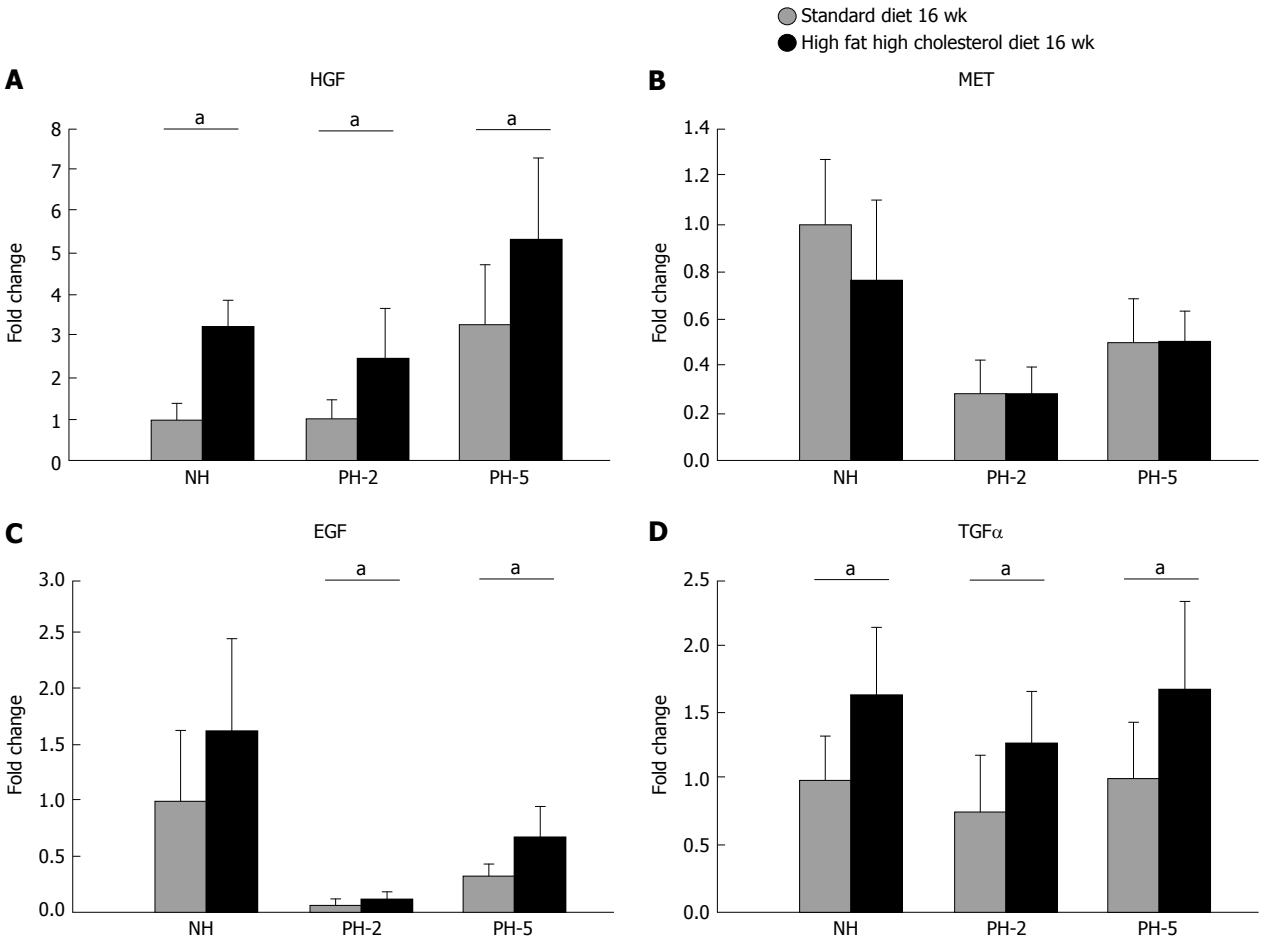

Figure 8 Relative mRNA expression. A: Hepatocyte growth factor (HGF); B: Proto-oncogene, tyrosine kinase (MET); C: Epidermal growth factor (EGF); and D: Transforming growth factor α (TGFα), standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Means and standard derivation displayed. Statistical 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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REFERENCES

1 **European Association for the Study of the Liver (EASL).** European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016; **64**: 1388-1402 [PMID: 27062661]

2 **Oda K**, Uto H, Mawatari S, Ido A. Clinical features of hepatocellular carcinoma associated with nonalcoholic fatty liver disease: a review of human studies. *Clin J Gastroenterol* 2015; **8**: 1-9 [PMID: 25575848 DOI: 10.1007/s12328-014-0548-5]

3 **Sanna C**, Rosso C, Marietti M, Bugianesi E. Non-Alcoholic Fatty Liver Disease and Extra-Hepatic Cancers. *Int J Mol Sci* 2016; **17**: pii: E717 [PMID: 27187365 DOI: 10.3390/ijms17050717]

4 **Landreau P**, Drouillard A, Launoy G, Ortega-Deballon P, Jooste V, Lepage C, Faivre J, Facy O, Bouvier AM. Incidence and survival in late liver metastases of colorectal cancer. *J Gastroenterol Hepatol* 2015; **30**: 82-85 [PMID: 25088563 DOI: 10.1111/jgh.12685]
5 **Smith JJ**, D'Angelica MI. Surgical management of hepatic metastases of colorectal cancer. *Hematol Oncol Clin North Am* 2015; **29**: 61-84 [PMID: 25475573 DOI: 10.1016/j.hoc.2014.09.003]
6 **de Meijer VE**, Kalish BT, Puder M, Ijzermans JN. Systematic review and meta-analysis of steatosis as a risk factor in major hepatic resection. *Br J Surg* 2010; **97**: 1331-1339 [PMID: 20641066 DOI: 10.1002/bjs.7194]
7 **Zhang BH**, Weltman M, Farrell GC. Does steatohepatitis impair liver regeneration? A study in a dietary model of non-alcoholic steatohepatitis in rats. *J Gastroenterol Hepatol* 1999; **14**: 133-137 [PMID: 10029293 DOI: 10.1046/j.1440-1746.1999.01822.x]
8 **Picard C**, Lambotte L, Starkel P, Sempoux C, Saliez A, Van den Berge V, Horsmans Y. Steatosis is not sufficient to cause an impaired regenerative response after partial hepatectomy in rats. *J Hepatol* 2002; **36**: 645-652 [PMID: 11983448 DOI: 10.1016/S0168-8278(02)00038-7]
9 **Marsman HA**, de Graaf W, Heger M, van Golen RF, Ten Kate FJ, Bennink R, van Gulik TM. Hepatic regeneration and functional recovery following partial liver resection in an experimental model of hepatic steatosis treated with omega-3 fatty acids. *Br J Surg* 2013; **100**: 674-683 [PMID: 23456631 DOI: 10.1002/bjs.9059]
10 **Hsiao IT**, Lin KJ, Chang SI, Yen TC, Chen TC, Yeh TS. Impaired liver regeneration of steatotic rats after portal vein ligation: a particular emphasis on (99m)Tc-DISIDA scintigraphy and adiponectin signaling. *J Hepatol* 2010; **52**: 540-549 [PMID: 20206399 DOI: 10.1016/j.jhep.2010.01.005]
11 **Boeykens N**, Ponsaerts P, Van der Linden A, Berneman Z, Ysebaert D, De Greef K. Injury-dependent retention of intraportally administered mesenchymal stromal cells following partial hepatectomy of steatotic liver does not lead to improved liver recovery. *PLoS One* 2013; **8**: e69092 [PMID: 23874878 DOI: 10.1371/journal.pone.0069092]
12 **Veteläinen R**, van Vliet AK, van Gulik TM. Severe steatosis increases hepatocellular injury and impairs liver regeneration in a rat model of partial hepatectomy. *Ann Surg* 2007; **245**: 44-50 [PMID: 17197964 DOI: 10.1097/01.sla.0000225253.84501.0e]
13 **Uzun MA**, Koksal N, Kadioglu H, Gunerhan Y, Aktas S, Dursun N, Sehirli AO. Effects of N-acetylcysteine on regeneration following partial hepatectomy in rats with nonalcoholic fatty liver disease. *Surg Today* 2009; **39**: 592-597 [PMID: 19562447 DOI: 10.1007/s00595-008-3930-4]
14 **Tsuchiya T**, Miyazawa M, Abe T, Saito T, Kanno H, Ishii S, Suzuki M, Kenjo A, Yamada F, Gunji T, Kimura T, Gotoh M. Hepatic regeneration and ischemia/reperfusion injury in fatty-liver rats. *Transplant Proc* 2000; **32**: 2324 [PMID: 11120184 DOI: 10.1016/S0041-1345(00)01683-3]
15 **Rao MS**, Papreddy K, Abecassis M, Hashimoto T. Regeneration of liver with marked fatty change following partial hepatectomy in rats. *Dig Dis Sci* 2001; **46**: 1821-1826 [PMID: 11575431 DOI: 10.1023/A:1010654908938]
16 **Ninomiya M**, Shimada M, Terashi T, Ijichi H, Yonemura Y, Harada N, Soejima Y, Suehiro T, Maehara Y. Sustained spatial disturbance of bile canalicular networks during regeneration of the steatotic rat liver. *Transplantation* 2004; **77**: 373-379 [PMID: 14966410 DOI: 10.1097/01.TP.0000109777.51902.09]
17 **Tsai CY**, Lin YS, Yeh TS, Cheong CF, Chang CH, Chen TC, Chen MF. Disrupted hepatic adiponectin signaling impairs liver regeneration of steatotic rats. *Chang Gung Med J* 2011; **34**: 248-259 [PMID: 21733354]
18 **Lai HS**, Lin WH, Chen PR, Wu HC, Lee PH, Chen WJ. Effects of a high-fiber diet on hepatocyte apoptosis and liver regeneration after partial hepatectomy in rats with fatty liver. *JPEN J Parenter Enteral Nutr* 2005; **29**: 401-407 [PMID: 16224031 DOI: 10.1177/0148607105029006401]
19 **Yang SQ**, Lin HZ, Mandal AK, Huang J, Diehl AM. Disrupted signaling and inhibited regeneration in obese mice with fatty livers: implications for nonalcoholic fatty liver disease pathophysiology. *Hepatology* 2001; **34**: 694-706 [PMID: 11584365 DOI: 10.1053/jhep.2001.28054]
20 **Abshagen K**, Mertens F, Eipel C, Vollmar B. Limited therapeutic efficacy of thrombopoietin on the regeneration of steatotic livers. *Int J Clin Exp Pathol* 2013; **6**: 1759-1769 [PMID: 24040440]
21 **Yamauchi H**, Uetsuka K, Okada T, Nakayama H, Doi K. Impaired liver regeneration after partial hepatectomy in db/db mice. *Exp Toxicol Pathol* 2003; **54**: 281-286 [PMID: 12710710 DOI: 10.1078/0940-2993-00265]
22 **Uetsuka K**, Shirai M, Yamauchi H, Nakayama H, Doi K. Impaired proliferation of non-parenchymal cells participates in an impairment of liver regeneration in db/db mice. *Exp Mol Pathol* 2005; **79**: 51-58 [PMID: 16005712 DOI: 10.1016/j.yexmp.2005.02.002]
23 **Redaelli CA**, Semela D, Carrick FE, Ledermann M, Candinas D, Sauter B, Dufour JF. Effect of vascular endothelial growth factor on functional recovery after hepatectomy in lean and obese mice. *J Hepatol* 2004; **40**: 305-312 [PMID: 14739103 DOI: 10.1016/j.jhep.2003.10.027]
24 **Selzner M**, Clavien PA. Failure of regeneration of the steatotic rat liver: disruption at two different levels in the regeneration pathway. *Hepatology* 2000; **31**: 35-42 [PMID: 10613725 DOI: 10.1002/hep.510310108]
25 **Leclercq IA**, Vansteenberghe M, Lebrun VB, VanHul NK, Abarca-Quinones J, Sempoux CL, Picard C, Stärkel P, Horsmans YL. Defective hepatic regeneration after partial hepatectomy in leptin-deficient mice is not rescued by exogenous leptin. *Lab Invest* 2006; **86**: 1161-1171 [PMID: 16983330 DOI: 10.1038/labinvest.3700474]
26 **Tanoue S**, Uto H, Kumamoto R, Arima S, Hashimoto S, Nasu Y, Takami Y, Moriuchi A, Sakiyama T, Oketani M, Ido A, Tsubouchi H. Liver regeneration after partial hepatectomy in rat is more impaired in a steatotic liver induced by dietary fructose compared to dietary fat. *Biochem Biophys Res Commun* 2011; **407**: 163-168 [PMID: 21371432 DOI: 10.1016/j.bbrc.2011.02.131]
27 **Heebøll S**, El-Houri RB, Hellberg YE, Haldrup D, Pedersen SB, Jessen N, Christensen LP, Grønbaek H. Effect of resveratrol on experimental non-alcoholic fatty liver disease depends on severity of pathology and timing of treatment. *J Gastroenterol Hepatol* 2016; **31**: 668-675 [PMID: 26312773 DOI: 10.1111/jgh.13151]
28 **Thomsen KL**, Grønbæk H, Glavind E, Hebbard L, Jessen N, Clouston A, George J, Vilstrup H. Experimental nonalcoholic steatohepatitis compromises ureagenesis, an essential hepatic metabolic function. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G295-G301 [PMID: 24924745 DOI: 10.1152/ajpgi.00036.2014]
29 **Xu ZJ**, Fan JG, Ding XD, Qiao L, Wang GL. Characterization of high-fat, diet-induced, non-alcoholic steatohepatitis with fibrosis in rats. *Dig Dis Sci* 2010; **55**: 931-940 [PMID: 19459046 DOI: 10.1007/s10620-009-0815-3]
30 **Andersen KJ**, Knudsen AR, Kannerup AS, Sasanuma H, Nyengaard JR, Hamilton-Dutoit S, Erlandsen EJ, Jørgensen B, Mortensen FV. The natural history of liver regeneration in rats: description of an animal model for liver regeneration studies. *Int J Surg* 2013; **11**: 903-908 [PMID: 23899538 DOI: 10.1016/j.ijsu.2013.07.009]
31 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]
32 **Bedossa P**, Poitou C, Veyrie N, Bouillot JL, Basdevant A, Paradis V, Tordjman J, Clement K. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* 2012; **56**: 1751-1759 [PMID: 22707395 DOI: 10.1002/hep.25889]
33 **Cruz-Orive LM.** Distribution-free estimation of sphere size distributions from slabs showing overprojection and truncation,

with a review of previous methods. *J Microsc* 1983; **131**: 265-290 [DOI: 10.1111/j.1365-2818.1983.tb04255.x]
34 **Sohlenius-Sternbeck AK**. Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. *Toxicol In Vitro* 2006; **20**: 1582-1586 [PMID: 16930941 DOI: 10.1016/j.tiv.2006.06.003]
35 **Brix-Christensen V**, Gjedsted J, Andersen SK, Vestergaard C, Nielsen J, Rix T, Nyboe R, Andersen NT, Larsson A, Schmitz O, Tønnesen E. Inflammatory response during hyperglycemia and hyperinsulinemia in a porcine endotoxemic model: the contribution of essential organs. *Acta Anaesthesiol Scand* 2005; **49**: 991-998 [PMID: 16045661 DOI: 10.1111/j.1399-6576.2005.00749.x]
36 **Chomczynski P**, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat Protoc* 2006; **1**: 581-585 [PMID: 17406285 DOI: 10.1038/nprot.2006.83]
37 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408 [PMID: 11846609 DOI: 10.1006/meth.2001.1262]
38 **Sydor S**, Gu Y, Schlattjan M, Bechmann LP, Rauen U, Best J, Paul A, Baba HA, Sowa JP, Gerken G, Canbay A. Steatosis does not impair liver regeneration after partial hepatectomy. *Lab Invest* 2013; **93**: 20-30 [PMID: 23069937 DOI: 10.1038/labinvest.2012.142]
39 **DeAngelis RA**, Markiewski MM, Taub R, Lambris JD. A high-fat diet impairs liver regeneration in C57BL/6 mice through overexpression of the NF-kappaB inhibitor, IkappaBalpha. *Hepatology* 2005; **42**: 1148-1157 [PMID: 16231352 DOI: 10.1002/hep.20879]
40 **Hamano M**, Ezaki H, Kiso S, Furuta K, Egawa M, Kizu T, Chatani N, Kamada Y, Yoshida Y, Takehara T. Lipid overloading during liver regeneration causes delayed hepatocyte DNA replication by increasing ER stress in mice with simple hepatic steatosis. *J Gastroenterol* 2014; **49**: 305-316 [PMID: 23512345 DOI: 10.1007/s00535-013-0780-7]
41 **Veteläinen R**, van Vliet A, van Gulik TM. Essential pathogenic and metabolic differences in steatosis induced by choline or methione-choline deficient diets in a rat model. *J Gastroenterol Hepatol* 2007; **22**: 1526-1533 [PMID: 17716355 DOI: 10.1111/j.1440-1746.2006.04701.x]
42 **Kucera O**, Cervinkova Z. Experimental models of non-alcoholic fatty liver disease in rats. *World J Gastroenterol* 2014; **20**: 8364-8376 [PMID: 25024595 DOI: 10.3748/wjg.v20.i26.8364]
43 **Stein TA**, Burns GP, Tropp BE, Wise L. Hepatic fat accumulation during liver regeneration. *J Surg Res* 1985; **39**: 338-343 [PMID: 4046590 DOI: 10.1016/0022-4804(85)90112-X]
44 **Farvid MS**, Ng TW, Chan DC, Barrett PH, Watts GF. Association of adiponectin and resistin with adipose tissue compartments, insulin resistance and dyslipidaemia. *Diabetes Obes Metab* 2005; **7**: 406-413 [PMID: 15955127 DOI: 10.1111/j.1463-1326.2004.00410.x]
45 **Shirai M**, Yamauchi H, Nakayama H, Doi K, Uetsuka K. Expression of epidermal growth factor receptor protein in the liver of db/db mice after partial hepatectomy. *Exp Toxicol Pathol* 2007; **59**: 157-162 [PMID: 17826083 DOI: 10.1016/j.etp.2007.06.003]
46 **Murata H**, Yagi T, Iwagaki H, Ogino T, Sadamori H, Matsukawa H, Umeda Y, Haga S, Takaka N, Ozaki M. Mechanism of impaired regeneration of fatty liver in mouse partial hepatectomy model. *J Gastroenterol Hepatol* 2007; **22**: 2173-2180 [PMID: 18031377 DOI: 10.1111/j.1440-1746.2006.04798.x]
47 **Yildirim SI**, Poulsen HE. Quantitative liver functions after 70% hepatectomy. *Eur J Clin Invest* 1981; **11**: 469-472 [PMID: 6800822 DOI: 10.1111/j.1365-2362.1981.tb02015.x]
48 **Antović J**, Djordjević V, Kocić G, Koraćević D, Bjelaković G, Bakić M. Blood coagulation factors changes during liver regeneration in rats. *Arch Int Physiol Biochim Biophys* 1993; **101**: 357-359 [PMID: 7511428 DOI: 10.3109/13813459309046992]
49 **Michalopoulos GK**. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am J Pathol* 2010; **176**: 2-13 [PMID: 20019184 DOI: 10.2353/ajpath.2010.090675]
50 **Kim MD**, Kim SS, Cha HY, Jang SH, Chang DY, Kim W, Suh-Kim H, Lee JH. Therapeutic effect of hepatocyte growth factor-secreting mesenchymal stem cells in a rat model of liver fibrosis. *Exp Mol Med* 2014; **46**: e110 [PMID: 25145391 DOI: 10.1038/emm.2014.49]
51 **Webber EM**, FitzGerald MJ, Brown PI, Bartlett MH, Fausto N. Transforming growth factor-alpha expression during liver regeneration after partial hepatectomy and toxic injury, and potential interactions between transforming growth factor-alpha and hepatocyte growth factor. *Hepatology* 1993; **18**: 1422-1431 [PMID: 8244268 DOI: 10.1002/hep.1840180622]

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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.

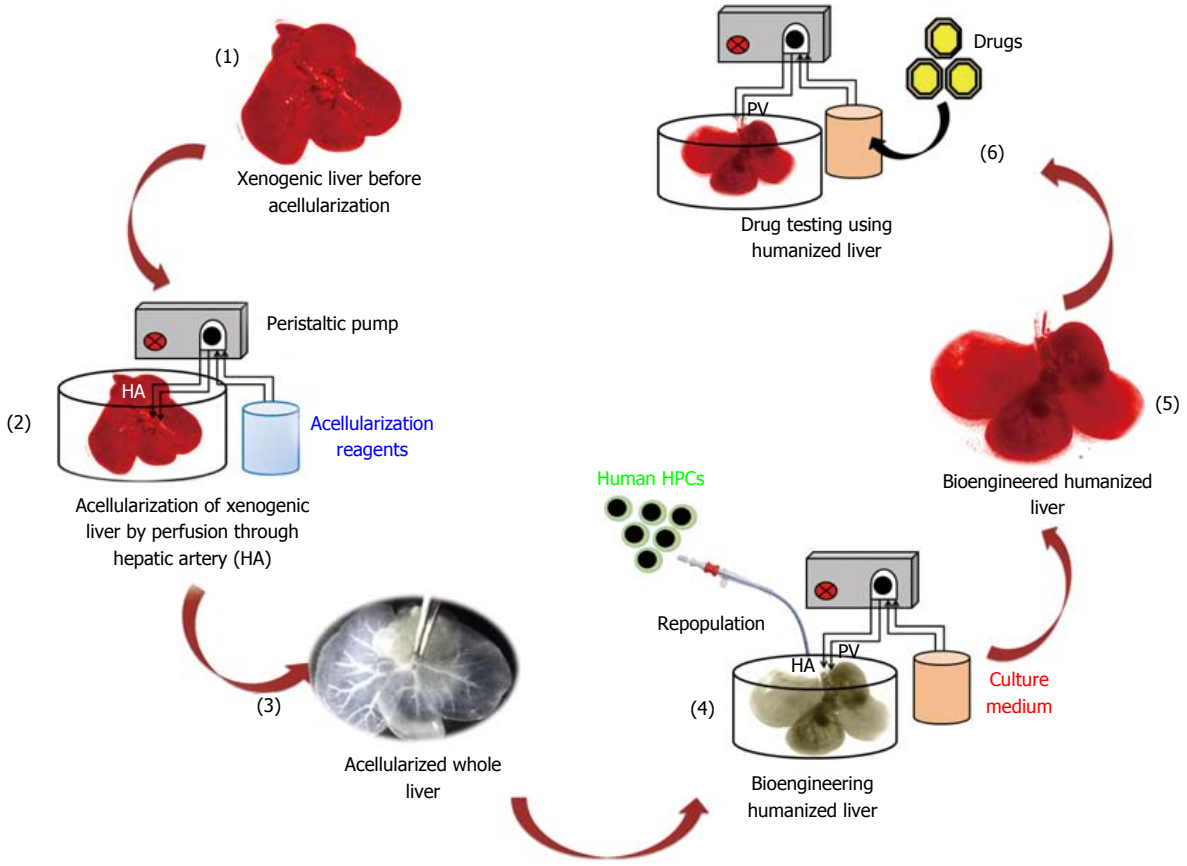

Figure 1 Schematic study plan. Representation of bioengineering technology to generate humanized livers using acellularization and human HPCs repopulation strategy for the development of three-dimensional platform for drug testing.

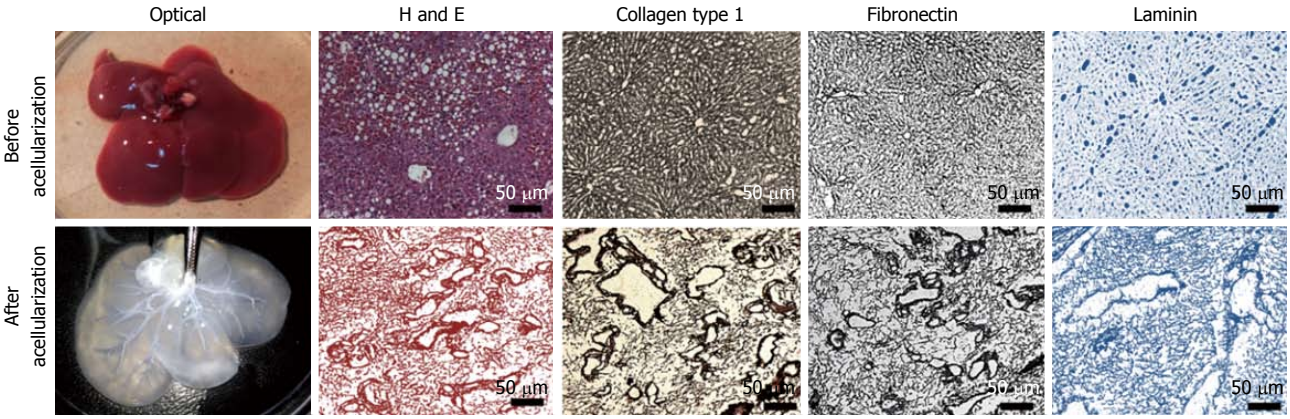

Figure 2 Xenogenic liver before and after perfusion acellularization. Macroscopic appearance (optical) of acellularized liver showing removal of hematopoietic, liver parenchyma and non-parenchyma cells while retaining the vascular tree. Histological comparison of native and acellularized liver. The expression 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

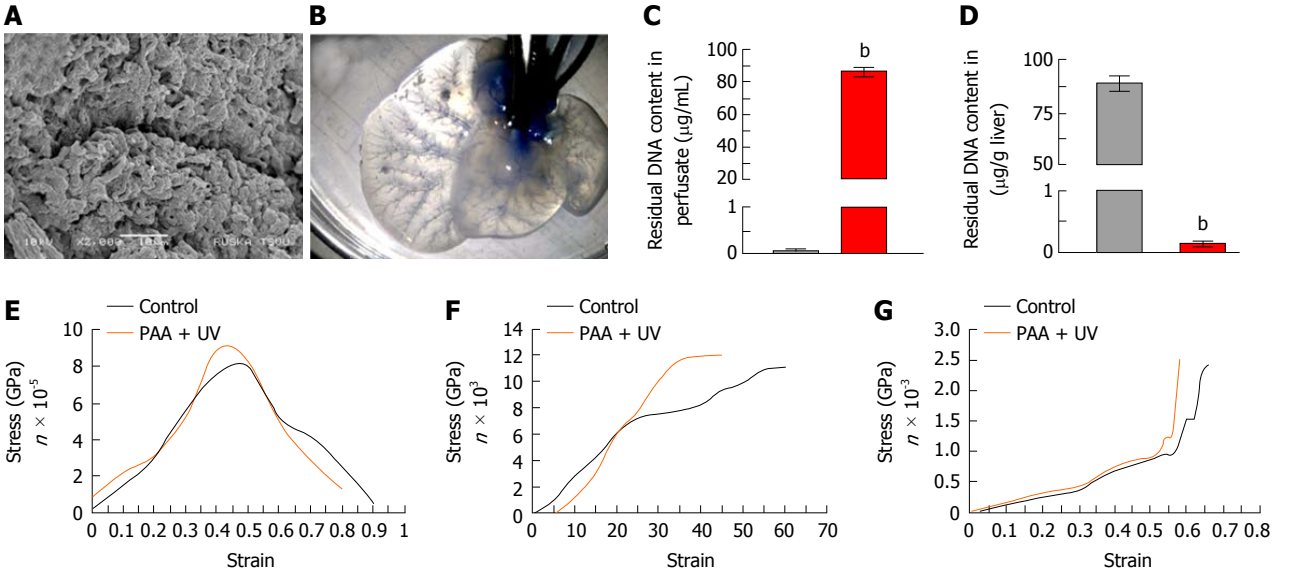

Figure 3 Characterization of acellularized liver scaffold. A: Ultra-structural characterization of acellularized xenogenic liver showing acellularity in the scaffold with preserved three-dimensional microanatomy of the portal tract surrounded by lobular structures. The parenchymal spaces were found enriched 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.

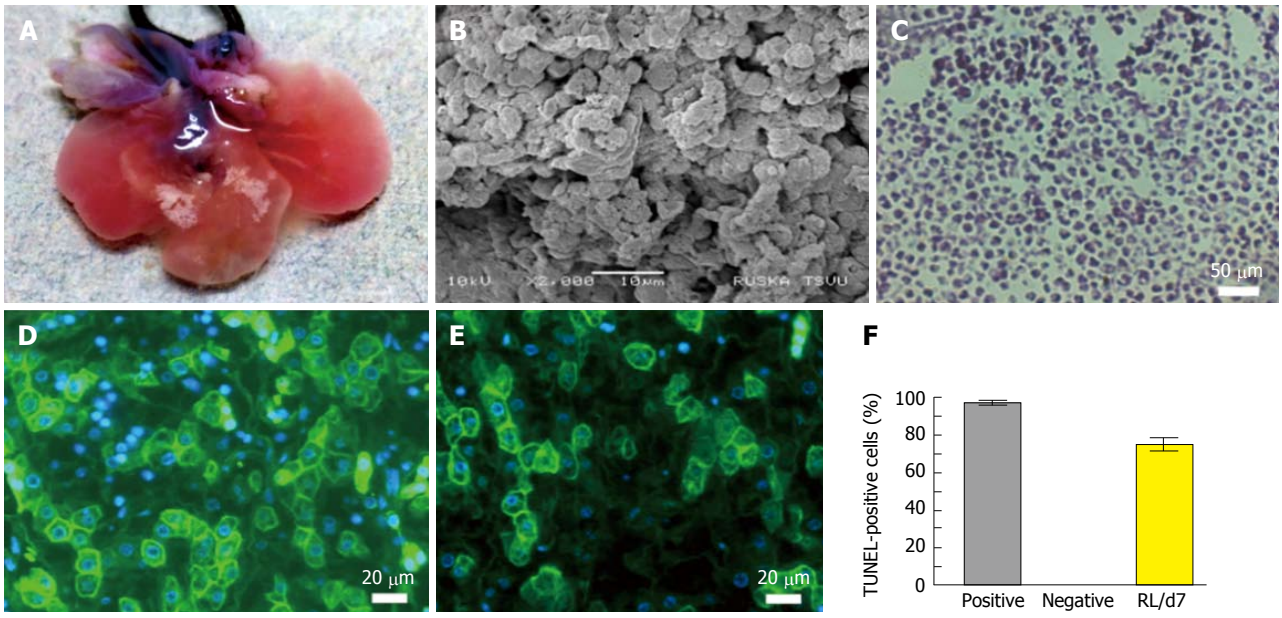

Figure 4 Characterization of humanized liver. A: Optical image of humanized liver after 7 d of repopulation with human HPCs into a customized bioreactor; B: SEM image of humanized liver at day 7 showing cellular proliferation and natural arrangements; C: H and E staining of thin micro-section of humanized 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

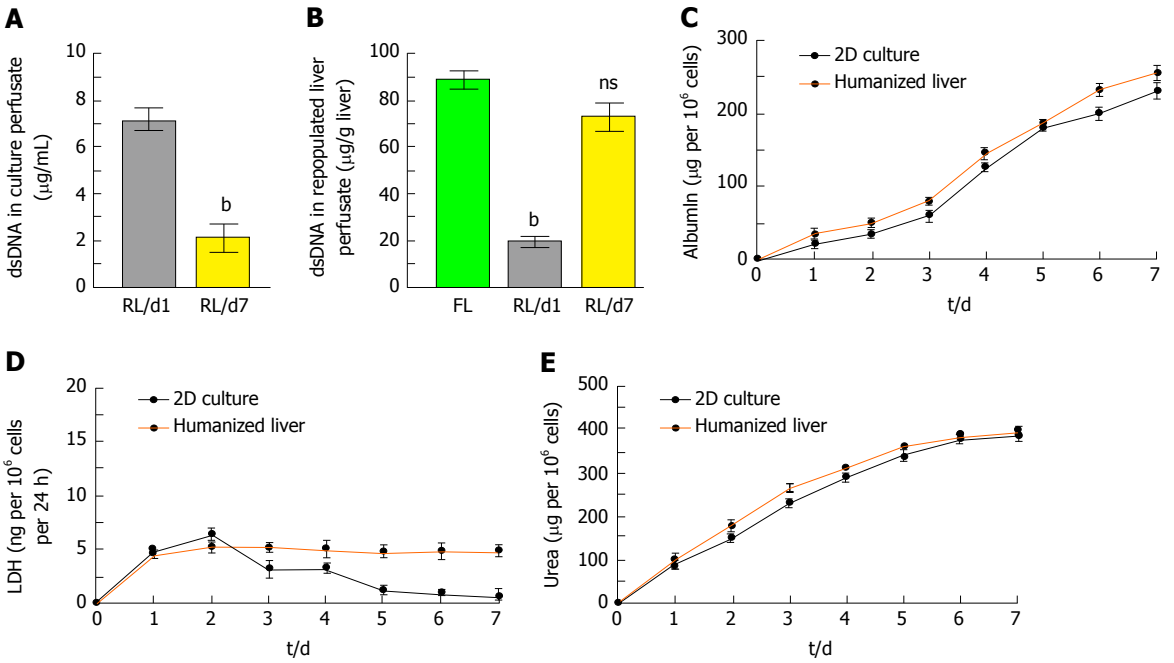

Figure 5 Functional assessment of humanized liver. A and B: Quantification of double stranded DNA (dsDNA) in (A) liver perfusate showing reduction in quantity at day 7 whereas (B) in humanized liver post-repopulation showed reciprocal relationship which was significantly high ($^bP < 0.001$) at day 7 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-

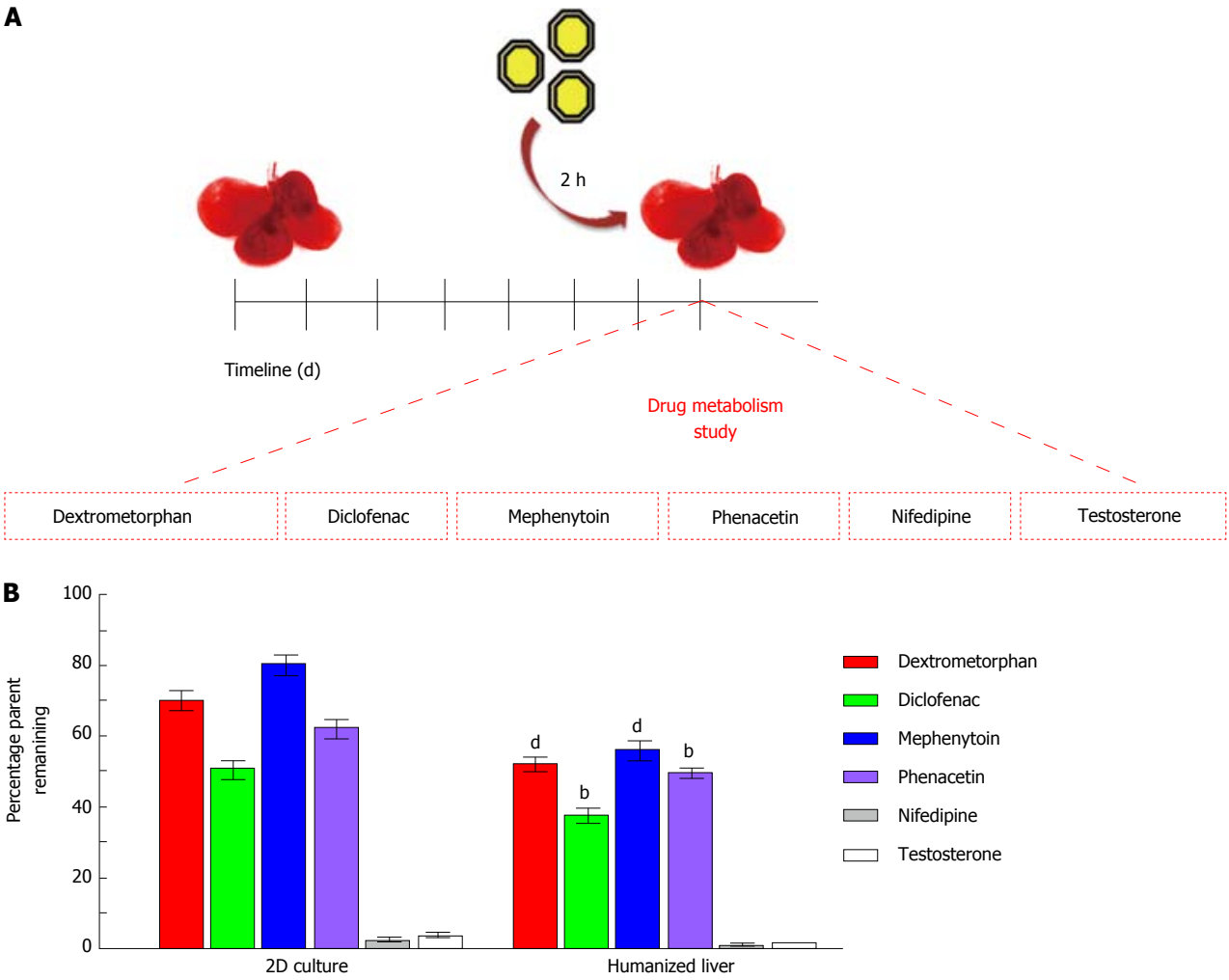

Figure 6 Drug metabolism studies using 3D humanized liver system. A: Schematic representation showing the timeline of humanized liver development and drug metabolism study; B: Bar graphs showing the rate of cellular metabolism of six cytochrome P-450 probe substrates in humanized liver as compared to 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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REFERENCES

1 **Khanna S**, Bajaj R, Khurana B, Srivastava K. Pharmacotherapeutic Principles of Ungual Drug Delivery System. *Int J Drug Dev Res* 2012; **3**: 9-18

2 **Kaitin KI**. Obstacles and opportunities in new drug development. *Clin Pharmacol Ther* 2008; **83**: 210-212 [PMID: 18202685 DOI: 10.1038/sj.clpt.6100462]

3 **Tiscornia G**, Monserrat N, Izpisua Belmonte JC. Modelling long QT syndrome with iPS cells: be still, my beating heart. *Circ Res* 2011; **108**: 648-649 [PMID: 21415406 DOI: 10.1161/RES.0b013e318216f0db]

4 **LeCluyse EL**, Witek RP, Andersen ME, Powers MJ. Organotypic liver culture models: meeting current challenges in toxicity testing. *Crit Rev Toxicol* 2012; **42**: 501-548 [PMID: 22582993 DOI: 10.31

09/10408444.2012.682115]
5 **Godoy P**, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Böttger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi G, Cho CS, Choi YJ, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT, Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R, Gibson A, Glanemann M, Goldring CE, Gómez-Lechón MJ, Groothuis GM, Gustavsson L, Guyot C, Hallifax D, Hammad S, Hayward A, Häussinger D, Hellerbrand C, Hewitt P, Hoehme S, Holzhütter HG, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM, Kevin Park B, Kordes C, Kullak-Ublick GA, LeCluyse EL, Lu P, Luebke-Wheeler J, Lutz A, Maltman DJ, Matz-Soja M, McMullen P, Merfort I, Messner S, Meyer C, Mwinyi J, Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J, Pluta L, Przyborski SA, Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B, Stelzer EH, Stieger B, Stöber R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken M, Weiss TS, Widera A, Woods CG, Xu JJ, Yarborough KM, Hengstler JG. Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol* 2013; **87**: 1315-1530 [PMID: 23974980 DOI: 10.1007/s00204-013-1078-5]
6 **Xu JJ**, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D. Cellular imaging predictions of clinical drug-induced liver injury. *Toxicol Sci* 2008; **105**: 97-105 [PMID: 18524759 DOI: 10.1093/toxsci/kfn109]
7 **Garreta E**, Oria R, Tarantino C, Pla-Roca M, Prado P, Fernandez-Aviles F, Campistol JM, Samitier J, Montserrat N. Tissue engineering by decellularization and 3D bioprinting. *Materials Today* 2017; **20**: 166-178 [DOI: 10.1016/j.mattod.2016.12.005]
8 **Baptista PM**, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S. The use of whole organ decellularization for the generation of a vascularized liver organoid. *Hepatology* 2011; **53**: 604-617 [PMID: 21274881 DOI: 10.1002/hep.24067]
9 **Mazza G**, Rombouts K, Rennie Hall A, Urbani L, Vinh Luong T, Al-Akkad W, Longato L, Brown D, Maghsoudlou P, Dhillon AP, Fuller B, Davidson B, Moore K, Dhar D, De Coppi P, Malago M, Pinzani M. Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. *Sci Rep* 2015; **5**: 13079 [PMID: 26248878 DOI: 10.1038/srep13079]
10 **Shaik AN**, Vishwakarma SK, Khan AA. Metabolism of six CYP probe substrates in fetal hepatocytes. *ADMET* 2016; **4**: 84-90 [DOI: 10.5599/admet.4.2.210]
11 **Bozzola JJ**, Russell LD. In: Electron microscopy principles and techniques for biologists. 2nd Edition. Sudbury, Massachusettes. Jones and Bartlett Publishers, 1998: 19-24, 54-55, 63-67
12 **Kajbafzadeh AM**, Javan-Farazmand N, Monajemzadeh M, Baghayee A. Determining the optimal decellularization and sterilization protocol for preparing a tissue scaffold of a human-sized liver tissue. *Tissue Eng Part C Methods* 2013; **19**: 642-651 [PMID: 23270591 DOI: 10.1089/ten.TEC.2012.0334]
13 **Habibullah CM**, Syed IH, Qamar A, Taher-Uz Z. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. *Transplantation* 1994; **58**: 951-952 [PMID: 7940741 DOI: 10.1097/00007890-199410270-00016]
14 **Vishwakarma SK**, Rahamathulla S, Bardia A, Tiwari SK, Srinivas G, Raj A, Tripura C, Sandhya A, Habeeb MA, Khan AA, Pande G, Reddy KP, Reddy PY. In vitro quantitative and relative gene expression analysis of pancreatic transcription factors Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1 in trans-differentiated human hepatic progenitors. *J Diabetes Investig* 2014; **5**: 492-500 [PMID: 25411615 DOI: 10.1111/jdi.12193]
15 **Khan AA**, Sivaram G, Vishwakarma SK, Lakki Reddy C, Srinivas G, Raj A, Nallari P, Habeeb MA, Venkateswarlu J. Transplantation of Epcam+Ve Human Hepatic Stem Cells in Liver Cirrhosis Patient and Cellular Immune Response. *J J Transplant Technol Res* 2015; **5**: 1-4
16 **Hoshino K**, Inouye H, Unokuchi T, Ito M, Tamaoki N, Tsuji K. HLA and diseases in Japanese patients [proceedings]. *Diabete Metab* 1976; **2**: 157-158 [PMID: 1010129]
17 **Yasar U**, Eliasson E, Forslund-Bergengren C, Tybring G, Gadd M, Sjöqvist F, Dahl ML. The role of CYP2C9 genotype in the metabolism of diclofenac in vivo and in vitro. *Eur J Clin Pharmacol* 2001; **57**: 729-735 [PMID: 11829203 DOI: 10.1007/s00228-001-0376-7]
18 **de Morais SM**, Wilkinson GR, Blaisdell J, Nakamura K, Meyer UA, Goldstein JA. The major genetic defect responsible for the polymorphism of S-mephenytoin metabolism in humans. *J Biol Chem* 1994; **269**: 15419-15422 [PMID: 8195181]
19 **Kerry NL**, Somogyi AA, Bochner F, Mikus G. The role of CYP2D6 in primary and secondary oxidative metabolism of dextromethorphan: in vitro studies using human liver microsomes. *Br J Clin Pharmacol* 1994; **38**: 243-248 [PMID: 7826826 DOI: 10.1111/j.1365-2125.1994.tb04348.x]
20 **Patki KC**, Von Moltke LL, Greenblatt DJ. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. *Drug Metab Dispos* 2003; **31**: 938-944 [PMID: 12814972 DOI: 10.1124/dmd.31.7.938]
21 **Rao MN**, Biju B, Ansar AK, Mujeeb S, Ramesh M, Srinivas NR. 'Open access' generic method for continuous determination of major human CYP450 probe substrates/metabolites and its application in drug metabolism studies. *Xenobiotica* 2003; **33**: 1233-1245 [PMID: 14742145 DOI: 10.1080/00498250310001636877]
22 **Jones HM**, Houston JB. Substrate depletion approach for determining in vitro metabolic clearance: time dependencies in hepatocyte and microsomal incubations. *Drug Metab Dispos* 2004; **32**: 973-982 [PMID: 15319339 DOI: 10.1124/dmd.104.000125]
23 **Olson H**, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Van Deun K, Smith P, Berger B, Heller A. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol* 2000; **32**: 56-67 [PMID: 11029269 DOI: 10.1006/rtph.2000.1399]
24 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]
25 **Medvedev SP**, Shevchenko AI, Zakian SM. Induced Pluripotent Stem Cells: Problems and Advantages when Applying them in Regenerative Medicine. *Acta Naturae* 2010; **2**: 18-28 [PMID: 22649638]
26 **Gore A**, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N, Panopoulos AD, Ruiz S, Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K. Somatic coding mutations in human induced pluripotent stem cells. *Nature* 2011; **471**: 63-67 [PMID: 21368825 DOI: 10.1038/nature09805]
27 **Hussein SM**, Batada NN, Vuoristo S, Ching RW, Autio R, Närvä E, Ng S, Sourour M, Hämäläinen R, Olsson C, Lundin K, Mikkola M, Trokovic R, Peitz M, Brüstle O, Bazett-Jones DP, Alitalo K, Lahesmaa R, Nagy A, Otonkoski T. Copy number variation and selection during reprogramming to pluripotency. *Nature* 2011; **471**: 58-62 [PMID: 21368824 DOI: 10.1038/nature09871]
28 **Scudellari M**. How iPS cells changed the world. *Nature* 2016; **534**: 310-312 [PMID: 27306170 DOI: 10.1038/534310a]
29 **Fields M**, Cai H, Gong J, Del Priore L. Potential of Induced Pluripotent Stem Cells (iPSCs) for Treating Age-Related Macular Degeneration (AMD). *Cells* 2016; **5**: pii: E44 [PMID: 27941641 DOI: 10.3390/cells5040044]
30 **Rao MS**, Khan AA, Parveen N, Habeeb MA, Habibullah CM, Pande G. Characterization of hepatic progenitors from human fetal liver during second trimester. *World J Gastroenterol* 2008; **14**: 5730-5737 [PMID: 18837092 DOI: 10.3748/wjg.14.5730]
31 **Vali SM**, Vishwakarma SK, Bardia A, Tiwari SK, Srinivas G, Raj A, Tripura C, Habeeb MA, Khan AA, Pande G. Isolation and

characterization of stem cells sub population within the human fetal liver. *Cell Biol Res Ther* 2014; **S1**: 1-6

32 **Khan AA**, Habeeb A, Parveen N, Naseem B, Babu RP, Capoor AK, Habibullah CM. Peritoneal transplantation of human fetal hepatocytes for the treatment of acute fatty liver of pregnancy: a case report. *Trop Gastroenterol* 2004; **25**: 141-143 [PMID: 15682663]

33 **Khan AA**, Parveen N, Habeeb MA, Paspala S, Rajendraprasad A, Mahaboob Vali S, Khaja M, Lakshmi N, Pramila R, Habibullah C. Cell Therapy for Acute Liver Failure - Ideal source of cell. *J Stem Cells Regen Med* 2008; **4**: 2-8 [PMID: 24693024]

34 **Khan AA**, Parveen N, Mahaboob VS, Rajendraprasad A, Ravindraprakash HR, Venkateswarlu J, Rao SG, Narusu ML, Khaja MN, Pramila R, Habeeb A, Habibullah CM. Safety and efficacy of autologous bone marrow stem cell transplantation through hepatic artery for the treatment of chronic liver failure: a preliminary study. *Transplant Proc* 2008; **40**: 1140-1144 [PMID: 18555134 DOI: 10.1016/j.transproceed.2008.03.111]

35 **Khan AA**, Shaik MV, Parveen N, Rajendraprasad A, Aleem MA, Habeeb MA, Srinivas G, Raj TA, Tiwari SK, Kumaresan K, Venkateswarlu J, Pande G, Habibullah CM. Human fetal liver-derived stem cell transplantation as supportive modality in the management of end-stage decompensated liver cirrhosis. *Cell Transplant* 2010; **19**: 409-418 [PMID: 20447340 DOI: 10.3727/096368910X498241]

36 **Habeeb MA**, Vishwakarma SK, Bardia A, Khan AA. Hepatic stem cells: A viable approach for the treatment of liver cirrhosis. *World J Stem Cells* 2015; **7**: 859-865 [PMID: 26131316 DOI: 10.4252/wjsc.v7.i5.859]

37 **Khan AA**, Parveen N, Mahaboob VS, Rajendraprasad A, Ravindraprakash HR, Venkateswarlu J, Rao P, Pande G, Narusu ML, Khaja MN, Pramila R, Habeeb A, Habibullah CM. Treatment of Crigler-Najjar Syndrome type 1 by hepatic progenitor cell transplantation: a simple procedure for management of hyperbilirubinemia. *Transplant Proc* 2008; **40**: 1148-1150 [PMID: 18555136 DOI: 10.1016/j.transproceed.2008.03.022]

38 **Strom SC**, Fisher RA, Thompson MT, Sanyal AJ, Cole PE, Ham JM, Posner MP. Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. *Transplantation* 1997; **63**: 559-569 [PMID: 9047152 DOI: 10.1097/00007890-199702270-00014]

39 **Fox IJ**, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV, Strom SC. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 1998; **338**: 1422-1426 [PMID: 9580649 DOI: 10.1056/NEJM199805143382004]

40 **Khetani SR**, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat Biotechnol* 2008; **26**: 120-126 [PMID: 18026090 DOI: 10.1038/nbt1361]

41 **Vernetti LA**, Senutovitch N, Boltz R, DeBiasio R, Shun TY, Gough A, Taylor DL. A human liver microphysiology platform for investigating physiology, drug safety, and disease models. *Exp Biol Med* (Maywood) 2016; **241**: 101-114 [PMID: 26202373 DOI: 10.1177/1535370215592121]

42 **Wang Z**, Samanipour R, Kim K. Organ-on-a-Chip Platforms for Drug Screeningand Tissue Engineering. Biomedical Engineering: Frontier Research and Converging Technologies, 2016; **9**: 209-233

43 **Low LA**, Tagle DA. Microphysiological Systems ("Organs-on-Chips") for Drug Efficacy and Toxicity Testing. *Clin Transl Sci* 2017; **10**: 237-239 [PMID: 28078768 DOI: 10.1111/cts.12444]

44 **Leclercq L**, Cuyckens F, Mannens GS, de Vries R, Timmerman P, Evans DC. Which human metabolites have we MIST? Retrospective analysis, practical aspects, and perspectives for metabolite identification and quantification in pharmaceutical development. *Chem Res Toxicol* 2009; **22**: 280-293 [PMID: 19183054 DOI: 10.1021/tx800432c]

45 **Rout S**, Vishwakarma SK, Khan AA. Decellularized heart: A step towards creating personalized bioengineered organs. *Cur Sci* 2014; **107**: 1

46 **Vishwakarma SK**, Bhavani PG, Bardia A, Abkari A, Murthy GS, Venkateshwarulu J, Khan AA. Preparation of natural three-dimensional goat kidney scaffold for the development of bioartificial organ. *Indian J Nephrol* 2014; **24**: 372-375 [PMID: 25484531 DOI: 10.4103/0971-4065.133008]

47 **Khan AA**. Emerging technologies for development of humanized bio-artificial organs. *J Med Allied Sci* 2016; **6**: 01-02 [DOI: 10.5455/jmas.216749]

48 **Jia L**, Liu X. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. *Curr Drug Metab* 2007; **8**: 822-829 [PMID: 18220563 DOI: 10.2174/138920007782798207]

49 **FDA**. Guidance for industry drug interaction studies-study design, data analysis, implications for dosing, and labeling recommendations. 2017. Available from: URL: https://www.fda.gov/downloads/drugs/guidances/ucm292362.pdf

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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.

REFERENCES

1 **Cheng SH**, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* 1990; **63**: 827-834 [PMID: 1699669 DOI: 10.1016/0092-8674(90)90148-8]

2 **Gilljam M**, Ellis L, Corey M, Zielenski J, Durie P, Tullis DE. Clinical manifestations of cystic fibrosis among patients with diagnosis in adulthood. *Chest* 2004; **126**: 1215-1224 [PMID: 15486385 DOI: 10.1378/chest.126.4.1215]

3 **Kobelska-Dubiel N**, Klincewicz B, Cichy W. Liver disease in cystic fibrosis. *Prz Gastroenterol* 2014; **9**: 136-141 [PMID: 25097709 DOI: 10.5114/pg.2014.43574]

4 **CF Foundation.** Patient registry annual data report. Bethesda: MD, 2015

5 **Bhardwaj S**, Canlas K, Kahi C, Temkit M, Molleston J, Ober M, Howenstine M, Kwo PY. Hepatobiliary abnormalities and disease in cystic fibrosis: epidemiology and outcomes through adulthood. *J Clin Gastroenterol* 2009; **43**: 858-864 [PMID: 19525864 DOI: 10.1097/MCG.0b013e31819e8bbd]

6 **Nash KL**, Allison ME, McKeon D, Lomas DJ, Haworth CS, Bilton D, Alexander GJ. A single centre experience of liver disease in adults with cystic fibrosis 1995-2006. *J Cyst Fibros* 2008; **7**: 252-257 [PMID: 18042441 DOI: 10.1016/j.jcf.2007.10.004]

7 **Sokol RJ**, Durie PR. Recommendations for management of liver and biliary tract disease in cystic fibrosis. Cystic Fibrosis Foundation Hepatobiliary Disease Consensus Group. *J Pediatr Gastroenterol Nutr* 1999; **28** Suppl 1: S1-13 [PMID: 9934970 DOI: 10.1097/00005176-199900001-00001]

8 **Debray D**, Kelly D, Houwen R, Strandvik B, Colombo C. Best practice guidance for the diagnosis and management of cystic fibrosis-associated liver disease. *J Cyst Fibros* 2011; **10** Suppl 2: S29-S36 [PMID: 21658639 DOI: 10.1016/S1569-1993(11)60006-4]

9 **Feranchak AP**, Sokol RJ. Cholangiocyte biology and cystic fibrosis liver disease. *Semin Liver Dis* 2001; **21**: 471-488 [PMID: 11745036 DOI: 10.1055/s-2001-19030]

10 **Colombo C**. Liver disease in cystic fibrosis. *Curr Opin Pulm Med* 2007; **13**: 529-536 [PMID: 17901760 DOI: 10.1097/MCP.0b013e3282f10a16]

11 **Colombo C**, Battezzati PM, Crosignani A, Morabito A, Costantini D, Padoan R, Giunta A. Liver disease in cystic fibrosis: A prospective study on incidence, risk factors, and outcome. *Hepatology* 2002; **36**: 1374-1382 [PMID: 12447862 DOI: 10.1002/hep.1840360613]

12 **Feigelson J**, Anagnostopoulos C, Poquet M, Pecau Y, Munck A, Navarro J. Liver cirrhosis in cystic fibrosis--therapeutic implications and long term follow up. *Arch Dis Child* 1993; **68**: 653-657 [PMID: 8280210 DOI: 10.1136/ADC.68.5.653]

13 **Lindblad A**, Glaumann H, Strandvik B. A two-year prospective study of the effect of ursodeoxycholic acid on urinary bile acid excretion and liver morphology in cystic fibrosis-associated liver disease. *Hepatology* 1998; **27**: 166-174 [PMID: 9425933 DOI: 10.1002/hep.510270126]

14 **Cantón R**, Cobos N, de Gracia J, Baquero F, Honorato J, Gartner S, Alvarez A, Salcedo A, Oliver A, García-Quetglas E; Spanish Consensus Group for Antimicrobial Therapy in the Cystic Fibrosis Patient. Antimicrobial therapy for pulmonary pathogenic colonisation and infection by Pseudomonas aeruginosa in cystic fibrosis patients. *Clin Microbiol Infect* 2005; **11**: 690-703 [PMID: 16104983]

15 **O'Sullivan BP**, Baker D, Leung KG, Reed G, Baker SS, Borowitz D. Evolution of pancreatic function during the first year in infants with cystic fibrosis. *J Pediatr* 2013; **162**: 808-812.e1 [PMID: 23245194 DOI: 10.1016/j.jpeds.2012.10.008]

16 **Moran A**, Brunzell C, Cohen RC, Katz M, Marshall BC, Onady G, Robinson KA, Sabadosa KA, Stecenko A, Slovis B; CFRD Guidelines Committee. Clinical care guidelines for cystic fibrosis-related diabetes: a position statement of the American Diabetes Association and a clinical practice guideline of the Cystic Fibrosis Foundation, endorsed by the Pediatric Endocrine Society. *Diabetes Care* 2010; **33**: 2697-2708 [PMID: 21115772 DOI: 10.2337/dc10-1768]

17 **Sanyal AJ**, Brunt EM, Kleiner DE, Kowdley KV, Chalasani N, Lavine JE, Ratziu V, McCullough A. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology* 2011; **54**: 344-353 [PMID: 21520200 DOI: 10.1002/hep.24376]

18 **Bohte AE**, Koot BG, van der Baan-Slootweg OH, van Werven JR, Bipat S, Nederveen AJ, Jansen PL, Benninga MA, Stoker J. US cannot be used to predict the presence or severity of hepatic steatosis in severely obese adolescents. *Radiology* 2012; **262**: 327-334 [PMID: 22106358 DOI: 10.1148/radiol.11111094]

19 **Friedrich-Rust M**, Schlueter N, Smaczny C, Eickmeier O, Rosewich M, Feifel K, Herrmann E, Poynard T, Gleiber W, Lais C, Zielen S, Wagner TO, Zeuzem S, Bojunga J. Non-invasive measurement of liver and pancreas fibrosis in patients with cystic fibrosis. *J Cyst Fibros* 2013; **12**: 431-439 [PMID: 23361108 DOI: 10.1016/j.jcf.2012.12.013]

20 **Gillespie CD**, O'Reilly MK, Allen GN, McDermott S, Chan VO, Ridge CA. Imaging the Abdominal Manifestations of Cystic Fibrosis. *Int J Hepatol* 2017; **2017**: 5128760 [PMID: 28250993 DOI: 10.1155/2017/5128760]

21 **Taylor KJ**, Gorelick FS, Rosenfield AT, Riely CA. Ultrasonography of alcoholic liver disease with histological correlation. *Radiology* 1981; **141**: 157-161 [PMID: 6270725 DOI: 10.1148/radiology.141.1.6270725]

22 **Limanond P**, Raman SS, Lassman C, Sayre J, Ghobrial RM, Busuttil RW, Saab S, Lu DS. Macrovesicular hepatic steatosis in living related liver donors: correlation between CT and histologic findings. *Radiology* 2004; **230**: 276-280 [PMID: 14695401 DOI: 10.1148/radiol.2301021176]

23 **Levenson H**, Greensite F, Hoefs J, Friloux L, Applegate G, Silva E, Kanel G, Buxton R. Fatty infiltration of the liver: quantification with phase-contrast MR imaging at 1.5 T vs biopsy. *AJR Am J Roentgenol* 1991; **156**: 307-312 [PMID: 1898804 DOI: 10.2214/ajr.156.2.1898804]

24 **McPherson S**, Stewart SF, Henderson E, Burt AD, Day CP. Simple non-invasive fibrosis scoring systems can reliably exclude advanced fibrosis in patients with non-alcoholic fatty liver disease. *Gut* 2010; **59**: 1265-1269 [PMID: 20801772 DOI: 10.1136/gut.2010.216077]

25 **Snyder N**, Gajula L, Xiao SY, Grady J, Luxon B, Lau DT, Soloway R, Petersen J. APRI: an easy and validated predictor of hepatic fibrosis in chronic hepatitis C. *J Clin Gastroenterol* 2006; **40**: 535-542 [PMID: 16825937 DOI: 10.1097/00004836-200607000-00013]

26 **Shin WG**, Park SH, Jang MK, Hahn TH, Kim JB, Lee MS, Kim DJ, Jun SY, Park CK. Aspartate aminotransferase to platelet ratio index (APRI) can predict liver fibrosis in chronic hepatitis B. *Dig Liver Dis* 2008; **40**: 267-274 [PMID: 18055281 DOI: 10.1016/j.dld.2007.10.011]

27 **Koh C**, Sakiani S, Surana P, Zhao X, Eccleston J, Kleiner DE, Herion D, Liang TJ, Hoofnagle JH, Chernick M, Heller T. Adult-onset cystic fibrosis liver disease: Diagnosis and characterization of an underappreciated entity. *Hepatology* 2017; **66**: 591-601 [PMID: 28422310 DOI: 10.1002/hep.29217]

28 **Williams CD**, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]

29 **Lindblad A**, Glaumann H, Strandvik B. Natural history of liver disease in cystic fibrosis. *Hepatology* 1999; **30**: 1151-1158 [PMID: 10534335 DOI: 10.1002/hep.510300527]

30 **Rinella ME**. Nonalcoholic fatty liver disease: a systematic review. *JAMA* 2015; **313**: 2263-2273 [PMID: 26057287 DOI: 10.1001/jama.2015.5370]

31 **Borowitz D**. Pathophysiology of Gastrointestinal Complications of Cystic Fibrosis. *Semin Respir Crit Care Med* 1994; **15**: 391-401 [DOI: 10.1055/s-2007-1006384]

32 **Herrmann U**, Dockter G, Lammert F. Cystic fibrosis-associated liver disease. *Best Pract Res Clin Gastroenterol* 2010; **24**: 585-592 [PMID: 20955961 DOI: 10.1016/j.bpg.2010.08.003]

33 **Wilroy RS Jr**, Crawford SE, Johnson WW. Cystic fibrosis with extensive fat replacement of the liver. *J Pediatr* 1966; **68**: 67-73 [PMID: 5948084 DOI: 10.1016/S0022-3476(66)80423-7]

34 **Strandvik B**, Hultcrantz R. Liver function and morphology during long-term fatty acid supplementation in cystic fibrosis. *Liver* 1994; **14**: 32-36 [PMID: 8177027 DOI: 10.1111/j.1600-0676.1994.tb00004.x]

35 **Zemel BS**, Jawad AF, FitzSimmons S, Stallings VA. Longitudinal relationship among growth, nutritional status, and pulmonary function in children with cystic fibrosis: analysis of the Cystic Fibrosis Foundation National CF Patient Registry. *J Pediatr* 2000; **137**: 374-380 [PMID: 10969263 DOI: 10.1067/mpd.2000.107891]

36 **Feingold KR**, Serio MK, Adi S, Moser AH, Grunfeld C. Tumor necrosis factor stimulates hepatic lipid synthesis and secretion. *Endocrinology* 1989; **124**: 2336-2342 [PMID: 2707158 DOI: 10.1210/endo-124-5-2336]

37 **Colombo C**, Apostolo MG, Ferrari M, Seia M, Genoni S, Giunta A, Sereni LP. Analysis of risk factors for the development of liver disease associated with cystic fibrosis. *J Pediatr* 1994; **124**: 393-399 [PMID: 8120708]

38 **Flass T**, Narkewicz MR. Cirrhosis and other liver disease in cystic fibrosis. *J Cyst Fibros* 2013; **12**: 116-124 [PMID: 23266093 DOI: 10.1016/j.jcf.2012.11.010]

39 **Haukeland JW**, Schreiner LT, Lorgen I, Frigstad SO, Bang C, Raknerud N, Konopski Z. ASAT/ALAT ratio provides prognostic information independently of Child-Pugh class, gender and age in non-alcoholic cirrhosis. *Scand J Gastroenterol* 2008; **43**: 1241-1248 [PMID: 18609128 DOI: 10.1080/00365520802158614]

40 **Sheth SG**, Flamm SL, Gordon FD, Chopra S. AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. *Am J Gastroenterol* 1998; **93**: 44-48 [PMID: 9448172 DOI: 10.1111/j.1572-0241.1998.044_c.x]

41 **Giannini E**, Risso D, Botta F, Chiarbonello B, Fasoli A, Malfatti F, Romagnoli P, Testa E, Ceppa P, Testa R. Validity and clinical utility of the aspartate aminotransferase-alanine aminotransferase ratio in assessing disease severity and prognosis in patients with hepatitis C virus-related chronic liver disease. *Arch Intern Med* 2003; **163**: 218-224 [PMID: 12546613 DOI: 10.1001/archinte.163.2.218]

42 **Van Biervliet S**, Van Biervliet JP, Robberecht E, Christophe A. Fatty acid composition of serum phospholipids in cystic fibrosis (CF) patients with or without CF related liver disease. *Clin Chem Lab Med* 2010; **48**: 1751-1755 [PMID: 20961201 DOI: 10.1515/CCLM.2010.336]

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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.

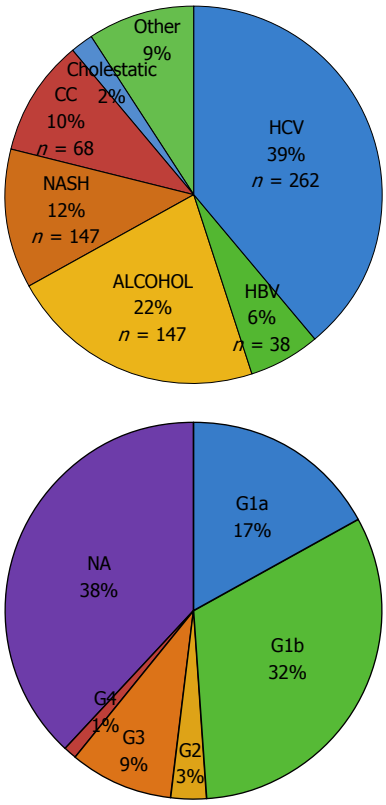

Figure 1 Etiologies oh hepatocellular carcinoma in the overall cohort and hepatitis C virus genotypes. HCV was the main cause of liver disease related with hepatocellular carcinoma including cirrhotic and non-cirrhotic patients. Distribution of known HCV genotypes (G) showed that G1b was the most frequent (*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

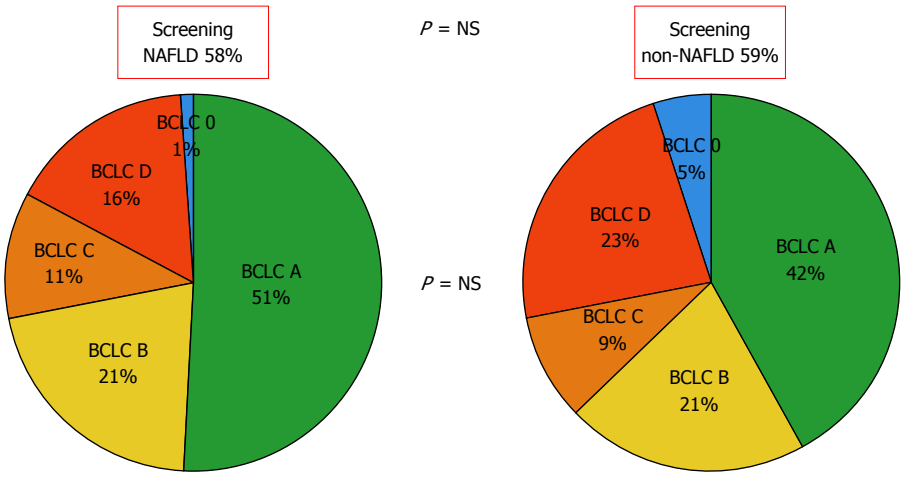

Figure 2 Comparative analysis regarding hepatocellular carcinoma previous surveillance and Barcelona Clinic Liver Cancer staging at diagnosis between non alcoholic fatty liver disease and other etiologies of liver disease. NAFLD: Non-alcoholic fatty liver disease: Non-NAFLD: Other than NAFLD (includes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

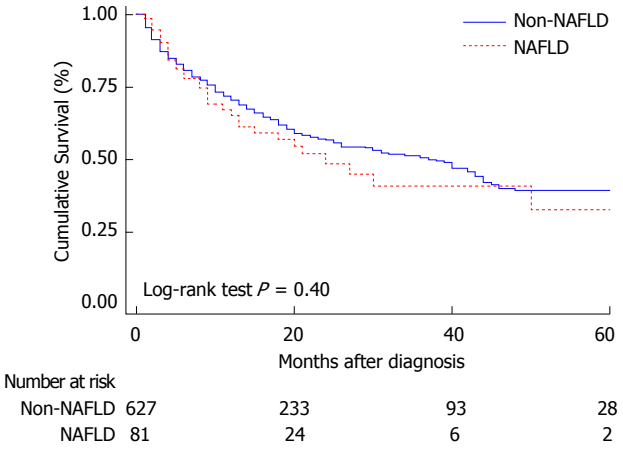

Figure 3 Comparative survival between non alcoholic fatty liver disease and other etiologies of liver disease. NAFLD: Non-alcoholic fatty liver disease; Non-NAFLD: Other than NAFLD (includes all other etiologies).

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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REFERENCES

1 **Alazawi W**, Cunningham M, Dearden J, Foster GR. Systematic review: outcome of compensated cirrhosis due to chronic hepatitis C infection. *Aliment Pharmacol Ther* 2010; **32**: 344-355 [PMID: 20497143 DOI: 10.1111/j.1365-2036.2010.04370.x]

2 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

3 **Flemming JA**, Kim WR, Brosgart CL, Terrault NA. Reduction in liver transplant wait-listing in the era of direct-acting antiviral therapy. *Hepatology* 2017; **65**: 804-812 [PMID: 28012259 DOI: 10.1002/hep.28923]

4 **Goldberg D**, Ditah IC, Saeian K, Lalehzari M, Aronsohn A, Gorospe EC, Charlton M. Changes in the Prevalence of Hepatitis C Virus Infection, Nonalcoholic Steatohepatitis, and Alcoholic Liver Disease Among Patients With Cirrhosis or Liver Failure on the Waitlist for Liver Transplantation. *Gastroenterology* 2017; **152**: 1090-1099.e1 [PMID: 28088461 DOI: 10.1053/j.gastro.2017.01.003]

5 **Piscaglia F**, Svegliati-Baroni G, Barchetti A, Pecorelli A, Marinelli S, Tiribelli C, Bellentani S; HCC-NAFLD Italian Study Group. Clinical patterns of hepatocellular carcinoma in nonalcoholic fatty liver disease: A multicenter prospective study. *Hepatology* 2016; **63**: 827-838 [PMID: 26599351 DOI: 10.1002/hep.28368]

6 **Fassio E**, Díaz S, Santa C, Reig ME, Martínez Artola Y, Alves de Mattos A, Míguez C, Galizzi J, Zapata R, Ridruejo E, de Souza FC, Hernández N, Pinchuk L; Multicenter Group for Study of Hepatocarcinoma in Latin America; Asociación Latinoamericana para el Estudio del Hígado (ALEH). Etiology of hepatocellular carcinoma in Latin America: a prospective, multicenter, international study. *Ann Hepatol* 2010; **9**: 63-69 [PMID: 20332549]

7 **Fassio E**, Míguez C, Soria S, Palazzo F, Gadano A, Adrover R, Landeira G, Fernández N, García D, Barbero R, Perelstein G, Ríos B, Isla R, Civetta E, Pérez Ravier R, Barzola S, Curciarello J, Colombato LA, Jmeniltzky A. Etiology of hepatocellular carcinoma in Argentina: results of a multicenter retrospective study. *Acta Gastroenterol Latinoam* 2009; **39**: 47-52 [PMID: 19408739]

8 **Debes JD**, Chan AJ, Balderramo D, Kikuchi L, Gonzalez Ballerga E, Prieto JE, Tapias M, Idrovo V, Davalos MB, Cairo F, Barreyro FJ, Paredes S, Hernandez N, Avendaño K, Diaz Ferrer J, Yang JD, Carrera E, Garcia JA, Mattos AZ, Hirsch BS, Gonçalves PT, Carrilho FJ, Roberts LR. Hepatocellular carcinoma in South America: Evaluation of risk factors, demographics and therapy. *Liver Int* 2018; **38**: 136-143 [PMID: 28640517 DOI: 10.1111/liv.13502]

9 **Piñero F**, Tisi Baña M, de Ataide EC, Hoyos Duque S, Marciano S, Varón A, Anders M, Zerega A, Menéndez J, Zapata R, Muñoz L, Padilla Machaca M, Soza A, McCormack L, Poniachik J, Podestá LG, Gadano A, Boin IS, Duvoux C, Silva M; Latin American Liver Research, Education and Awareness Network (LALREAN). Liver transplantation for hepatocellular carcinoma: evaluation of the alpha-fetoprotein model in a multicenter cohort from Latin America. *Liver Int* 2016; **36**: 1657-1667 [PMID: 27169841 DOI: 10.1111/liv.13159]

10 **Gabrielli M**, Vivanco M, Hepp J, Martínez J, Pérez R, Guerra J, Arrese M, Figueroa E, Soza A, Yáñes R, Humeres R, Rios H, Palacios JM, Zapata R, Sanhueza E, Contreras J, Rencoret G, Rossi R, Jarufe N. Liver transplantation results for hepatocellular carcinoma in Chile. *Transplant Proc* 2010; **42**: 299-301 [PMID: 20172336 DOI: 10.1016/j.transproceed.2009.11.034]

11 **Costa PE**, Vasconcelos JB, Coelho GR, Barros MA, Neto BA, Pinto DS, Júnior JT, Correia FG, Garcia JH. Ten-year experience with liver transplantation for hepatocellular carcinoma in a Federal University Hospital in the Northeast of Brazil. *Transplant Proc* 2014; **46**: 1794-1798 [PMID: 25131039 DOI: 10.1016/j.transproceed.2014.05.016]

12 **Salvalaggio PR**, Caicedo JC, de Albuquerque LC, Contreras A, Garcia VD, Felga GE, Maurette RJ, Medina-Pestana JO, Niño-Murcia A, Pacheco-Moreira LF, Rocca J, Rodriguez-Davalos M, Ruf A, Rusca LA, Vilatoba M. Liver transplantation in Latin America: the state-of-the-art and future trends. *Transplantation* 2014; **98**: 241-246 [PMID: 25093292 DOI: 10.1097/TP.0000000000000198]

13 **Piñero F**, Marciano S, Anders M, Orozco F, Zerega A, Cabrera CR, Baña MT, Gil O, Andriani O, de Santibañes E, McCormack L, Gadano A, Silva M. Screening for liver cancer during transplant waiting list: a multicenter study from South America. *Eur J*

Gastroenterol Hepatol 2015; **27**: 355-360 [PMID: 25563142 DOI: 10.1097/MEG.0000000000000272]

14 **Piñero F**, Marciano S, Anders M, Orozco Ganem F, Zerega A, Cagliani J, Andriani O, de Santibañes E, Gil O, Podestá LG, McCormack L, Gadano A, Silva M. Identifying patients at higher risk of hepatocellular carcinoma recurrence after liver transplantation in a multicenter cohort study from Argentina. *Eur J Gastroenterol Hepatol* 2016; **28**: 421-427 [PMID: 26684693 DOI: 10.1097/MEG.0000000000000551]

15 **European Association for the Study of the Liver.** European Organisation For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438 DOI: 10.1016/j.jhep.2011.12.001]

16 **Bruix J**, Reig M, Sherman M. Evidence-Based Diagnosis, Staging, and Treatment of Patients With Hepatocellular Carcinoma. *Gastroenterology* 2016; **150**: 835-853 [PMID: 26795574 DOI: 10.1053/j.gastro.2015.12.041]

17 **European Association for the Study of the Liver.** European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol* 2016; **64**: 1388-1402 [PMID: 27062661 DOI: 10.1016/j.jhep.2015.11.004.]

18 **Oken MM**, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, Carbone PP. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982; **5**: 649-655 [PMID: 7165009]

19 **Duvoux C**, Roudot-Thoraval F, Decaens T, Pessione F, Badran H, Piardi T, Francoz C, Compagnon P, Vanlemmens C, Dumortier J, Dharancy S, Gugenheim J, Bernard PH, Adam R, Radenne S, Muscari F, Conti F, Hardwigsen J, Pageaux GP, Chazouillères O, Salame E, Hilleret MN, Lebray P, Abergel A, Debette-Gratien M, Kluger MD, Mallat A, Azoulay D, Cherqui D; Liver Transplantation French Study Group. Liver transplantation for hepatocellular carcinoma: a model including α-fetoprotein improves the performance of Milan criteria. *Gastroenterology* 2012; **143**: 986-994.e3; quiz e14-15 [PMID: 22750200 DOI: 10.1053/j.gastro.2012.05.052]

20 **von Elm E**, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *J Clin Epidemiol* 2008; **61**: 344-349 [PMID: 18313558 DOI: 10.1016/j.jclinepi.2007.11.008]

21 **Carrilho FJ**, Kikuchi L, Branco F, Goncalves CS, Mattos AA; Brazilian HCC Study Group. Clinical and epidemiological aspects of hepatocellular carcinoma in Brazil. *Clinics* (Sao Paulo) 2010; **65**: 1285-1290 [PMID: 21340216]

22 **Ascha MS**, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 1972-1978 [PMID: 20209604 DOI: 10.1002/hep.23527]

23 **Hassan MM**, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, Patt YZ. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; **36**: 1206-1213 [PMID: 12395331 DOI: 10.1053/jhep.2002.36780]

24 **Wong RJ**, Cheung R, Ahmed A. Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. *Hepatology* 2014; **59**: 2188-2195 [PMID: 24375711 DOI: 10.1002/hep.26986]

25 **Siriwardana RC**, Niriella MA, Dassanayake AS, Liyanage C, Gunathilaka B, Jayathunge S, de Silva HJ. Clinical characteristics and outcome of hepatocellular carcinoma in alcohol related and cryptogenic cirrhosis: a prospective study. *Hepatobiliary Pancreat Dis Int* 2015; **14**: 401-405 [PMID: 26256085]

26 **Perumpail RB**, Wong RJ, Ahmed A, Harrison SA. Hepatocellular Carcinoma in the Setting of Non-cirrhotic Nonalcoholic Fatty Liver Disease and the Metabolic Syndrome: US Experience. *Dig Dis Sci* 2015; **60**: 3142-3148 [PMID: 26250831 DOI: 10.1007/s10620-015-3821-7]

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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,

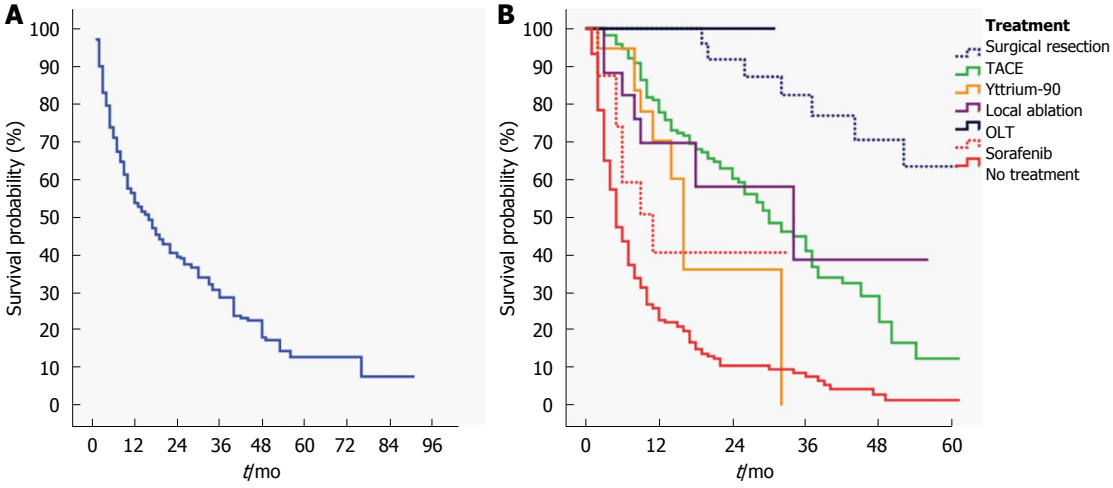

Figure 1 Overall survival in patients with hepatocellular carcinoma. A: Overall median survival in the whole cohort; B: Survival curves was stratified by primary treatment modality.

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]

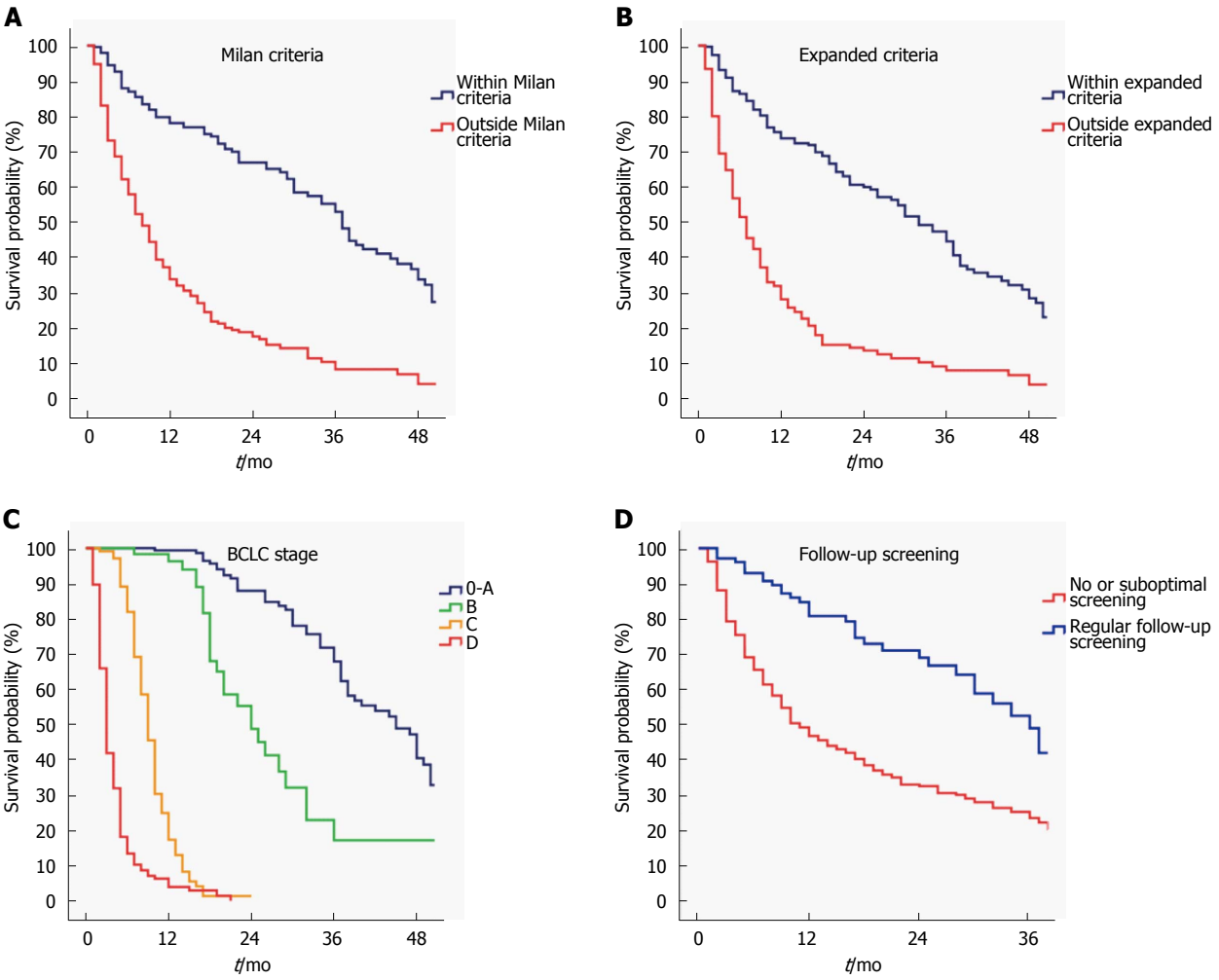

Figure 2 Overall survival according to oncological stage and pre-diagnostic screening characteristics. Patients within Milan (A) or expanded criteria (B), patients who were diagnosed at earlier BCLC stages (C) and patients who had regular follow-up screening for HCC (D) had a significantly better survival (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.

REFERENCES

1 **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538 [PMID: 16879891 DOI: 10.1016/j.jhep.2006.05.013]

2 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

3 **Sherman M**. Hepatocellular carcinoma: epidemiology, surveillance, and diagnosis. *Semin Liver Dis* 2010; **30**: 3-16 [PMID: 20175029 DOI: 10.1055/s-0030-1247128]

4 **Tandon P**, Garcia-Tsao G. Prognostic indicators in hepatocellular carcinoma: a systematic review of 72 studies. *Liver Int* 2009; **29**: 502-510 [PMID: 19141028 DOI: 10.1111/j.1478-3231.2008.01957.x]

5 **Llovet JM**, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338 [PMID: 10518312 DOI: 10.1055/s-2007-1007122]

6 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J; EASL Panel of Experts on HCC. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430 [PMID: 11592607]

7 **Pugh RN**, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649 [PMID: 4541913]

8 **Yao FY**, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. *Hepatology* 2001; **33**: 1394-1403 [PMID: 11391528 DOI: 10.1053/jhep.2001.24563]

9 **Benvegnù L**, Noventa F, Bernardinello E, Pontisso P, Gatta A, Alberti A. Evidence for an association between the aetiology of cirrhosis and pattern of hepatocellular carcinoma development. *Gut* 2001; **48**: 110-115 [PMID: 11115831]

10 **Shijo H**, Okazaki M, Koganemaru F, Higashi M, Sakaguchi S, Okumura M. Influence of hepatitis B virus infection and age on mode of growth of hepatocellular carcinoma. *Cancer* 1991; **67**: 2626-2632 [PMID: 1707748]

11 **Cantarini MC**, Trevisani F, Morselli-Labate AM, Rapaccini G, Farinati F, Del Poggio P, Di Nolfo MA, Benvegnù L, Zoli M, Borzio F, Bernardi M; Italian Liver Cancer (ITA.LI.CA) group. Effect of the etiology of viral cirrhosis on the survival of patients with hepatocellular carcinoma. *Am J Gastroenterol* 2006; **101**: 91-98 [PMID: 16405539 DOI: 10.1111/j.1572-0241.2006.00364.x]

12 **Bozorgzadeh A**, Orloff M, Abt P, Tsoulfas G, Younan D, Kashyap R, Jain A, Mantry P, Maliakkal B, Khorana A, Schwartz S. Survival outcomes in liver transplantation for hepatocellular carcinoma, comparing impact of hepatitis C versus other etiology of cirrhosis. *Liver Transpl* 2007; **13**: 807-813 [PMID: 17539001 DOI: 10.1002/lt.21054]

13 **Franssen B**, Alshebeeb K, Tabrizian P, Marti J, Pierobon ES, Lubezky N, Roayaie S, Florman S, Schwartz ME. Differences in surgical outcomes between hepatitis B- and hepatitis C-related hepatocellular carcinoma: a retrospective analysis of a single North American center. *Ann Surg* 2014; **260**: 650-656; discussion 656-658 [PMID: 25203882 DOI: 10.1097/SLA.0000000000000917]

14 **Huang YH**, Wu JC, Chen CH, Chang TT, Lee PC, Chau GY, Lui WY, Chang FY, Lee SD. Comparison of recurrence after hepatic resection in patients with hepatitis B vs. hepatitis C-related small hepatocellular carcinoma in hepatitis B virus endemic area. *Liver Int* 2005; **25**: 236-241 [PMID: 15780044 DOI: 10.1111/j.1478-3231.2005.01081.x]

15 **Li Q**, Li H, Qin Y, Wang PP, Hao X. Comparison of surgical outcomes for small hepatocellular carcinoma in patients with hepatitis B versus hepatitis C: a Chinese experience. *J Gastroenterol Hepatol* 2007; **22**: 1936-1941 [PMID: 17914973 DOI: 10.1111/j.1440-1746.2006.04619.x]

16 **Sasaki Y**, Yamada T, Tanaka H, Ohigashi H, Eguchi H, Yano M, Ishikawa O, Imaoka S. Risk of recurrence in a long-term follow-up after surgery in 417 patients with hepatitis B- or hepatitis C-related hepatocellular carcinoma. *Ann Surg* 2006; **244**: 771-780 [PMID: 17060771 DOI: 10.1097/01.sla.0000225126.56483.b3]

17 **Roayaie S**, Haim MB, Emre S, Fishbein TM, Sheiner PA, Miller CM, Schwartz ME. Comparison of surgical outcomes for hepatocellular carcinoma in patients with hepatitis B versus hepatitis C: a western experience. *Ann Surg Oncol* 2000; **7**: 764-770 [PMID: 11129425]

18 **Utsunomiya T**, Shimada M, Kudo M, Ichida T, Matsui O, Izumi N, Matsuyama Y, Sakamoto M, Nakashima O, Ku Y, Takayama T, Kokudo N; Liver Cancer Study Group of Japan. A comparison of the surgical outcomes among patients with HBV-positive, HCV-positive, and non-B non-C hepatocellular carcinoma: a nationwide study of 11,950 patients. *Ann Surg* 2015; **261**: 513-520 [PMID: 25072437 DOI: 10.1097/SLA.0000000000000821]

19 **Trevisani F**, Magini G, Santi V, Morselli-Labate AM, Cantarini MC, Di Nolfo MA, Del Poggio P, Benvegnù L, Rapaccini G, Farinati F, Zoli M, Borzio F, Giannini EG, Caturelli E, Bernardi M; Italian Liver Cancer (ITA.LI.CA) Group. Impact of etiology of cirrhosis on the survival of patients diagnosed with hepatocellular carcinoma during surveillance. *Am J Gastroenterol* 2007; **102**: 1022-1031 [PMID: 17313497 DOI: 10.1111/j.1572-0241.2007.01100.x]

20 **Chen PH**, Kao WY, Chiou YY, Hung HH, Su CW, Chou YH, Huo TI, Huang YH, Wu WC, Chao Y, Lin HC, Wu JC. Comparison of prognosis by viral etiology in patients with hepatocellular carcinoma after radiofrequency ablation. *Ann Hepatol* 2013; **12**: 263-273 [PMID: 23396738]

21 **Yang JD**, Kim WR, Park KW, Chaiteerakij R, Kim B, Sanderson SO, Larson JJ, Pedersen RA, Therneau TM, Gores GJ, Roberts LR, Park JW. Model to estimate survival in ambulatory patients with hepatocellular carcinoma. *Hepatology* 2012; **56**: 614-621 [PMID: 22370914 DOI: 10.1002/hep.25680]

22 **Toso C**, Trotter J, Wei A, Bigam DL, Shah S, Lancaster J, Grant DR, Greig PD, Shapiro AM, Kneteman NM. Total tumor volume predicts risk of recurrence following liver transplantation in patients with hepatocellular carcinoma. *Liver Transpl* 2008; **14**: 1107-1115 [PMID: 18668667 DOI: 10.1002/lt.21484]

23 **Toso C**, Meeberg G, Hernandez-Alejandro R, Dufour JF, Marotta P, Majno P, Kneteman NM. Total tumor volume and alpha-fetoprotein for selection of transplant candidates with hepatocellular carcinoma: A prospective validation. *Hepatology* 2015; **62**: 158-165 [PMID: 25777590 DOI: 10.1002/hep.27787]

24 **Trevisani F**, D'Intino PE, Morselli-Labate AM, Mazzella G, Accogli E, Caraceni P, Domenicali M, De Notariis S, Roda E, Bernardi M. Serum alpha-fetoprotein for diagnosis of hepatocellular carcinoma in patients with chronic liver disease: influence of HBsAg and anti-HCV status. *J Hepatol* 2001; **34**: 570-575 [PMID: 11394657]

25 **Tangkijvanich P**, Anukulkarnkusol N, Suwangool P, Lertmaharit S, Hanvivatvong O, Kullavanijaya P, Poovorawan Y. Clinical characteristics and prognosis of hepatocellular carcinoma: analysis based on serum alpha-fetoprotein levels. *J Clin Gastroenterol* 2000; **31**: 302-308 [PMID: 11129271]

26 **Ebara M**, Ohto M, Shinagawa T, Sugiura N, Kimura K, Matsutani S, Morita M, Saisho H, Tsuchiya Y, Okuda K. Natural history of

minute hepatocellular carcinoma smaller than three centimeters complicating cirrhosis. A study in 22 patients. *Gastroenterology* 1986; **90**: 289-298 [PMID: 2416627]

27 **Hakeem AR**, Young RS, Marangoni G, Lodge JP, Prasad KR. Systematic review: the prognostic role of alpha-fetoprotein following liver transplantation for hepatocellular carcinoma. *Aliment Pharmacol Ther* 2012; **35**: 987-999 [PMID: 22429190 DOI: 10.1111/j.1365-2036.2012.05060.x]

28 **Hanazaki K**, Kajikawa S, Koide N, Adachi W, Amano J. Prognostic factors after hepatic resection for hepatocellular carcinoma with hepatitis C viral infection: univariate and multivariate analysis. *Am J Gastroenterol* 2001; **96**: 1243-1250 [PMID: 11316177 DOI: 10.1111/j.1572-0241.2001.03634.x]

29 **Wang CC**, Iyer SG, Low JK, Lin CY, Wang SH, Lu SN, Chen CL. Perioperative factors affecting long-term outcomes of 473 consecutive patients undergoing hepatectomy for hepatocellular carcinoma. *Ann Surg Oncol* 2009; **16**: 1832-1842 [PMID: 19365625 DOI: 10.1245/s10434-009-0448-y]

30 **Zhang Q**, Shang L, Zang Y, Chen X, Zhang L, Wang Y, Wang L, Liu Y, Mao S, Shen Z. α-Fetoprotein is a potential survival predictor in hepatocellular carcinoma patients with hepatitis B selected for liver transplantation. *Eur J Gastroenterol Hepatol* 2014; **26**: 544-552 [PMID: 24614696 DOI: 10.1097/MEG.0000000000000029]

31 **Duvoux C**, Roudot-Thoraval F, Decaens T, Pessione F, Badran H, Piardi T, Francoz C, Compagnon P, Vanlemmens C, Dumortier J, Dharancy S, Gugenheim J, Bernard PH, Adam R, Radenne S, Muscari F, Conti F, Hardwigsen J, Pageaux GP, Chazouillères O, Salame E, Hilleret MN, Lebray P, Abergel A, Debette-Gratien M, Kluger MD, Mallat A, Azoulay D, Cherqui D; Liver Transplantation French Study Group. Liver transplantation for hepatocellular carcinoma: a model including α-fetoprotein improves the performance of Milan criteria. *Gastroenterology* 2012; **143**: 986-994.e3; quiz e14-15 [PMID: 22750200 DOI: 10.1053/j.gastro.2012.05.052]

32 **Suh SW**, Lee KW, Lee JM, You T, Choi Y, Kim H, Lee HW, Lee JM, Yi NJ, Suh KS. Prediction of aggressiveness in early-stage hepatocellular carcinoma for selection of surgical resection. *J Hepatol* 2014; **60**: 1219-1224 [PMID: 24548529 DOI: 10.1016/j.jhep.2014.01.027]

33 **Grieco A**, Pompili M, Caminiti G, Miele L, Covino M, Alfei B, Rapaccini GL, Gasbarrini G. Prognostic factors for survival in patients with early-intermediate hepatocellular carcinoma undergoing non-surgical therapy: comparison of Okuda, CLIP, and BCLC staging systems in a single Italian centre. *Gut* 2005; **54**: 411-418 [PMID: 15710992 DOI: 10.1136/gut.2004.048124]

34 **op den Winkel M**, Nagel D, Sappl J, op den Winkel P, Lamerz R, Zech CJ, Straub G, Nickel T, Rentsch M, Stieber P, Göke B, Kolligs FT. Prognosis of patients with hepatocellular carcinoma. Validation and ranking of established staging-systems in a large western HCC-cohort. *PLoS One* 2012; **7**: e45066 [PMID: 23071507 DOI: 10.1371/journal.pone.0045066]

35 **Lee YH**, Hsu CY, Hsia CY, Huang YH, Su CW, Lin HC, Chiou YY, Huo TI. A prognostic model for patients with hepatocellular carcinoma within the Milan criteria undergoing non-transplant therapies, based on 1106 patients. *Aliment Pharmacol Ther* 2012; **36**: 551-559 [PMID: 22817677 DOI: 10.1111/j.1365-2036.2012.05226.x]

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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.

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Data sharing statement: The dataset is available from the corresponding author at ari.joffe@ahs.ca upon reasonable request. Consent was not obtained but the presented data are anonymized and risk of identification is low.

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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.

REFERENCES

1 **Kim WR**, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB, Harper AM, Wainright JL, Snyder JJ, Israni AK, Kasiske BL. OPTN/SRTR 2013 Annual Data Report: liver. *Am J Transplant* 2015; **15** Suppl 2: 1-28 [PMID: 25626341 DOI: 10.1111/ajt.13197]

2 **Sundaram SS**, Alonso EM, Anand R; Study of Pediatric Liver Transplantation Research Group. Outcomes after liver transplantation in young infants. *J Pediatr Gastroenterol Nutr* 2008; **47**: 486-492 [PMID: 18852642 DOI: 10.1097/MPG.0b013e318175d7d2]

3 **Robertson CM**, Dinu IA, Joffe AR, Alton GY, Yap JY, Asthana S, Acton BV, Sauve RS, Martin SR, Kneteman NM, Gilmour SM; Western Canadian Therapies Follow-up Group. Neurocognitive outcomes at kindergarten entry after liver transplantation at <3 yr of age. *Pediatr Transplant* 2013; **17**: 621-630 [PMID: 23961979 DOI: 10.1111/petr.12134]

4 **Johnson SB**, Riley AW, Granger DA, Riis J. The science of early life toxic stress for pediatric practice and advocacy. *Pediatrics* 2013; **131**: 319-327 [PMID: 23339224 DOI: 10.1542/peds.2012-0469]

5 **Garner AS**, Shonkoff JP; Committee on Psychosocial Aspects of Child and Family Health; Committee on Early Childhood, Adoption, and Dependent Care; Section on Developmental and Behavioral Pediatrics. Early childhood adversity, toxic stress, and the role of the pediatrician: translating developmental science into lifelong health. *Pediatrics* 2012; **129**: e224-e231 [PMID: 22201148 DOI: 10.1542/peds.2011-2662]

6 **Campbell F**, Conti G, Heckman JJ, Moon SH, Pinto R, Pungello E, Pan Y. Early childhood investments substantially boost adult health. *Science* 2014; **343**: 1478-1485 [PMID: 24675955 DOI: 10.1126/science.1248429]

7 **Walhovd KB**, Krogsrud SK, Amlien IK, Bartsch H, Bjørnerud A, Due-Tønnessen P, Grydeland H, Hagler DJ Jr, Håberg AK, Kremen WS, Ferschmann L, Nyberg L, Panizzon MS, Rohani DA, Skranes J, Storsve AB, Sølsnes AE, Tamnes CK, Thompson WK, Reuter C, Dale AM, Fjell AM. Neurodevelopmental origins of lifespan changes in brain and cognition. *Proc Natl Acad Sci USA* 2016; **113**: 9357-9362 [PMID: 27432992 DOI: 10.1073/pnas.1524259113]

8 **Bekker J**, Ploem S, de Jong KP. Early hepatic artery thrombosis after liver transplantation: a systematic review of the incidence, outcome and risk factors. *Am J Transplant* 2009; **9**: 746-757 [PMID: 19298450 DOI: 10.1111/j.1600-6143.2008.02541.x]

9 **Hackl C**, Schlitt HJ, Melter M, Knoppke B, Loss M. Current developments in pediatric liver transplantation. *World J Hepatol* 2015; **7**: 1509-1520 [PMID: 26085910 DOI: 10.4254/wjh.v7.i11.1509]

10 **Feier FH**, da Fonseca EA, Seda-Neto J, Chapchap P. Biliary complications after pediatric liver transplantation: Risk factors, diagnosis and management. *World J Hepatol* 2015; **7**: 2162-2170 [PMID: 26328028 DOI: 10.4254/wjh.v7.i18.2162]

11 **Diem HV**, Evrard V, Vinh HT, Sokal EM, Janssen M, Otte JB, Reding R. Pediatric liver transplantation for biliary atresia: results of primary grafts in 328 recipients. *Transplantation* 2003; **75**: 1692-1697 [PMID: 12777858 DOI: 10.1097/01.TP.0000062570.83203.A3]

12 **Utterson EC**, Shepherd RW, Sokol RJ, Bucuvalas J, Magee JC, McDiarmid SV, Anand R; Split Research Group. Biliary atresia: clinical profiles, risk factors, and outcomes of 755 patients listed for liver transplantation. *J Pediatr* 2005; **147**: 180-185 [PMID: 16126046 DOI: 10.1016/j.jpeds.2005.04.073]

13 **McDiarmid SV**, Anand R, Martz K, Millis MJ, Mazariegos G. A multivariate analysis of pre-, peri-, and post-transplant factors affecting outcome after pediatric liver transplantation. *Ann Surg* 2011; **254**: 145-154 [PMID: 21606838 DOI: 10.1097/SLA.0b013e31821ad86a]

14 **Diamond IR**, Fecteau A, Millis JM, Losanoff JE, Ng V, Anand R, Song C; SPLIT Research Group. Impact of graft type on outcome in pediatric liver transplantation: a report From Studies of Pediatric

Liver Transplantation (SPLIT). *Ann Surg* 2007; **246**: 301-310 [PMID: 17667510 DOI: 10.1097/SLA.0b013e3180caa415]
15 **Ye H**, Zhao Q, Wang Y, Wang D, Zheng Z, Schroder PM, Lu Y, Kong Y, Liang W, Shang Y, Guo Z, He X. Outcomes of Technical Variant Liver Transplantation versus Whole Liver Transplantation for Pediatric Patients: A Meta-Analysis. *PLoS One* 2015; **10**: e0138202 [PMID: 26368552 DOI: 10.1371/journal.pone.0138202]
16 **Laurence JM**, Sapisochin G, DeAngelis M, Seal JB, Miserachs MM, Marquez M, Zair M, Fecteau A, Jones N, Hrycko A, Avitzur Y, Ling SC, Ng V, Cattral M, Grant D, Kamath BM, Ghanekar A. Biliary complications in pediatric liver transplantation: Incidence and management over a decade. *Liver Transpl* 2015; **21**: 1082-1090 [PMID: 25991054 DOI: 10.1002/lt.24180]
17 **Farmer DG**, Venick RS, McDiarmid SV, Ghobrial RM, Gordon SA, Yersiz H, Hong J, Candell L, Cholakians A, Wozniak L, Martin M, Vargas J, Ament M, Hiatt J, Busuttil RW. Predictors of outcomes after pediatric liver transplantation: an analysis of more than 800 cases performed at a single institution. *J Am Coll Surg* 2007; **204**: 904-914; discussion 914-916 [PMID: 17481508 DOI: 10.1016/j.jamcollsurg.2007.01.061]
18 **Evrard V**, Otte JB, Sokal E, Rochet JS, Haccourt F, Gennari F, Latinne D, Jamart J, Reding R. Impact of surgical and immunological parameters in pediatric liver transplantation: a multivariate analysis in 500 consecutive recipients of primary grafts. *Ann Surg* 2004; **239**: 272-280 [PMID: 14745337 DOI: 10.1097/01.sla.0000108681.24374.02]
19 **Harris PA**, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009; **42**: 377-381 [PMID: 18929686 DOI: 10.1016/j.jbi.2008.08.010]
20 **Emre S**, Umman V, Cimsit B, Rosencrantz R. Current concepts in pediatric liver transplantation. *Mt Sinai J Med* 2012; **79**: 199-213 [PMID: 22499491 DOI: 10.1002/msj.21305]
21 **Boudi FB**. Pediatric Liver Transplantation. In: Medscape. 2015. Available from: URL: http://emedicine.medscape.com/article/1012910-overview#showall
22 **Ooi CY**, Brandão LR, Zolpys L, De Angelis M, Drew W, Jones N, Ling SC, Fecteau A, Ng VL. Thrombotic events after pediatric liver transplantation. *Pediatr Transplant* 2010; **14**: 476-482 [PMID: 19849808 DOI: 10.1111/j.1399-3046.2009.01252.x]
23 **Uchida Y**, Sakamoto S, Egawa H, Ogawa K, Ogura Y, Taira K, Kasahara M, Uryuhara K, Takada Y, Kamiyama Y, Tanaka K, Uemoto S. The impact of meticulous management for hepatic artery thrombosis on long-term outcome after pediatric living donor liver transplantation. *Clin Transplant* 2009; **23**: 392-399 [PMID: 19191812 DOI: 10.1111/j.1399-0012.2008.00924.x]
24 **Tiao GM**, Alonso M, Bezerra J, Yazigi N, Heubi J, Balistreri W, Bucuvalas J, Ryckman F. Liver transplantation in children younger than 1 year--the Cincinnati experience. *J Pediatr Surg* 2005; **40**: 268-273 [PMID: 15868596 DOI: 10.1016/j.jpedsurg.2004.09.021]
25 **Venick RS**, Farmer DG, McDiarmid SV, Duffy JP, Gordon SA, Yersiz H, Hong JC, Vargas JH, Ament ME, Busuttil RW. Predictors of survival following liver transplantation in infants: a single-center analysis of more than 200 cases. *Transplantation* 2010; **89**: 600-605 [PMID: 19997060 DOI: 10.1097/TP.0b013e3181c5cdc1]
26 **Oh SH**, Kim KM, Kim DY, Lee YJ, Rhee KW, Jang JY, Chang SH, Lee SY, Kim JS, Choi BH, Park SJ, Yoon CH, Ko GY, Sung KB, Hwang GS, Choi KT, Yu E, Song GW, Ha TY, Moon DB, Ahn CS, Kim KH, Hwang S, Park KM, Lee YJ, Lee SG. Long-term outcomes of pediatric living donor liver transplantation at a single institution. *Pediatr Transplant* 2010; **14**: 870-878 [PMID: 20609169 DOI: 10.1111/j.1399-3046.2010.01357.x]
27 **Seda-Neto J**, Antunes da Fonseca E, Pugliese R, Candido HL, Benavides MR, Carballo Afonso R, Neiva R, Porta G, Miura IK, Teng HW, Iwase FC, Rodrigues ML, Carneiro de Albuquerque LA, Kondo M, Chapchap P. Twenty Years of Experience in Pediatric Living Donor Liver Transplantation: Focus on Hepatic Artery Reconstruction, Complications, and Outcomes. *Transplantation* 2016; **100**: 1066-1072 [PMID: 27014791 DOI: 10.1097/TP.0000000000001135]
28 **Bourdeaux C**, Darwish A, Jamart J, Tri TT, Janssen M, Lerut J, Otte JB, Sokal E, de Ville de Goyet J, Reding R. Living-related versus deceased donor pediatric liver transplantation: a multivariate analysis of technical and immunological complications in 235 recipients. *Am J Transplant* 2007; **7**: 440-447 [PMID: 17173657 DOI: 10.1111/j.1600-6143.2006.01626.x]
29 **Yankol Y**, Fernandez LA, Kanmaz T, Leverson GE, Mezrich JD, Foley D, Mecit N, D'Alessandro AM, Acarli K, Kalayoglu M. Results of pediatric living donor compared to deceased donor liver transplantation in the PELD/MELD era: Experience from two centers on two different continents. *Pediatr Transplant* 2016; **20**: 72-82 [PMID: 26861217 DOI: 10.1111/petr.12641]
30 **Anderson CD**, Turmelle YP, Lowell JA, Nadler M, Millis M, Anand R, Martz K, Shepherd RW; SPLIT Research Group. The effect of recipient-specific surgical issues on outcome of liver transplantation in biliary atresia. *Am J Transplant* 2008; **8**: 1197-1204 [PMID: 18444930 DOI: 10.1111/j.1600-6143.2008.02223.x]
31 **Yang SC**, Huang CJ, Chen CL, Wang CH, Wu SC, Shih TH, Juang SE, Lee YE, Jawan B, Cheng YF, Cheng KW. Living donor liver transplantation with body-weight more or less than 10 kilograms. *World J Gastroenterol* 2015; **21**: 7248-7253 [PMID: 26109812 DOI: 10.3748/wjg.v21.i23.7248]
32 **Englesbe MJ**, Kelly B, Goss J, Fecteau A, Mitchell J, Andrews W, Krapohl G, Magee JC, Mazariegos G, Horslen S, Bucuvalas J. Reducing pediatric liver transplant complications: a potential roadmap for transplant quality improvement initiatives within North America. *Am J Transplant* 2012; **12**: 2301-2306 [PMID: 22883313 DOI: 10.1111/j.1600-6143.2012.04204.x]
33 **Cramm SL**, Waits SA, Englesbe MJ, Bucuvalas JC, Horslen SP, Mazariegos GV, Soltys KA, Anand R, Magee JC. Failure to Rescue as a Quality Improvement Approach in Transplantation: A First Effort to Evaluate This Tool in Pediatric Liver Transplantation. *Transplantation* 2016; **100**: 801-807 [PMID: 26910329 DOI: 10.1097/TP.0000000000001121]
34 **Allingstrup M**, Wetterslev J, Ravn FB, Møller AM, Afshari A. Antithrombin III for critically ill patients: a systematic review with meta-analysis and trial sequential analysis. *Intensive Care Med* 2016; **42**: 505-520 [PMID: 26862016 DOI: 10.1007/s00134-016-4225-7]
35 **Kamran Hejazi Kenari S**, Mirzakhani H, Eslami M, Saidi RF. Current state of the art in management of vascular complications after pediatric liver transplantation. *Pediatr Transplant* 2015; **19**: 18-26 [PMID: 25425338 DOI: 10.1111/petr.12407]
36 **Alvarez F**. Portal vein complications after pediatric liver transplantation. *Curr Gastroenterol Rep* 2012; **14**: 270-274 [PMID: 22434261 DOI: 10.1007/s11894-012-0257-5]
37 **Lisman T**, Porte RJ. Rebalanced hemostasis in patients with liver disease: evidence and clinical consequences. *Blood* 2010; **116**: 878-885 [PMID: 20400681 DOI: 10.1182/blood-2010-02-261891]
38 **Lisman T**, Stravitz RT. Rebalanced Hemostasis in Patients with Acute Liver Failure. *Semin Thromb Hemost* 2015; **41**: 468-473 [PMID: 26049071 DOI: 10.1055/s-0035-1550430]
39 **Roberts LN**, Bernal W. Management of Bleeding and Thrombosis in Critically Ill Patients with Liver Disease. *Semin Thromb Hemost* 2015; **41**: 520-526 [PMID: 26080305 DOI: 10.1055/s-0035-1550431]
40 **Lisman T**, Caldwell SH, Burroughs AK, Northup PG, Senzolo M, Stravitz RT, Tripodi A, Trotter JF, Valla DC, Porte RJ; Coagulation in Liver Disease Study Group. Hemostasis and thrombosis in patients with liver disease: the ups and downs. *J Hepatol* 2010; **53**: 362-371 [PMID: 20546962 DOI: 10.1016/j.jhep.2010.01.042]
41 **Magnusson M**, Ignjatovic V, Hardikar W, Monagle P. A conceptual and practical approach to haemostasis in paediatric liver disease. *Arch Dis Child* 2016; **101**: 854-859 [PMID: 27013527 DOI: 10.1136/archdischild-2015-309535]
42 **Alonso EM**, Ng VL, Anand R, Anderson CD, Ekong UD, Fredericks EM, Furuya KN, Gupta NA, Lerret SM, Sundaram S, Tiao G; Studies of Pediatric Liver Transplantation (SPLIT)

Research Group. The SPLIT research agenda 2013. *Pediatr Transplant* 2013; **17**: 412-422 [PMID: 23718800 DOI: 10.1111/petr.12090]
43 **Iglesias J**, López JA, Ortega J, Roqueta J, Asensio M, Margarit C. Liver transplantation in infants weighing under 7 kilograms: management and outcome of PICU. *Pediatr Transplant* 2004; **8**: 228-232 [PMID: 15176958 DOI: 10.1111/j.1399-3046.2004.00128.x]
44 **Ciria R**, Sánchez-Hidalgo JM, Briceño J, Naranjo A, Pleguezuelo M, Díaz-Nieto R, Luque A, Jiménez J, García-Menor E, Gilbert JJ, de la Mata M, Pérez-Navero JL, Solórzano G, Rufián S, Pera C, López-Cillero P. Establishment of a pediatric liver transplantation program: experience with 100 transplantation procedures. *Transplant Proc* 2009; **41**: 2444-2446 [PMID: 19715946 DOI: 10.1016/j.transproceed.2009.06.072]
45 **Mohamed El Moghazy W**, Ogura Y, Mutsuko M, Harada K, Koizumi A, Uemoto S. Pediatric living-donor liver transplantation for acute liver failure: analysis of 57 cases. *Transpl Int* 2010; **23**: 823-830 [PMID: 20158695 DOI: 10.1111/j.1432-2277.2010.01059.x]
46 **Gelas T**, McKiernan PJ, Kelly DA, Mayer DA, Mirza DF, Sharif K. ABO-incompatible pediatric liver transplantation in very small recipients: Birmingham's experience. *Pediatr Transplant* 2011; **15**: 706-711 [PMID: 21762327 DOI: 10.1111/j.1399-3046.2011.01541.x]
47 **Mekeel KL**, Langham MR Jr, Gonzalez-Peralta RP, Hemming AW. Liver transplantation in very small infants. *Pediatr Transplant* 2007; **11**: 66-72 [PMID: 17239125 DOI: 10.1111/j.1399-3046.2006.00610.x]
48 **Wagenaar AE**, Tashiro J, Sola JE, Ekwenna O, Tekin A, Perez EA. Pediatric liver transplantation: predictors of survival and resource utilization. *Pediatr Surg Int* 2016; **32**: 439-449 [PMID: 27001031 DOI: 10.1007/s00383-016-3881-6]
49 **Roberts JP**, Hulbert-Shearon TE, Merion RM, Wolfe RA, Port FK. Influence of graft type on outcomes after pediatric liver transplantation. *Am J Transplant* 2004; **4**: 373-377 [PMID: 14961989]
50 **Berg CL**, Steffick DE, Edwards EB, Heimbach JK, Magee JC, Washburn WK, Mazariegos GV. Liver and intestine transplantation in the United States 1998-2007. *Am J Transplant* 2009; **9**: 907-931 [PMID: 19341415 DOI: 10.1111/j.1600-6143.2009.02567.x]
51 **Alexopoulos SP**, Nekrasov V, Cao S, Groshen S, Kaur N, Genyk YS, Matsuoka L. Effects of recipient size and allograft type on pediatric liver transplantation for biliary atresia. *Liver Transpl* 2017; **23**: 221-233 [PMID: 27862929 DOI: 10.1002/lt.24675]
52 **Dindo D**, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213 [PMID: 15273542]

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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.

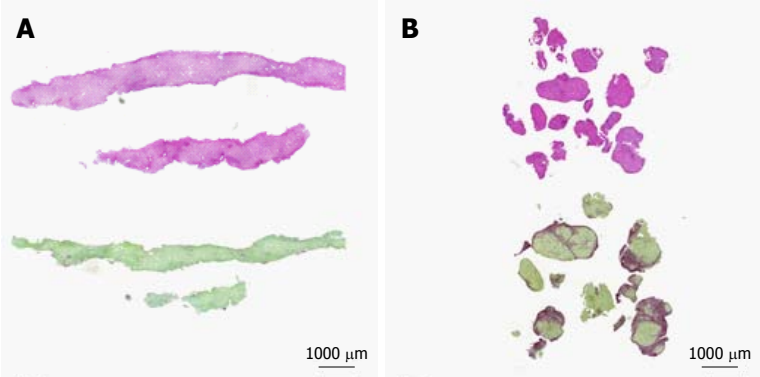

Figure 1 Variability of histological specimens in HE (top) and PicroSirius red (bottom) stained transjugular biopsy specimens. A: Non-cirrhotic sample with 0.01% fibrosis proportion; B: Cirrhotic sample with 21% of fibrosis proportion.

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

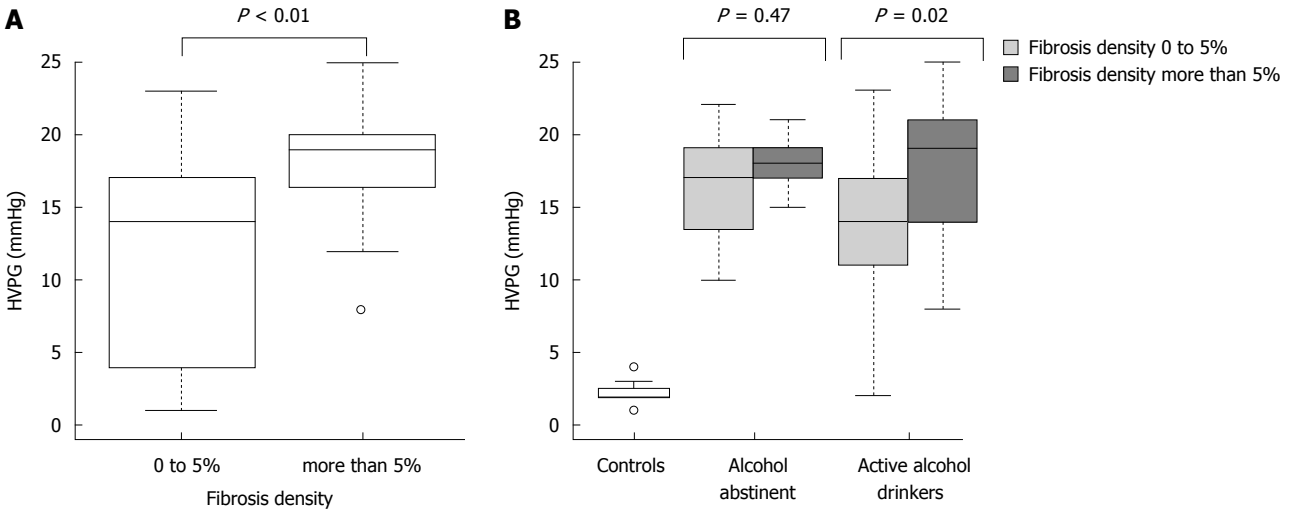

Figure 2 Correlation between hepatic venous pressure gradient and fibrosis density measured by collagen proportionate area. A: Correlation between HVPG and fibrosis density measured by CPA in the whole group of patients; B: Subgroup analysis showing a positive correlation between CPA and HVPG restricted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

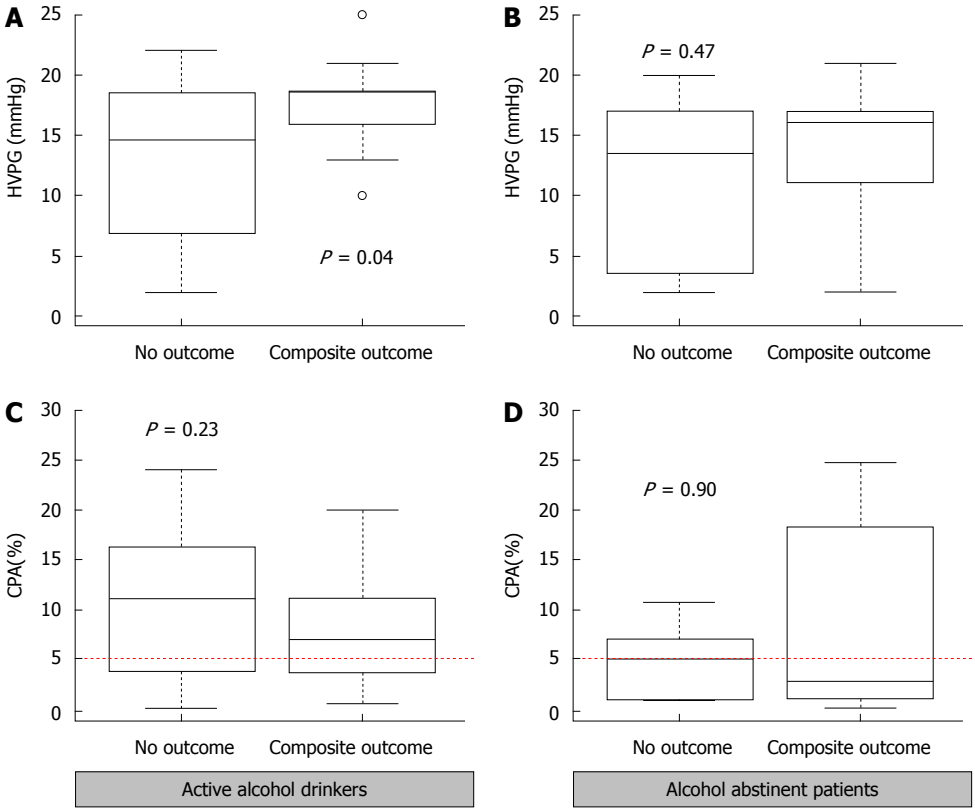

Figure 3 Relationship between hepatic venous pressure gradient (A), collagen proportionate area (B) and development of clinical complications related to portal hypertension during follow-up both in active alcohol drinkers (C) and abstinent patients (D).

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.

REFERENCES

1 **Trautwein C**, Friedman SL, Schuppan D, Pinzani M. Hepatic fibrosis: Concept to treatment. *J Hepatol* 2015; **62**: S15-S24 [PMID: 25920084 DOI: 10.1016/j.jhep.2015.02.039S0168-8278(15)00149-X]

2 **Ripoll C**, Bañares R, Rincón D, Catalina MV, Lo Iacono O, Salcedo M, Clemente G, Núñez O, Matilla A, Molinero LM. Influence of hepatic venous pressure gradient on the prediction of survival of patients with cirrhosis in the MELD Era. *Hepatology* 2005; **42**: 793-801 [PMID: 16175621 DOI: 10.1002/hep.20871]

3 **Rehm J**, Samokhvalov AV, Shield KD. Global burden of alcoholic liver diseases. *J Hepatol* 2013; **59**: 160-168 [PMID: 23511777 DOI: 10.1016/j.jhep.2013.03.007S0168-8278(13)00184-0]

4 **Lackner C**, Bataller R, Burt A, Miquel R, Schuppan D, Tiniakos D, Trauner M. Fibrosis evaluation by transient elastography in alcoholic liver disease: Is the histological scoring system impacting cutoff values? *Hepatology* 2017; **65**: 1758-1761 [PMID: 28120459 DOI: 10.1002/hep.29075]

5 **Savolainen V**, Perola M, Lalu K, Penttilä A, Virtanen I, Karhunen PJ. Early perivenular fibrogenesis--precirrhotic lesions among moderate alcohol consumers and chronic alcoholics. *J Hepatol* 1995; **23**: 524-531 [PMID: 8583139 DOI: 0168-8278(95)80057-3]

6 **Michalak S**, Rousselet MC, Bedossa P, Pilette C, Chappard D, Oberti F, Gallois Y, Calès P. Respective roles of porto-septal fibrosis and centrilobular fibrosis in alcoholic liver disease. *J Pathol* 2003; **201**: 55-62 [PMID: 12950017 DOI: 10.1002/path.1412]

7 **Hall A**, Germani G, Isgrò G, Burroughs AK, Dhillon AP. Fibrosis distribution in explanted cirrhotic livers. *Histopathology* 2012; **60**: 270-277 [PMID: 22211285 DOI: 10.1111/j.1365-2559.2011.04094.x]

8 **Mathurin P**, Beuzin F, Louvet A, Carrié-Ganne N, Balian A, Trinchet JC, Dalsoglio D, Prevot S, Naveau S. Fibrosis progression occurs in a subgroup of heavy drinkers with typical histological features. *Aliment Pharmacol Ther* 2007; **25**: 1047-1054 [PMID: 17439505 DOI: 10.1111/j.1365-2036.2007.03302.x]

9 **Castera L**. Noninvasive methods to assess liver disease in patients with hepatitis B or C. *Gastroenterology* 2012; **142**: 1293-1302.e4 [PMID: 22537436 DOI: 10.1053/j.gastro.2012.02.017S0016-5085(12)00230-2]

10 **Lombardi R**, Buzzetti E, Roccarina D, Tsochatzis EA. Non-invasive assessment of liver fibrosis in patients with alcoholic liver disease. *World J Gastroenterol* 2015; **21**: 11044-11052 [PMID: 26494961 DOI: 10.3748/wjg.v21.i39.11044]

11 **Merkel C**, Montagnese S. Hepatic venous pressure gradient measurement in clinical hepatology. *Dig Liver Dis* 2011; **43**: 762-767 [PMID: 21549649 DOI: 10.1016/j.dld.2011.03.002S1590-8658(11)00092-2]

12 **Poynard T**, Degott C, Munoz C, Lebrec D. Relationship between degree of portal hypertension and liver histologic lesions in patients with alcoholic cirrhosis. Effect of acute alcoholic hepatitis on portal hypertension. *Dig Dis Sci* 1987; **32**: 337-343 [PMID: 3829877]

13 **Luca A**, García-Pagán JC, Bosch J, Feu F, Caballería J, Groszmann RJ, Rodés J. Effects of ethanol consumption on hepatic hemodynamics in patients with alcoholic cirrhosis. *Gastroenterology* 1997; **112**: 1284-1289 [PMID: 9098014 DOI: S0016508597001789]

14 **Hu LS**, George J, Wang JH. Current concepts on the role of nitric oxide in portal hypertension. *World J Gastroenterol* 2013; **19**: 1707-1717 [PMID: 23555159 DOI: 10.3748/wjg.v19.i11.1707]

15 **Chen SH**, Peng CY, Lai HC, Chang IP, Lee CJ, Su WP, Lin CH, Kao JT, Chuang PH. Head-to-Head Comparison between Collagen Proportionate Area and Acoustic Radiation Force Impulse Elastography in Liver Fibrosis Quantification in Chronic Hepatitis C. *PLoS One* 2015; **10**: e0140554 [PMID: 26461105 DOI: 10.1371/journal.pone.0140554PONE-D-15-32031]

16 **Tsochatzis E**, Bruno S, Isgro G, Hall A, Theocharidou E, Manousou P, Dhillon AP, Burroughs AK, Luong TV. Collagen proportionate area is superior to other histological methods for sub-classifying cirrhosis and determining prognosis. *J Hepatol* 2014; **60**: 948-954 [PMID: 24412606 DOI: 10.1016/j.jhep.2013.12.023]

17 **Manousou P**, Burroughs AK, Tsochatzis E, Isgro G, Hall A, Green A, Calvaruso V, Ma GL, Gale J, Burgess G, O'Beirne J, Patch D, Thorburn D, Leandro G, Dhillon AP. Digital image analysis of collagen assessment of progression of fibrosis in recurrent HCV after liver transplantation. *J Hepatol* 2013; **58**: 962-968 [PMID: 23262247 DOI: 10.1016/j.jhep.2012.12.016]

18 **de Lédinghen V**, Wong VW, Vergniol J, Wong GL, Foucher J, Chu SH, Le Bail B, Choi PC, Chermak F, Yiu KK, Merrouche W, Chan HL. Diagnosis of liver fibrosis and cirrhosis using liver stiffness measurement: comparison between M and XL probe of FibroScan ®. *J Hepatol* 2012; **56**: 833-839 [PMID: 22173167 DOI: 10.1016/j.jhep.2011.10.017S0168-8278(11)00855-5]

19 **Spahr L**, Rubbia-Brandt L, Frossard JL, Giostra E, Rougemont AL, Pugin J, Fischer M, Egger H, Hadengue A. Combination of steroids with infliximab or placebo in severe alcoholic hepatitis: a randomized controlled pilot study. *J Hepatol* 2002; **37**: 448-455 [PMID: 12217597 DOI: S0168827802002301]

20 **Bedossa P**, Poitou C, Veyrie N, Bouillot JL, Basdevant A, Paradis V, Tordjman J, Clement K. Histopathological algorithm and scoring system for evaluation of liver lesions in morbidly obese patients. *Hepatology* 2012; **56**: 1751-1759 [PMID: 22707395 DOI: 10.1002/hep.25889]

21 **Kalambokis G**, Manousou P, Vibhakorn S, Marelli L, Cholongitas E, Senzolo M, Patch D, Burroughs AK. Transjugular liver biopsy--indications, adequacy, quality of specimens, and complications--a systematic review. *J Hepatol* 2007; **47**: 284-294 [PMID: 17561303 DOI: 10.1016/j.jhep.2007.05.001]

22 **Elphick DA**, Dube AK, McFarlane E, Jones J, Gleeson D. Spectrum of liver histology in presumed decompensated alcoholic liver disease. *Am J Gastroenterol* 2007; **102**: 780-788 [PMID: 17222323 DOI: 10.1111/j.1572-0241.2006.01034.x]

23 **Liao WC**, Hou MC, Chang CJ, Lee FY, Lin HC, Lee SD. Potential precipitating factors of esophageal variceal bleeding: a case-control study. *Am J Gastroenterol* 2011; **106**: 96-103 [PMID: 20823836 DOI: 10.1038/ajg.2010.342ajg2010342]

24 **Francque S**, Laleman W, Verbeke L, Van Steenkiste C, Casteleyn C, Kwanten W, Van Dyck C, D'Hondt M, Ramon A, Vermeulen W, De Winter B, Van Marck E, Van Marck V, Pelckmans P, Michielsen P. Increased intrahepatic resistance in severe steatosis: endothelial dysfunction, vasoconstrictor overproduction and altered microvascular architecture. *Lab Invest* 2012; **92**: 1428-1439 [PMID: 22890552 DOI: 10.1038/labinvest.2012.103labinvest2012103]

25 **Bolognesi M**, Verardo A, Di Pascoli M. Peculiar characteristics of portal-hepatic hemodynamics of alcoholic cirrhosis. *World J Gastroenterol* 2014; **20**: 8005-8010 [PMID: 25009370 DOI: 10.3748/wjg.v20.i25.8005]

26 **Kim MY**, Cho MY, Baik SK, Park HJ, Jeon HK, Im CK, Won CS, Kim JW, Kim HS, Kwon SO, Eom MS, Cha SH, Kim YJ, Chang SJ, Lee SS. Histological subclassification of cirrhosis using the Laennec fibrosis scoring system correlates with clinical stage and grade of portal hypertension. *J Hepatol* 2011; **55**: 1004-1009 [PMID: 21354227 DOI: 10.1016/j.jhep.2011.02.012]

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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.

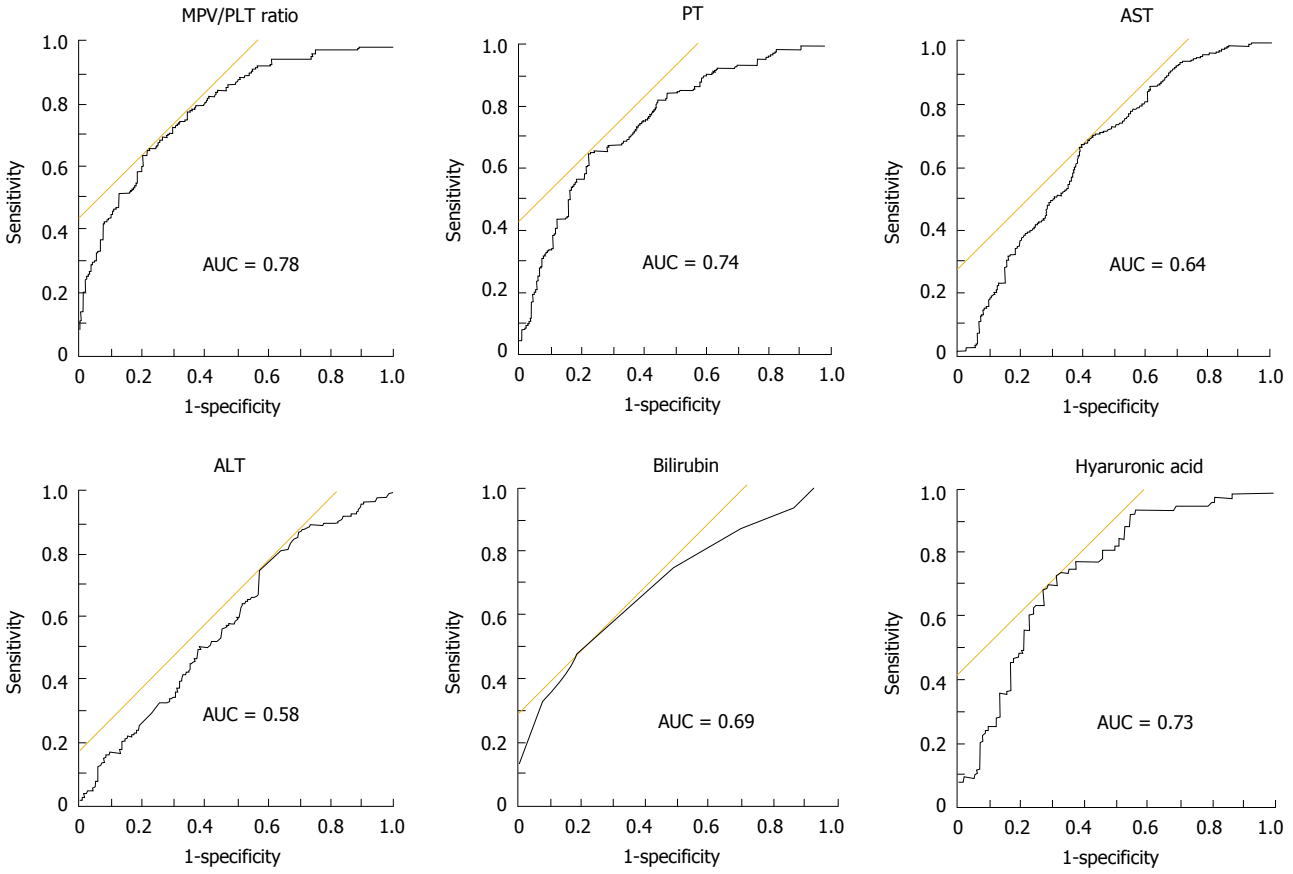

Figure 1 Receiver operating characteristic curve analysis of parameters differing significantly in the liver cirrhosis and non-liver cirrhosis groups on multivariate analysis. The area under the curve of MPV/PLT was the highest. A MPV/PLT ratio of 0.8 had a sensitivity of 65% and a specificity of 78%. 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

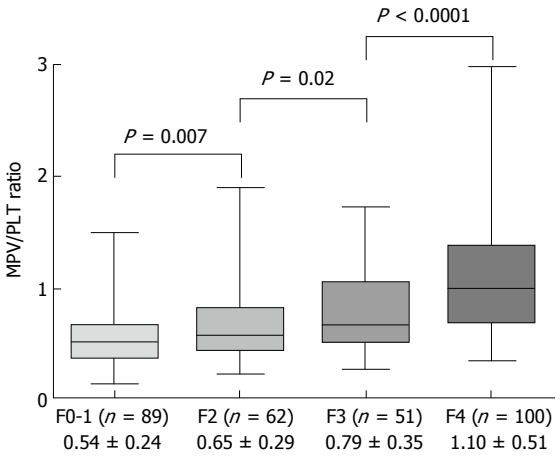

Figure 2 Relationship between MPV/PLT ratio and fibrosis stage. There were significant differences between each pair of stages. MPV/PLT: Mean platelet volume to platelet count.

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.

REFERENCES

1 **Liu S**, Ren J, Han G, Wang G, Gu G, Xia Q, Li J. Mean platelet volume: a controversial marker of disease activity in Crohn's disease. *Eur J Med Res* 2012; **17**: 27 [PMID: 23058104 DOI: 10.1186/2047-783X-17-27]

2 **Lippi G**, Filippozzi L, Salvagno GL, Montagnana M, Franchini M, Guidi GC, Targher G. Increased mean platelet volume in patients with acute coronary syndromes. *Arch Pathol Lab Med* 2009; **133**: 1441-1443 [PMID: 19722752 DOI: 10.1043/1543-2165-133.9.1441]

3 **Karagoz E**, Ulcay A, Tanoglu A, Kara M, Turhan V, Erdem H, Oncul O, Gorenek L. Clinical usefulness of mean platelet volume and red blood cell distribution width to platelet ratio for predicting the severity of hepatic fibrosis in chronic hepatitis B virus patients. *Eur J Gastroenterol Hepatol* 2014; **26**: 1320-1324 [PMID: 25210777 DOI: 10.1097/MEG.0000000000000203]

4 **Qi XT**, Wan F, Lou Y, Ye B, Wu D. The mean platelet volume is a potential biomarker for cirrhosis in chronic hepatitis B virus infected patients. *Hepatogastroenterology* 2014; **61**: 456-459 [PMID: 24901161]

5 **Ekiz F**, Yüksel O, Koçak E, Yılmaz B, Altınbaş A, Çoban S, Yüksel I, Üsküdar O, Köklü S. Mean platelet volume as a fibrosis marker in patients with chronic hepatitis B. *J Clin Lab Anal* 2011; **25**: 162-165 [PMID: 21567462 DOI: 10.1002/jcla.20450]

6 **Purnak T**, Olmez S, Torun S, Efe C, Sayilir A, Ozaslan E, Tenlik I, Kalkan IH, Beyazit Y, Yuksel O. Mean platelet volume is increased in chronic hepatitis C patients with advanced fibrosis. *Clin Res Hepatol Gastroenterol* 2013; **37**: 41-46 [PMID: 22572524 DOI: 10.1016/j.clinre.2012.03.035]

7 **Ichida F,** Tsuji T, Omata M, Ichida T, Inoue K, Kamimura T, Yamada G, Hino K, Yokosuka O, Suzuki H. New Inuyama classification: new criteria for histological assess- ment of chronic hepatitis. Int Hepatol Commun. 1996: 112

8 **Ha-Kawa SK**, Tanaka Y. A quantitative model of technetium-99m-DTPA-galactosyl-HSA for the assessment of hepatic blood flow and hepatic binding receptor. *J Nucl Med* 1991; **32**: 2233-2240 [PMID: 1744708]

9 **Kwon AH**, Matsui Y, Ha-Kawa SK, Kamiyama Y. Functional hepatic volume measured by technetium-99m-galactosyl-human serum albumin liver scintigraphy: comparison between hepatocyte volume and liver volume by computed tomography. *Am J Gastroenterol* 2001; **96**: 541-546 [PMID: 11232703 DOI: 10.1111/j.1572-0241.2001.03556.x]

10 **Edmondson HA**, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer* 1954; **7**: 462-503 [PMID: 13160935]

11 **Castéra L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Lédinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350 [PMID: 15685546]

12 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713 [PMID: 14698338]

13 **Foucher J**, Chanteloup E, Vergniol J, Castéra L, Le Bail B, Adhoute X, Bertet J, Couzigou P, de Lédinghen V. Diagnosis of cirrhosis by transient elastography (FibroScan): a prospective study. *Gut* 2006; **55**: 403-408 [PMID: 16020491 DOI: 10.1136/gut.2005.069153]

14 **Turhan O**, Coban E, Inan D, Yalcin AN. Increased mean platelet volume in chronic hepatitis B patients with inactive disease. *Med Sci Monit* 2010; **16**: CR202-CR205 [PMID: 20357720]

15 **Ceylan B**, Fincanci M, Yardimci C, Eren G, Tözalgan Ü, Müderrisoğlu C, Paşaoğlu E. Can mean platelet volume determine the severity of liver fibrosis or inflammation in patients with chronic hepatitis B? *Eur J Gastroenterol Hepatol* 2013; **25**: 606-612 [PMID: 23325286 DOI: 10.1097/MEG.0b013e32835d08da]

16 **Shin WY**, Jung DH, Shim JY, Lee HR. The association between non-alcoholic hepatic steatosis and mean platelet volume in an obese Korean population. *Platelets* 2011; **22**: 442-446 [PMID: 21751850 DOI: 10.3109/09537104.2010.540049]

17 **Ozhan H**, Aydin M, Yazici M, Yazgan O, Basar C, Gungor A, Onder E. Mean platelet volume in patients with non-alcoholic fatty liver disease. *Platelets* 2010; **21**: 29-32 [PMID: 19947902 DOI: 10.3109/09537100903391023]

18 **Kilciler G**, Genc H, Tapan S, Ors F, Kara M, Karadurmus N, Ercin CN, Karslioglu Y, Kilic S, Bagci S, Erbil MK, Dogru T. Mean platelet volume and its relationship with carotid atherosclerosis in subjects with non-alcoholic fatty liver disease. *Ups J Med Sci* 2010; **115**: 253-259 [PMID: 20731535 DOI: 10.3109/03009734.2010.500062]

19 **Arslan N**, Makay B. Mean platelet volume in obese adolescents with nonalcoholic fatty liver disease. *J Pediatr Endocrinol Metab* 2010; **23**: 807-813 [PMID: 21073123]

20 **Celikbilek M**, Gürsoy S, Deniz K, Karaman A, Zararsiz G, Yurci A. Mean platelet volume in biopsy-proven non-alcoholic fatty liver disease. *Platelets* 2013; **24**: 194-199 [PMID: 22646469 DOI: 10.3109/09537104.2012.688898]

21 **Inagaki N**, Kibata K, Tamaki T, Shimizu T, Nomura S. Prognostic impact of the mean platelet volume/platelet count ratio in terms of survival in advanced non-small cell lung cancer. *Lung Cancer* 2014; **83**: 97-101 [PMID: 24189108 DOI: 10.1016/j.lungcan.2013.08.020]

22 **Cho SY**, Yang JJ, You E, Kim BH, Shim J, Lee HJ, Lee WI, Suh JT, Park TS. Mean platelet volume/platelet count ratio in hepatocellular carcinoma. *Platelets* 2013; **24**: 375-377 [PMID: 22835043 DOI: 10.3109/09537104.2012.701028]

23 **Kurt M**, Onal IK, Sayilir AY, Beyazit Y, Oztas E, Kekilli M, Turhan N, Karaman K, Akdogan M. The role of mean platelet volume in the diagnosis of hepatocellular carcinoma in patients with chronic liver disease. *Hepatogastroenterology* 2012; **59**: 1580-1582 [PMID: 22683976 DOI: 10.5754/hge10444]

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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.

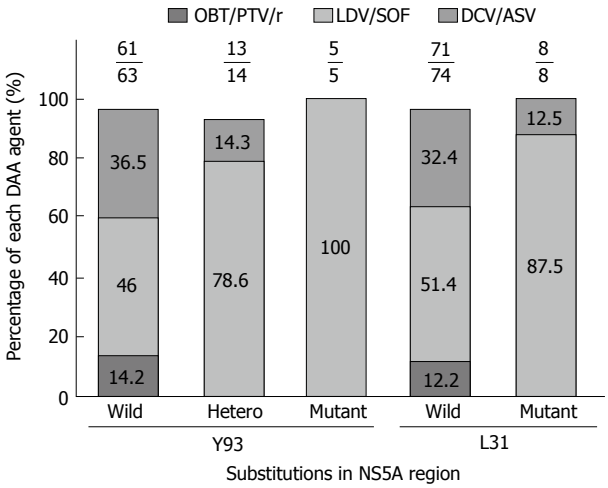

Figure 1 SVR rates for NS5A resistance-associated substitutions and each interferon-free agent. The number above each column is the number of cases with SVR (numerator) and total cases (denominator). Two relapsed patients with wild-type Y93, 1 with Y93 hetero and 3 relapsed patients with wild-type L31 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.

REFERENCES

1 **Ray Kim W.** Global epidemiology and burden of hepatitis C. *Microbes Infect* 2002; **4**: 1219-1225 [PMID: 12467763]

2 **Lavanchy D.** Evolving epidemiology of hepatitis C virus. *Clin Microbiol Infect* 2011; **17**: 107-115 [PMID: 21091831 DOI: 10.1111/j.1469-0691.2010.03432.x]

3 **Zhu RX,** Seto WK, Lai CL, Yuen MF. Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region. *Gut Liver* 2016; **10**: 332-339 [PMID: 27114433 DOI: 10.5009/gnl15257]

4 **Nishiguchi S,** Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, Shiomi S, Seki S, Kobayashi K, Otani S. Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995; **346**: 1051-1055 [PMID: 7564784]

5 **Tanaka H,** Imai Y, Hiramatsu N, Ito Y, Imanaka K, Oshita M, Hijioka T, Katayama K, Yabuuchi I, Yoshihara H, Inoue A, Kato M, Takehara T, Tamura S, Kasahara A, Hayashi N, Tsukuma H. Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. *Ann Intern Med* 2008; **148**: 820-826 [PMID: 18519928]

6 **Umemura T,** Ichijo T, Yoshizawa K, Tanaka E, Kiyosawa K. Epidemiology of hepatocellular carcinoma in Japan. *J Gastroenterol* 2009; **44** Suppl 19: 102-107 [PMID: 19148802 DOI: 10.1007/s00535-008-2251-0]

7 **Goh GB,** Chang PE, Tan CK. Changing epidemiology of hepatocellular carcinoma in Asia. *Best Pract Res Clin Gastroenterol* 2015; **29**: 919-928 [PMID: 26651253 DOI: 10.1016/j.bpg.2015.09.007]

8 **Kumada H,** Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. *J Hepatol* 2012; **56**: 78-84 [PMID: 21827730 DOI: 10.1016/j.jhep.2011.07.016]

9 **Hayashi N,** Izumi N, Kumada H, Okanoue T, Tsubouchi H, Yatsuhashi H, Kato M, Ki R, Komada Y, Seto C, Goto S. Simeprevir with peginterferon/ribavirin for treatment-naive hepatitis C genotype 1 patients in Japan: CONCERTO-1, a phase III trial. *J Hepatol* 2014; **61**: 219-227 [PMID: 24727123 DOI: 10.1016/j.jhep.2014.04.004]

10 **Izumi N,** Hayashi N, Kumada H, Okanoue T, Tsubouchi H, Yatsuhashi H, Kato M, Ki R, Komada Y, Seto C, Goto S. Once-daily simeprevir with peginterferon and ribavirin for treatment-experienced HCV genotype 1-infected patients in Japan: the CONCERTO-2 and CONCERTO-3 studies. *J Gastroenterol* 2014; **49**: 941-953 [PMID: 24626851 DOI: 10.1007/s00535-014-0949-8]

11 **Pawlotsky JM.** NS5A inhibitors in the treatment of hepatitis C. *J Hepatol* 2013; **59**: 375-382 [PMID: 23567084 DOI: 10.1016/j.jhep.2013.03.030]

12 **Mizokami M,** Yokosuka O, Takehara T, Sakamoto N, Korenaga M, Mochizuki H, Nakane K, Enomoto H, Ikeda F, Yanase M, Toyoda H, Genda T, Umemura T, Yatsuhashi H, Ide T, Toda N, Nirei K, Ueno Y, Nishigaki Y, Betular J, Gao B, Ishizaki A, Omote M, Mo H, Garrison K, Pang PS, Knox SJ, Symonds WT, McHutchison JG, Izumi N, Omata M. Ledipasvir and sofosbuvir fixed-dose combination with and without ribavirin for 12 weeks in treatment-naive and previously treated Japanese patients with genotype 1 hepatitis C: an open-label, randomised, phase 3 trial. *Lancet Infect Dis* 2015; **15**: 645-653 [PMID: 25863559 DOI: 10.1016/s1473-3099(15)70099-x]

13 **Suzuki Y,** Ikeda K, Suzuki F, Toyota J, Karino Y, Chayama K, Kawakami Y, Ishikawa H, Watanabe H, Hu W, Eley T, McPhee F, Hughes E, Kumada H. Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infection and limited treatment options. *J Hepatol* 2013; **58**: 655-662 [PMID: 23183526 DOI: 10.1016/j.jhep.2012.09.037]

14 **Izumi N.** Diagnostic and treatment algorithm of the Japanese society of hepatology: a consensus-based practice guideline. *Oncology* 2010; **78** Suppl 1: 78-86 [PMID: 20616588 DOI: 10.1159/000315234]

15 **Itakura J,** Kurosaki M, Takada H, Nakakuki N, Matsuda S, Gondou K, Asano Y, Hattori N, Itakura Y, Tamaki N, Yasui Y, Suzuki S, Hosokawa T, Tsuchiya K, Nakanishi H, Takahashi Y, Maekawa S, Enomoto N, Izumi N. Naturally occurring,

resistance-associated hepatitis C virus NS5A variants are linked to interleukin-28B genotype and are sensitive to interferon-based therapy. *Hepatol Res* 2015; **45**: E115-E121 [PMID: 25564756 DOI: 10.1111/hepr.12474]

16 **Kinugasa H,** Ikeda F, Takaguchi K, Mori C, Matsubara T, Shiraha H, Takaki A, Iwasaki Y, Toyooka S, Yamamoto K. Low frequency of drug-resistant virus did not affect the therapeutic efficacy in daclatasvir plus asunaprevir therapy in patients with chronic HCV genotype-1 infection. *Antivir Ther* 2016; **21**: 37-44 [PMID: 26115551 DOI: 10.3851/imp2976]

17 **Shiffman ML,** Reddy KR, Smith C, Marinos G, Goncales FL, Jr., Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982 [PMID: 12324553 DOI: 10.1056/NEJMoa020047]

18 **Hadziyannis SJ,** Sette H, Jr., Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Jr., Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355 [PMID: 14996676]

19 **Manns MP,** McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965 [PMID: 11583749]

20 **Asselah T,** Marcellin P. New direct-acting antivirals' combination for the treatment of chronic hepatitis C. *Liver Int* 2011; **31** Suppl 1: 68-77 [PMID: 21205141 DOI: 10.1111/j.1478-3231.2010.02411.x]

21 **Kumada H,** Suzuki Y, Ikeda K, Toyota J, Karino Y, Chayama K, Kawakami Y, Ido A, Yamamoto K, Takaguchi K, Izumi N, Koike K, Takehara T, Kawada N, Sata M, Miyagoshi H, Eley T, McPhee F, Damokosh A, Ishikawa H, Hughes E. Daclatasvir plus asunaprevir for chronic HCV genotype 1b infection. *Hepatology* 2014; **59**: 2083-2091 [PMID: 24604476 DOI: 10.1002/hep.27113]

22 **Kumada H,** Chayama K, Rodrigues L, Jr., Suzuki F, Ikeda K, Toyoda H, Sato K, Karino Y, Matsuzaki Y, Kioka K, Setze C, Pilot-Matias T, Patwardhan M, Vilchez RA, Burroughs M, Redman R. Randomized phase 3 trial of ombitasvir/paritaprevir/ritonavir for hepatitis C virus genotype 1b-infected Japanese patients with or without cirrhosis. *Hepatology* 2015; **62**: 1037-1046 [PMID: 26147154 DOI: 10.1002/hep.27972]

23 **Omata M,** Nishiguchi S, Ueno Y, Mochizuki H, Izumi N, Ikeda F, Toyoda H, Yokosuka O, Nirei K, Genda T, Umemura T, Takehara T, Sakamoto N, Nishigaki Y, Nakane K, Toda N, Ide T, Yanase M, Hino K, Gao B, Garrison KL, Dvory-Sobol H, Ishizaki A, Omote M, Brainard D, Knox S, Symonds WT, McHutchison JG, Yatsuhashi H, Mizokami M. Sofosbuvir plus ribavirin in Japanese patients with chronic genotype 2 HCV infection: an open-label, phase 3 trial. *J Viral Hepat* 2014; **21**: 762-768 [PMID: 25196837 DOI: 10.1111/jvh.12312]

24 **Shafran SD,** Shaw D, Charafeddine M, Agarwal K, Foster GR, Abunimeh M, Pilot-Matias T, Pothacamury RK, Fu B, Cohen E, Cohen DE, Gane E. Efficacy and safety results of patients with HCV genotype 2 or 3 infection treated with ombitasvir/paritaprevir/ritonavir and sofosbuvir with or without ribavirin (QUARTZ II-III). *J Viral Hepat* 2017 [PMID: 28833938 DOI: 10.1111/jvh.12782]

25 **Schnell G,** Tripathi R, Krishnan P, Beyer J, Reisch T, Irvin M, Dekhtyar T, Setze C, Rodrigues-Jr L, Alves K, Burroughs M, Redman R, Chayama K, Kumada H, Collins C, Pilot-Matias T. Resistance characterization of hepatitis C virus genotype 2 from Japanese patients treated with ombitasvir and paritaprevir/ritonavir. *J Med Virol* 2018; **90**: 109-119 [PMID: 28842997 DOI: 10.1002/jmv.24923]

26 **Mizokami M,** Dvory-Sobol H, Izumi N, Nishiguchi S, Doehle B, Svarovskaia ES, De-Oertel S, Knox S, Brainard DM, Miller MD, Mo H, Sakamoto N, Takehara T, Omata M. Resistance Analyses of Japanese Hepatitis C-Infected Patients Receiving Sofosbuvir or Ledipasvir/Sofosbuvir Containing Regimens in Phase 3 Studies. *J Viral Hepat* 2016; **23**: 780-788 [PMID: 27196675 DOI: 10.1111/jvh.12549]

27 **Backus LI,** Belperio PS, Shahoumian TA, Loomis TP, Mole LA. Real-world effectiveness of ledipasvir/sofosbuvir in 4,365 treatment-naive, genotype 1 hepatitis C-infected patients. *Hepatology* 2016; **64**: 405-414 [PMID: 27115523 DOI: 10.1002/hep.28625]

28 **Giliead.** Post-marketing surveillance of ledipasvir/sofosbuvir 2015-2016. Available from: URL: https://www.harvoni.jp/~/media/files/gilead/harvoni/proper/hvn_post_marketing_surveillance_final_report.pdf?la=ja-jp

29 **Reig M,** Marino Z, Perello C, Inarrairaegui M, Ribeiro A, Lens S, Diaz A, Vilana R, Darnell A, Varela M, Sangro B, Calleja JL, Forns X, Bruix J. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. *J Hepatol* 2016; **65**: 719-726 [PMID: 27084592 DOI: 10.1016/j.jhep.2016.04.008]

30 **Akuta N,** Sezaki H, Suzuki F, Fujiyama S, Kawamura Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Suzuki Y, Arase Y, Ikeda K, Kumada H. Ledipasvir plus sofosbuvir as salvage therapy for HCV genotype 1 failures to prior NS5A inhibitors regimens. *J Med Virol* 2017; **89**: 1248-1254 [PMID: 28079269 DOI: 10.1002/jmv.24767]

31 **Iio E,** Shimada N, Takaguchi K, Senoh T, Eguchi Y, Atsukawa M, Tsubota A, Abe H, Kato K, Kusakabe A, Miyaki T, Matsuura K, Matsunami K, Shinkai N, Fujiwara K, Nojiri S, Tanaka Y. Clinical evaluation of sofosbuvir/ledipasvir in patients with chronic hepatitis C genotype 1 with and without prior daclatasvir/asunaprevir therapy. *Hepatol Res* 2017 [PMID: 28332272 DOI: 10.1111/hepr.12898]

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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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Informed consent statement: Informed consent was obtained from all patients for being included in the study.

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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-

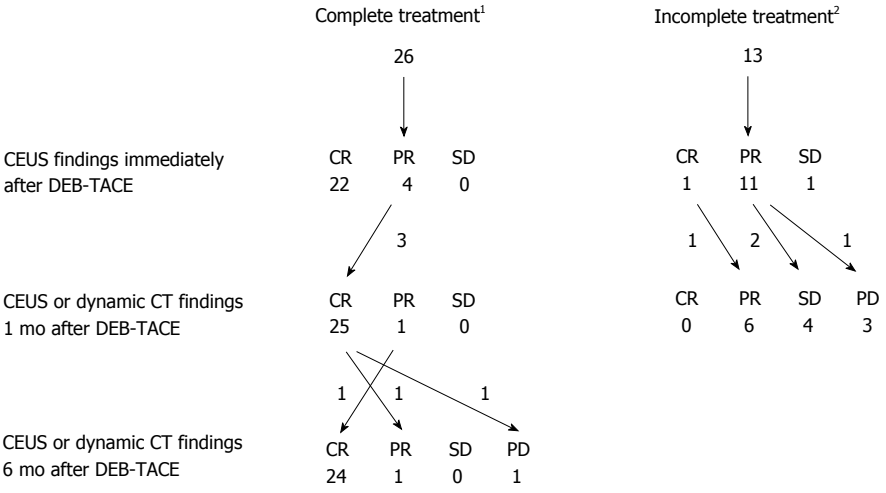

Figure 1 Patient flow diagram. In this diagram, treatment response evaluated by contrast-enhanced ultrasonography (CEUS) and/or dynamic computed tomography (CT) immediately, one month and six months after DEB-TACE for hepatocellular carcinoma (HCC) using mRECIST. Findings on CEUS or dynamic CT were evaluated 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

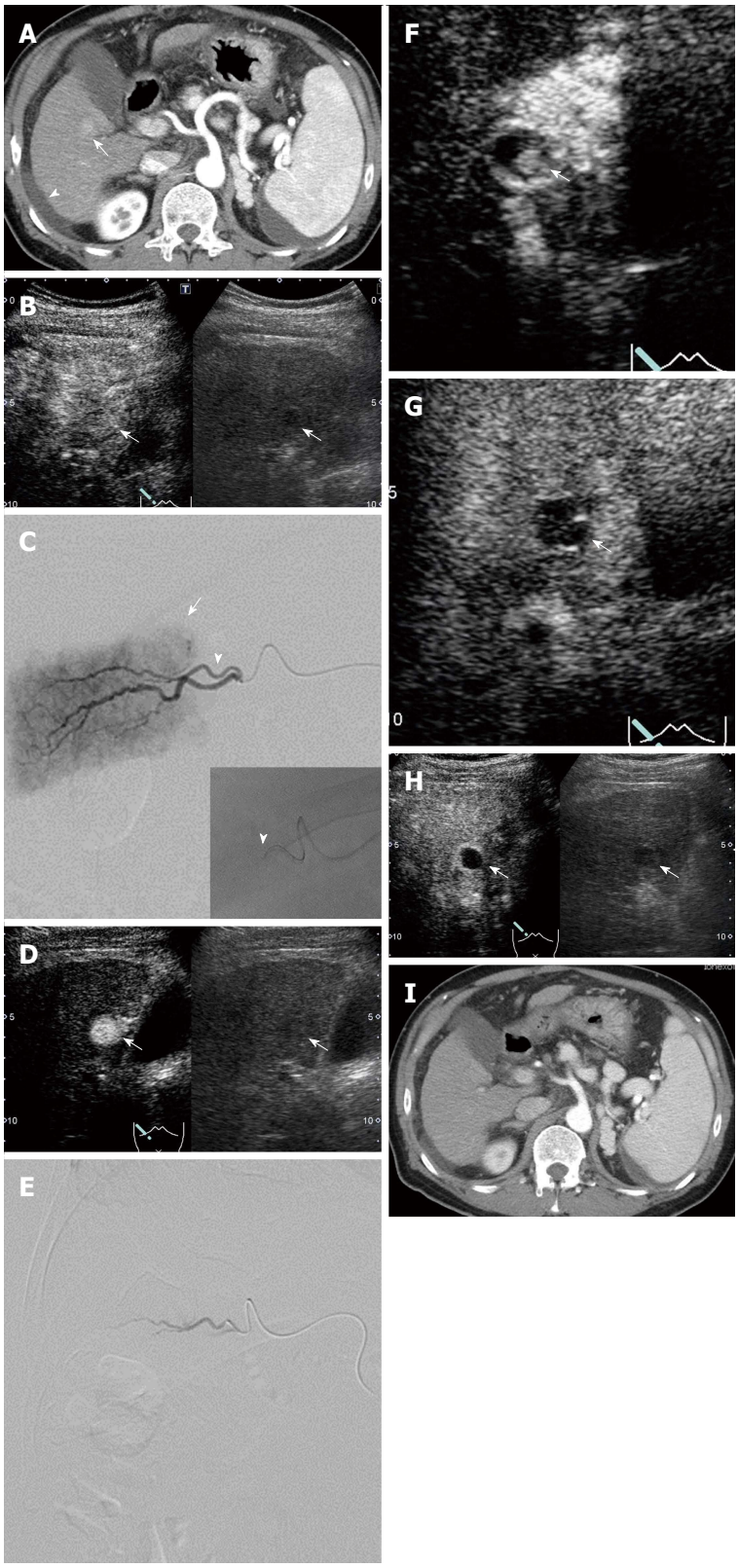

Figure 2 The patient was a 59-year-old male with alcoholic liver cirrhosis. DEB-TACE and transcatheter CEUS (IAUS) using Sonazoid for HCC in S5 with a diameter of 15 mm were performed. A: Dynamic CT in the arterial phase before DEB-TACE showed a hypervascular lesion in S5 (arrow) and ascites (arrow head); 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.

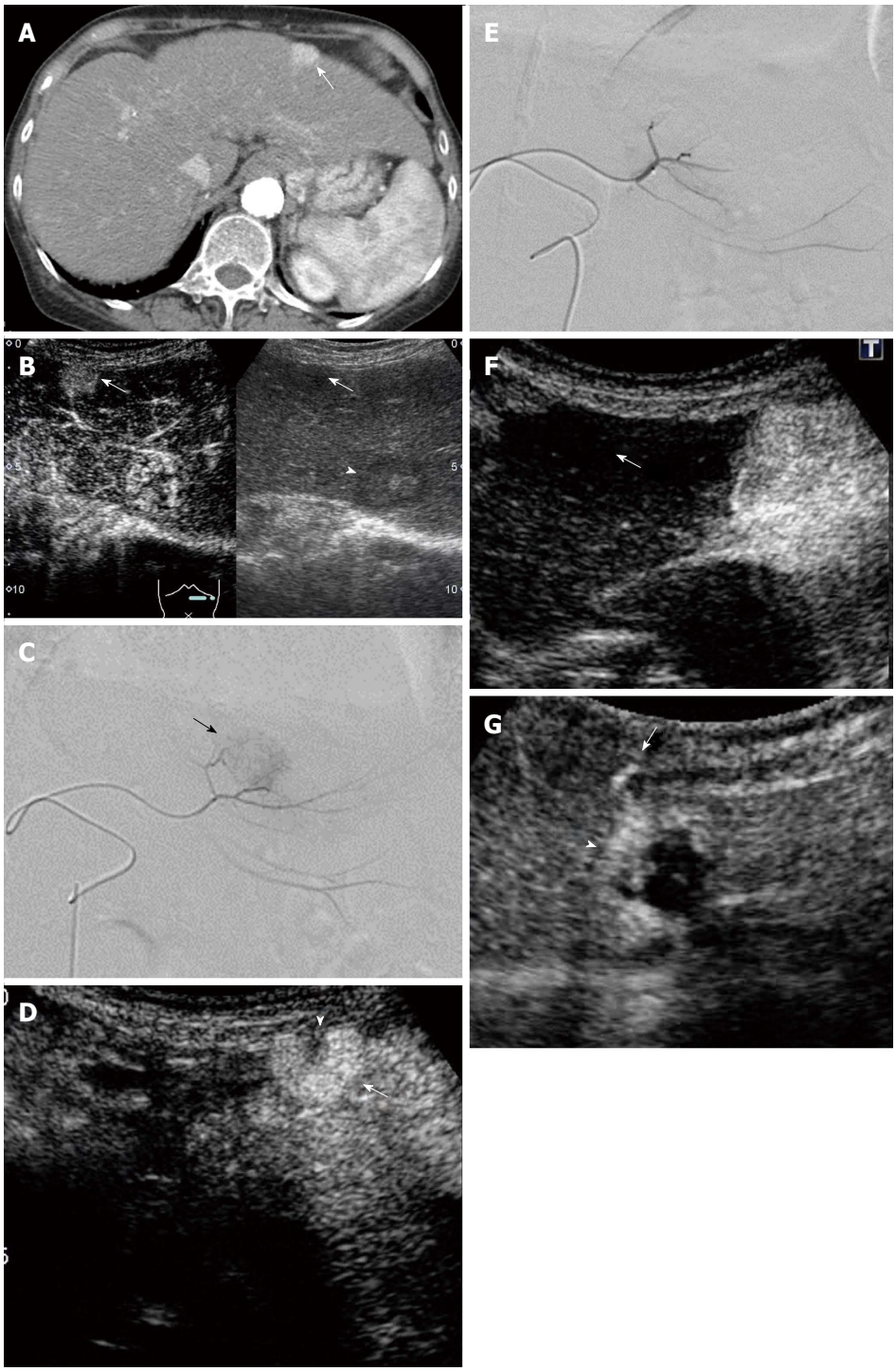

Figure 3 The patient was a 76-year-old female with hepatitis C virus cirrhosis. DEB-TACE and IAUS for HCC in S3 with a diameter of 17 mm were performed. A: Dynamic CT in the arterial phase before DEB-TACE showed a hypervascular lesion in S3 (arrow); B: CEUS in the arterial phase before DEB-TACE showed 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

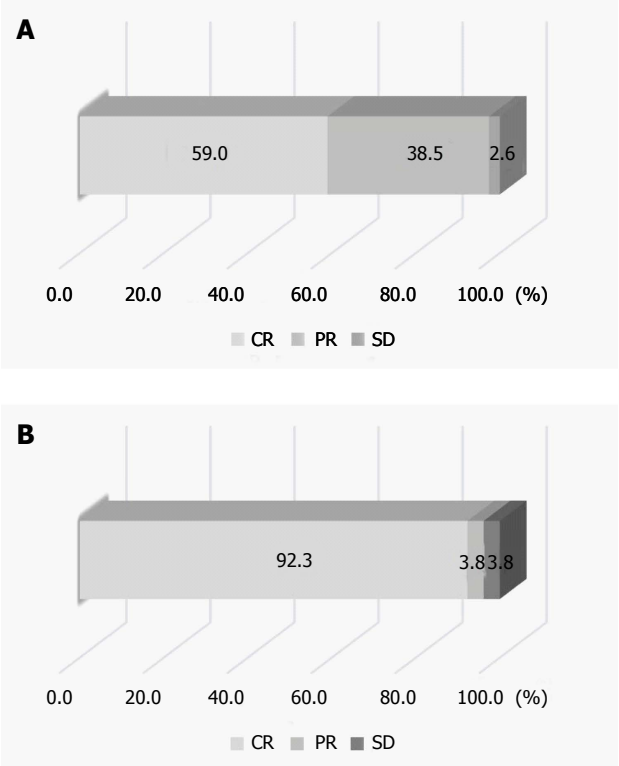

Figure 4 Treatment response. A: The overall complete response (CR) rate of all thirty-nine lesions at one month after treatment; B: The overall CR rate of twenty-six lesions treated completely at six months after treatment. PR: Partial response; SD: Stable disease; PD: Progressive disease.

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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REFERENCES

1 **Bruix J**, Sala M, Llovet JM. Chemoembolization for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S179-S188 [PMID: 15508083]

2 **Llovet JM**, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; **37**: 429-442 [PMID: 12540794 DOI: 10.1053/jhep.2003.50047]

3 **Lewis AL**, Gonzalez MV, Leppard SW, Brown JE, Stratford PW, Phillips GJ, Lloyd AW. Doxorubicin eluting beads - 1: effects of drug loading on bead characteristics and drug distribution. *J Mater Sci Mater Med* 2007; **18**: 1691-1699 [PMID: 17483878 DOI: 10.1007/s10856-007-3068-8]

4 **Maluccio M**, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin* 2012; **62**: 394-399 [PMID: 23070690 DOI: 10.3322/caac.21161]

5 **Cazejust J**, Bessoud B, Colignon N, Garcia-Alba C, Planché O, Menu Y. Hepatocellular carcinoma vascularization: from the most common to the lesser known arteries. *Diagn Interv Imaging* 2014; **95**: 27-36 [PMID: 23978434 DOI: 10.1016/j.diii.2013.04.015]

6 **Hong K**, Khwaja A, Liapi E, Torbenson MS, Georgiades CS, Geschwind JF. New intra-arterial drug delivery system for the treatment of liver cancer: preclinical assessment in a rabbit model of liver cancer. *Clin Cancer Res* 2006; **12**: 2563-2567 [PMID: 16638866 DOI: 10.1158/1078-0432.CCR-05-2225]

7 **Lencioni R**, de Baere T, Burrel M, Caridi JG, Lammer J, Malagari K, Martin RC, O'Grady E, Real MI, Vogl TJ, Watkinson A, Geschwind JF. Transcatheter treatment of hepatocellular carcinoma with Doxorubicin-loaded DC Bead (DEBDOX): technical recommendations. *Cardiovasc Intervent Radiol* 2012; **35**: 980-985 [PMID: 22009576 DOI: 10.1007/s00270-011-0287-7]

8 **Kakeda S**, Korogi Y, Ohnari N, Moriya J, Oda N, Nishino K, Miyamoto W. Usefulness of cone-beam volume CT with flat panel detectors in conjunction with catheter angiography for transcatheter arterial embolization. *J Vasc Interv Radiol* 2007; **18**: 1508-1516 [PMID: 18057285 DOI: 10.1016/j.jvir.2007.08.003]

9 **Claudon M**, Dietrich CF, Choi BI, Cosgrove DO, Kudo M, Nolsøe CP, Piscaglia F, Wilson SR, Barr RG, Chammas MC, Chaubal NG, Chen MH, Clevert DA, Correas JM, Ding H, Forsberg F, Fowlkes JB, Gibson RN, Goldberg BB, Lassau N, Leen EL, Mattrey RF, Moriyasu F, Solbiati L, Weskott HP, Xu HX; World Federation for Ultrasound in Medicine; European Federation of Societies for Ultrasound. Guidelines and good clinical practice recommendations for Contrast Enhanced Ultrasound (CEUS) in the liver - update 2012: A WFUMB-EFSUMB initiative in cooperation with representatives of AFSUMB, AIUM, ASUM, FLAUS and ICUS. *Ultrasound Med Biol* 2013; **39**: 187-210 [PMID: 23137926 DOI: 10.1016/j.ultrasmedbio.2012.09.002]

10 **Shiozawa K**, Watanabe M, Kikuchi Y, Kudo T, Maruyama K, Sumino Y. Evaluation of sorafenib for hepatocellular carcinoma by contrast-enhanced ultrasonography: a pilot study. *World J Gastroenterol* 2012; **18**: 5753-5758 [PMID: 23155317 DOI: 10.3748/wjg.v18.i40.5753]

11 **Shiozawa K**, Watanabe M, Ikehara T, Kobayashi K, Ochi Y, Suzuki Y, Fuchinoue K, Yoneda M, Kenmochi T, Okubo Y, Mori T, Makino H, Tsukamoto N, Igarashi Y, Sumino Y. Evaluation of contrast-enhanced ultrasonography for hepatocellular carcinoma prior to and following stereotactic body radiation therapy using the CyberKnife® system: A preliminary report. *Oncol Lett* 2016; **11**: 208-212 [PMID: 26870190 DOI: 10.3892/ol.2015.3874]

12 **Schacherer D**, Girlich C, Zorger N, Wiest R, Schoelmerich J, Feuerbach S, Jung EM. Sono-hepatic-arteriography (Sono-HA) in the assessment of hepatocellular carcinoma in patients undergoing transcatheter arterial chemoembolization (TACE). *Ultraschall Med* 2010; **31**: 270-275 [PMID: 20408118 DOI: 10.1055/s-0029-1245242]

13 **Uller W**, Wiggermann P, Gössmann H, Klebl F, Salzberger B, Stroszczynski C, Jung EM. Evaluation of the microcirculation of hepatocellular carcinomas using contrast-enhanced ultrasound with intraarterial and intravenous contrast application during transarterial chemoembolization with drug-eluting beads (DEB-TACE): preliminary data. *Clin Hemorheol Microcirc* 2011; **49**: 55-66 [PMID: 22214678 DOI: 10.3233/CH-2011-1457]

14 **Moschouris H**, Malagari K, Kalokairinou M, Stamatiou K, Marinis A, Papadaki MG. Contrast-enhanced ultrasonography with intraarterial administration of SonoVue for guidance of transarterial chemoembolization: an initial experience. *Med Ultrason* 2011; **13**: 296-301 [PMID: 22132402]

15 **Burgmans MC**, van Erkel AR, Too CW, Coenraad M, Lo RH, Tan BS. Pilot study evaluating catheter-directed contrast-enhanced ultrasound compared to catheter-directed computed tomography arteriography as adjuncts to digital subtraction angiography to guide transarterial chemoembolization. *Clin Radiol* 2014; **69**: 1056-1061 [PMID: 25017449 DOI: 10.1016/j.crad.2014.06.001]

16 **Lekht I**, Nayyar M, Luu B, Guichet PL, Ho J, Ter-Oganesyan R, Katz M, Gulati M. Intra-arterial contrast-enhanced ultrasound (IA CEUS) for localization of hepatocellular carcinoma (HCC) supply during transarterial chemoembolization (TACE): a case series. *Abdom Radiol* (NY) 2017; **42**: 1400-1407 [PMID: 28008454 DOI: 10.1007/s00261-016-1016-0]

17 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

18 **Kokudo N**, Hasegawa K, Akahane M, Igaki H, Izumi N, Ichida T, Uemoto S, Kaneko S, Kawasaki S, Ku Y, Kudo M, Kubo S, Takayama T, Tateishi R, Fukuda T, Matsui O, Matsuyama Y, Murakami T, Arii S, Okazaki M, Makuuchi M. Evidence-based Clinical Practice Guidelines for Hepatocellular Carcinoma: The Japan Society of Hepatology 2013 update (3rd JSH-HCC Guidelines). *Hepatol Res* 2015; **45**: 123-127 [PMID: 25625806 DOI: 10.1111/hepr.12464]

19 **Oken MM**, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, Carbone PP. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982; **5**: 649-655 [PMID: 7165009]

20 **Kudo M**, Hatanaka K, Maekawa K. Newly developed novel

ultrasound technique, defect reperfusion ultrasound imaging, using sonazoid in the management of hepatocellular carcinoma. *Oncology* 2010; **78** Suppl 1: 40-45 [PMID: 20616583 DOI: 10.1159/000315229]

21 **Lencioni R**, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis* 2010; **30**: 52-60 [PMID: 20175033 DOI: 10.1055/s-0030-1247132]

22 **Zhong X**, Lim EA, Hershman DL, Moinpour CM, Unger J, Lee SM. Identifying Severe Adverse Event Clusters Using the National Cancer Institute's Common Terminology Criteria for Adverse Events. *J Oncol Pract* 2016; **12**: e270-e280, 245-246 [PMID: 26907453 DOI: 10.1200/JOP.2015.006106]

23 **Zorger N**, Jung EM, Schreyer AG, Heiss P, Mueller-Wille R, Wiest R, Feuerbach S, Rennert J. Ultrasound-arterioportography (US-AP): A new technical approach to perform detection of liver lesions. *Clin Hemorheol Microcirc* 2010; **46**: 117-126 [PMID: 21135487 DOI: 10.3233/CH-2010-1338]

24 **Varela M**, Real MI, Burrel M, Forner A, Sala M, Brunet M, Ayuso C, Castells L, Montañá X, Llovet JM, Bruix J. Chemoembolization of hepatocellular carcinoma with drug eluting beads: efficacy and doxorubicin pharmacokinetics. *J Hepatol* 2007; **46**: 474-481 [PMID: 17239480 DOI: 10.1016/j.jhep.2006.10.020]

25 **Poon RT**, Tso WK, Pang RW, Ng KK, Woo R, Tai KS, Fan ST. A phase I/II trial of chemoembolization for hepatocellular carcinoma using a novel intra-arterial drug-eluting bead. *Clin Gastroenterol Hepatol* 2007; **5**: 1100-1108 [PMID: 17627902 DOI: 10.1016/j.cgh.2007.04.021]

26 **Malagari K**, Chatzimichael K, Alexopoulou E, Kelekis A, Hall B, Dourakis S, Delis S, Gouliamos A, Kelekis D. Transarterial chemoembolization of unresectable hepatocellular carcinoma with drug eluting beads: results of an open-label study of 62 patients. *Cardiovasc Intervent Radiol* 2008; **31**: 269-280 [PMID: 17999110 DOI: 10.1007/s00270-007-9226-z]

27 **Golfieri R**, Giampalma E, Renzulli M, Cioni R, Bargellini I, Bartolozzi C, Breatta AD, Gandini G, Nani R, Gasparini D, Cucchetti A, Bolondi L, Trevisani F; PRECISION ITALIA STUDY GROUP. Randomised controlled trial of doxorubicin-eluting beads vs conventional chemoembolisation for hepatocellular carcinoma. *Br J Cancer* 2014; **111**: 255-264 [PMID: 24937669 DOI: 10.1038/bjc.2014.199]

28 **Manini MA**, Sangiovanni A, Martinetti L, Viganò D, La Mura V, Aghemo A, Iavarone M, Crespi S, Nicolini A, Colombo M. Transarterial chemoembolization with drug-eluting beads is effective for the maintenance of the Milan-in status in patients with a small hepatocellular carcinoma. *Liver Transpl* 2015; **21**: 1259-1269 [PMID: 26074360 DOI: 10.1002/lt.24196]

29 **Gunsar F**. Liver Transplantation for Hepatocellular Carcinoma Beyond the Milan Criteria. *Exp Clin Transplant* 2017; **15**: 59-64 [PMID: 28302001 DOI: 10.6002/ect.TOND16.L16]

30 **Kakeda S**, Korogi Y, Hatakeyama Y, Ohnari N, Oda N, Nishino K, Miyamoto W. The usefulness of three-dimensional angiography with a flat panel detector of direct conversion type in a transcatheter arterial chemoembolization procedure for hepatocellular carcinoma: initial experience. *Cardiovasc Intervent Radiol* 2008; **31**: 281-288 [PMID: 18026792 DOI: 10.1007/s00270-007-9114-6]

31 **Watanabe R**, Matsumura M, Munemasa T, Fujimaki M, Suematsu M. Mechanism of hepatic parenchyma-specific contrast of microbubble-based contrast agent for ultrasonography: microscopic studies in rat liver. *Invest Radiol* 2007; **42**: 643-651 [PMID: 17700280 DOI: 10.1097/RLI.0b013e31805f2682]

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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

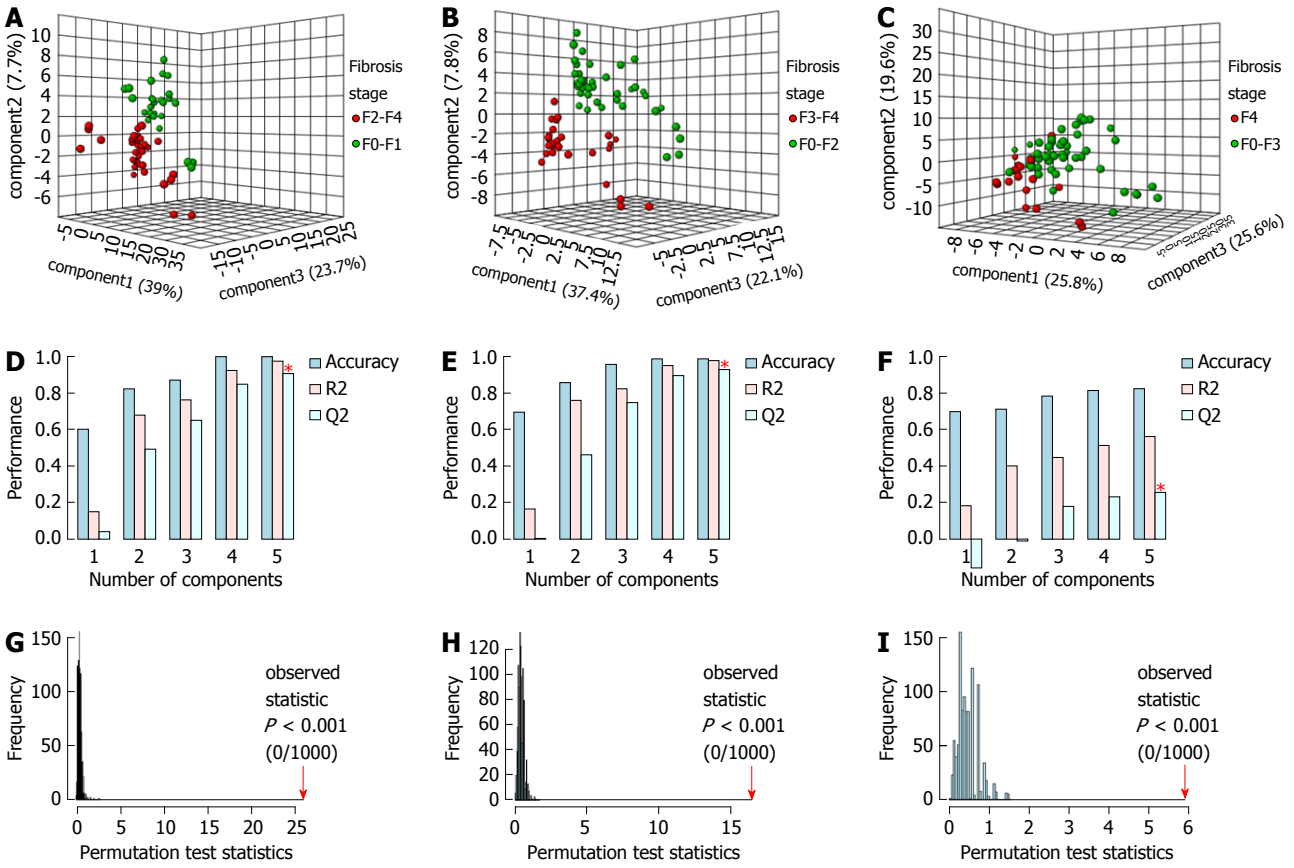

Figure 1 Partial least squares discriminant analysis metabonomic models to predict significant fibrosis (A, D, G) advanced fibrosis (B, E, H) and cirrhosis (C, F, I) in 69 chronic hepatitis C patients from Pernambuco/Brazil. Three-dimensional score plot (A, B, C). Classification of MM using different 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

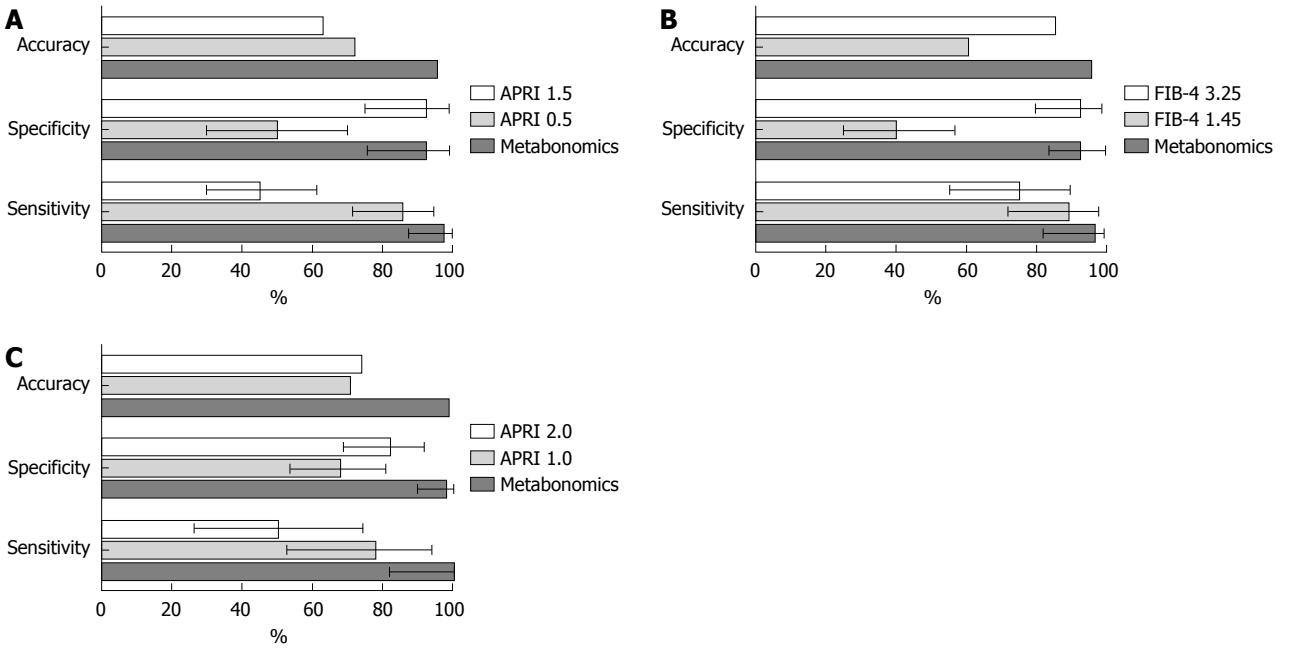

Figure 2 Comparison of performance of the linear discriminant analysis metabonomic models, aspartate aminotransferase to platelet ratio index and fibrosis index based on four factors, in 69 chronic hepatitis C patients from Pernambuco/Brazil. A and C: Performance of metabonomic models (MM) and APRI to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

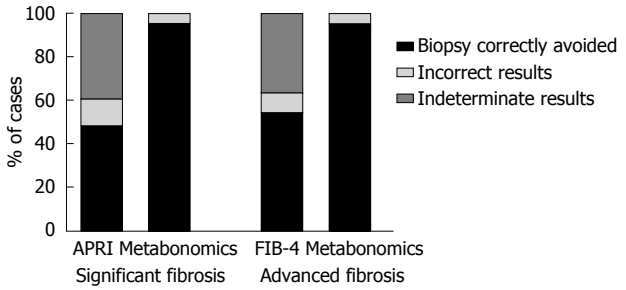

Figure 3 Comparison of biopsy correctly avoided by metabonomic models, aspartate aminotransferase to platelet ratio index and fibrosis index based on four factors, in 69 chronic hepatitis C patients from Pernambuco/Brazil. APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis index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

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REFERENCES

1 **Global Burden of Hepatitis C Working Group**. Global burden of disease (GBD) for hepatitis C. *J Clin Pharmacol* 2004; **44**: 20-29 [PMID: 14681338 DOI: 10.1177/0091270003258669]

2 **Lozano R**, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans

V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De León FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2095-2128 [PMID: 23245604 DOI: 10.1016/S0140-6736(12)61728-0]

3 **Brasil**. Ministério da Saúde. Boletim Epidemiológico-Hepatites Virais. 2015; 1-29. Avaiable from: URL: http://www.aids.gov.br/es/node/90

4 **Ferreira PR**, Brandão-Mello CE, Estes C, Gonçales Júnior FL, Coelho HS, Razavi H, Cheinquer H, Wolff FH, Ferraz ML, Pessoa MG, Mendes-Correa MC. Disease burden of chronic hepatitis C in Brazil. *Braz J Infect Dis* 2015; **19**: 363-368 [PMID: 26051505 DOI: 10.1016/j.bjid.2015.04.004]

5 **Rockey DC**, Caldwell SH, Goodman ZD, Nelson RC, Smith AD; American Association for the Study of Liver Diseases. Liver biopsy. *Hepatology* 2009; **49**: 1017-1044 [PMID: 19243014 DOI: 10.1002/hep.22742]

6 **Regev A**, Berho M, Jeffers LJ, Milikowski C, Molina EG, Pyrsopoulos NT, Feng ZZ, Reddy KR, Schiff ER. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am J Gastroenterol* 2002; **97**: 2614-2618 [PMID: 12385448 DOI: 10.1111/j.1572-0241.2002.06038.x]

7 **Wai CT**, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 518-526 [PMID: 12883497 DOI: 10.1053/jhep.2003.50346]

8 **Sterling RK**, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, S Sulkowski M, Torriani FJ, Dieterich DT, Thomas DL, Messinger D, Nelson M; APRICOT Clinical Investigators. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006; **43**: 1317-1325 [PMID: 16729309 DOI: 10.1002/hep.21178]

9 **European Association for Study of Liver.** Asociacion Latinoamericana para el Estudio del Higado. EASL-ALEH Clinical Practice Guidelines: Non-invasive tests for evaluation of liver disease severity and prognosis. *J Hepatol* 2015; **63**: 237-264 [PMID: 25911335 DOI: 10.1016/j.jhep.2015.04.006]

10 **Nicholson JK**, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 1999; **29**: 1181-1189 [PMID: 10598751 DOI: 10.1080/004982599238047]

11 **Dunn WB,** Ellis DI. Metabolomics: Current analytical platforms and methodologies. *Trends Anal Chem* 2005; **24**: 285–294 [DOI:10.1016/j.trac.2004.11.021]

12 **Nicholson JK**, Lindon JC. Systems biology: Metabonomics. *Nature* 2008; **455**: 1054-1056 [PMID: 18948945 DOI: 10.1038/4551054a]

13 **Amathieu R**, Triba MN, Goossens C, Bouchemal N, Nahon P, Savarin P, Le Moyec L. Nuclear magnetic resonance based metabolomics and liver diseases: Recent advances and future clinical applications. *World J Gastroenterol* 2016; **22**: 417-426 [PMID: 26755887 DOI: 10.3748/wjg.v22.i1.417]

14 **Godoy MM**, Lopes EP, Silva RO, Hallwass F, Koury LC, Moura IM, Gonçalves SM, Simas AM. Hepatitis C virus infection diagnosis using metabonomics. *J Viral Hepat* 2010; **17**: 854-858 [PMID: 20070502 DOI: 10.1111/j.1365-2893.2009.01252.x]

15 **Munshi SU,** Taneja S, Bhavesh NS, Shastri J, Aggarwal R, Jameel S. Metabonomic analysis of hepatitis e patients shows deregulated metabolic cycles and abnormalities in amino acid metabolism. *J Viral Hepat* 2011; **18**: e591-e602 [DOI: 10.1111/j.1365-2893.2011.01488.x]

16 **Shariff MI**, Ladep NG, Cox IJ, Williams HR, Okeke E, Malu A, Thillainayagam AV, Crossey MM, Khan SA, Thomas HC, Taylor-Robinson SD. Characterization of urinary biomarkers of hepatocellular carcinoma using magnetic resonance spectroscopy in a Nigerian population. *J Proteome Res* 2010; **9**: 1096-1103 [PMID: 19968328 DOI: 10.1021/pr901058t]

17 **Shariff MIF,** Gomaa AI, Cox IJ, Patel M, Williams HRT, Crossey MME. Urinary metabolic biomarkers of hepatocellular carcinoma in an Egyptian population: A validation study. *J Proteome Res* 2011; **10**: 1828-1836 [DOI: 10.1021/pr101096f]

18 **Gao H**, Lu Q, Liu X, Cong H, Zhao L, Wang H, Lin D. Application of 1H NMR-based metabonomics in the study of metabolic profiling of human hepatocellular carcinoma and liver cirrhosis. *Cancer Sci* 2009; **100**: 782-785 [PMID: 19469021]

19 **Gouveia LR**, Santos JC, Silva RD, Batista AD, Domingues ALC, Lopes EPA, Silva RO. Diagnosis of coinfection by schistosomiasis and viral hepatitis B or C using 1H NMR-based metabonomics. *PLoS One* 2017; **12**: e0182196 [PMID: 28763497 DOI: 10.1371/journal.pone.0182196]

20 **Sackett DL**, Haynes RB. The architecture of diagnostic research. *BMJ* 2002; **324**: 539-541 [PMID: 11872558]

21 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293 [PMID: 8690394 DOI: 10.1002/hep.510240201]

22 **Xia J** Sinelnikov I V., Han B, Wishart DS. MetaboAnalyst 3.0--making metabolomics more meaningful. *Nucleic Acids Res* 2015; **43**: W251-W257 [DOI: 10.1093/nar/gkv380]

23 **Poynard T**, Ratziu V, Benmanov Y, Di Martino V, Bedossa P, Opolon P. Fibrosis in patients with chronic hepatitis C: detection and significance. *Semin Liver Dis* 2000; **20**: 47-55 [PMID: 10895431]

24 **Matsue Y,** Tsutsumi M, Hayashi N, Saito T, Tsuchishima M, Toshikuni N. Serum Osteopontin predicts degree of hepatic fibrosis and serves as a biomarker in patients with hepatitis C virus infection. *PLoS One* 2015; **10**: e0118744 [DOI: 10.1371/journal.pone.0118744]

25 **Boursier J**, de Ledinghen V, Zarski JP, Fouchard-Hubert I, Gallois Y, Oberti F, Calès P; multicentric groups from SNIFF 32, VINDIAG 7, and ANRS/HC/EP23 FIBROSTAR studies. Comparison of eight diagnostic algorithms for liver fibrosis in hepatitis C: new algorithms are more precise and entirely

noninvasive. *Hepatology* 2012; **55**: 58-67 [PMID: 21898504 DOI: 10.1002/hep.24654]
26 **Le Moyec L,** Triba M, Nahon P, Bouchemal N, Hantz E, Goossens C. Nuclear magnetic resonance metabolomics and human liver diseases: The principles and evidence associated with protein and carbohydrate metabolism (Review). *Biomed Reports* 2017; **6**: 387-395 [DOI: 10.3892/br.2017.868]
27 **Beyoğlu D,** Idle, JR. The metabolomic window into hepatobiliary disease. *J Hepatol* 2013; **59**: 842-858 [DOI: 10.1016/j.jhep.2013.05.030]
28 **Amathieu R**, Nahon P, Triba M, Bouchemal N, Trinchet JC, Beaugrand M, Dhonneur G, Le Moyec L. Metabolomic approach by 1H NMR spectroscopy of serum for the assessment of chronic liver failure in patients with cirrhosis. *J Proteome Res* 2011; **10**: 3239-3245 [PMID: 21568267 DOI: 10.1021/pr200265z]
29 **Amathieu R**, Triba MN, Nahon P, Bouchemal N, Kamoun W, Haouache H, Trinchet JC, Savarin P, Le Moyec L, Dhonneur G. Serum 1H-NMR metabolomic fingerprints of acute-on-chronic liver failure in intensive care unit patients with alcoholic cirrhosis. *PLoS One* 2014; **9**: e89230 [PMID: 24586615 DOI: 10.1371/journal.pone.0089230]
30 **Jiménez B**, Montoliu C, MacIntyre DA, Serra MA, Wassel A, Jover M, Romero-Gomez M, Rodrigo JM, Pineda-Lucena A, Felipo V. Serum metabolic signature of minimal hepatic encephalopathy by (1)H-nuclear magnetic resonance. *J Proteome Res* 2010; **9**: 5180-5187 [PMID: 20690770 DOI: 10.1021/pr100486e]
31 **Qi SW**, Tu ZG, Peng WJ, Wang LX, Ou-Yang X, Cai AJ, Dai Y. ^{1}H NMR-based serum metabolic profiling in compensated and decompensated cirrhosis. *World J Gastroenterol* 2012; **18**: 285-290 [PMID: 22294833 DOI: 10.3748/wjg.v18.i3.285]
32 **Sands CJ**, Guha IN, Kyriakides M, Wright M, Beckonert O, Holmes E, Rosenberg WM, Coen M. Metabolic phenotyping for enhanced mechanistic stratification of chronic hepatitis C-induced liver fibrosis. *Am J Gastroenterol* 2015; **110**: 159-169 [PMID: 25533003 DOI: 10.1038/ajg.2014.370]
33 **Embade N,** Mariño Z, Diercks T, Cano A, Lens S, Cabrera D. Metabolic characterization of advanced liver fibrosis in HCV patients as studied by serum 1H-NMR spectroscopy. *PLoS One* 2016; **11**: e0155094 [DOI: 10.1371/journal.pone.0155094]
34 **Sarfaraz MO**, Myers RP, Coffin CS, Gao ZH, Shaheen AA, Crotty PM, Zhang P, Vogel HJ, Weljie AM. A quantitative metabolomics profiling approach for the noninvasive assessment of liver histology in patients with chronic hepatitis C. *Clin Transl Med* 2016; **5**: 33 [PMID: 27539580 DOI: 10.1186/s40169-016-0109-2]
35 **Gabbani T**, Marsico M, Bernini P, Lorefice E, Grappone C, Biagini MR, Milani S, Annese V. Metabolomic analysis with 1H-NMR for non-invasive diagnosis of hepatic fibrosis degree in patients with chronic hepatitis C. *Dig Liver Dis* 2017 [PMID: 28625405 DOI: 10.1016/j.dld.2017.05.018]

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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.

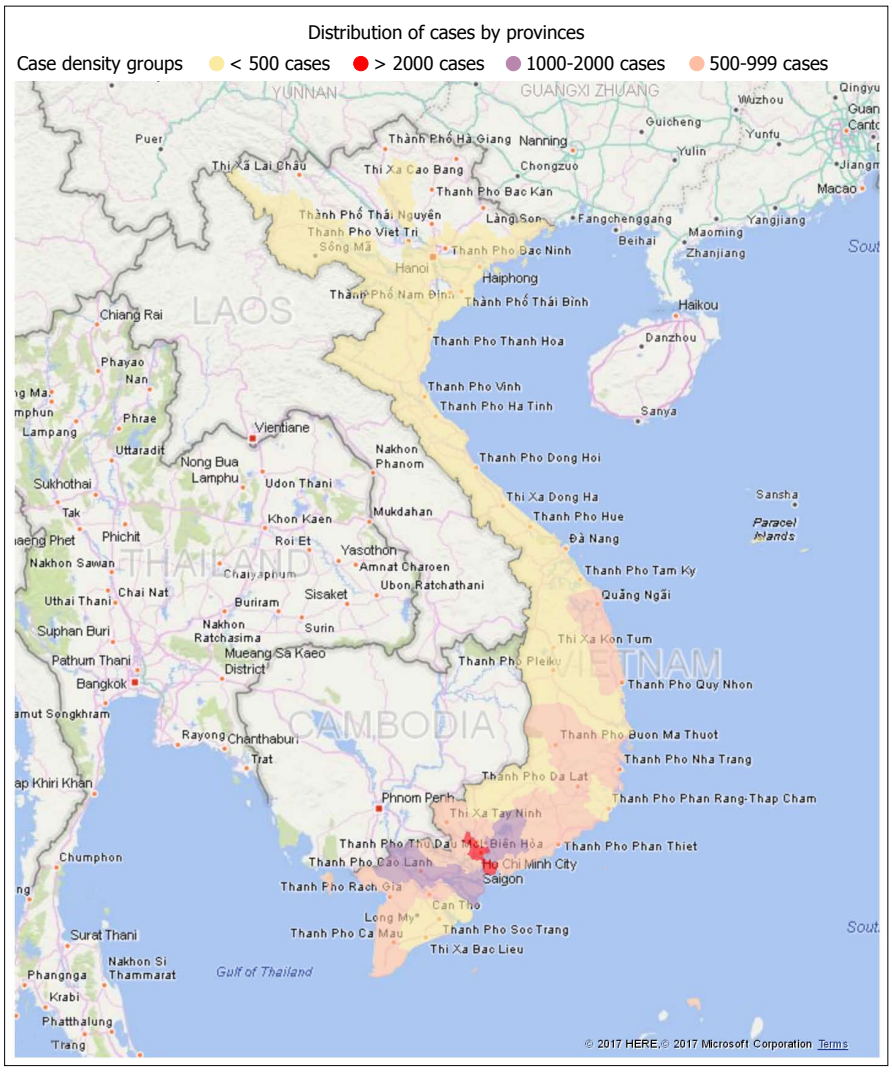

Figure 1 Geographic distribution of patients with hepatocellular carcinoma receiving care at Cho Ray Hospital, Vietnam.

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.

REFERENCES

1 **Ferlay J**, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; **136**: E359-E386 [PMID: 25220842 DOI: 10.1002/ijc.29210]

2 **El-Serag HB**. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; **142**: 1264-1273.e1 [PMID: 22537432 DOI: 10.1053/j.gastro.2011.12.061]

3 **Kuper H**, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. *J Intern Med* 2000; **248**: 171-183 [PMID: 10971784 DOI: 10.1046/j.1365-2796.2000.00742.x]

4 **Nordenstedt H**, White DL, El-Serag HB. The changing pattern of epidemiology in hepatocellular carcinoma. *Dig Liver Dis* 2010; **42** Suppl **3**: S206-S214 [PMID: 20547305 DOI: 10.1016/S1590-8658(10)60507-5]

5 **Zhu RX**, Seto WK, Lai CL, Yuen MF. Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region. *Gut Liver* 2016; **10**: 332-339 [PMID: 27114433 DOI: 10.5009/gnl15257]

6 **El-Serag HB**. Hepatocellular carcinoma. *N Engl J Med* 2011; **365**: 1118-1127 [PMID: 21992124 DOI: 10.1056/NEJMra1001683]

7 A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. *Hepatology* 1998; **28**: 751-755 [PMID: 9731568 DOI: 10.1002/hep.510280322]

8 **Sereno L**, Mesquita F, Kato M, Jacka D, Nguyen TT, Nguyen TN. Epidemiology, responses, and way forward: the silent epidemic of viral hepatitis and HIV coinfection in Vietnam. *J Int Assoc Physicians AIDS Care* (Chic) 2012; **11**: 311-320 [PMID: 22828983 DOI: 10.1177/1545109712453939]

9 **Anh PT**, Parkin DM, Hanh NT, Duc NB. Cancer in the population of Hanoi, Vietnam, 1988-1990. *Br J Cancer* 1993; **68**: 1236-1242 [PMID: 8260379 DOI: 10.1038/bjc.1993.511]

10 **Do SH**, Yamada H, Fujimoto M, Ohisa M, Matsuo J, Akita T, Katayama K, Van Nguyen N, Miyakawa Y, Tanaka J. High prevalences of hepatitis B and C virus infections among adults living in Binh Thuan province, Vietnam. *Hepatol Res* 2015; **45**: 259-268 [PMID: 24799322 DOI: 10.1111/hepr.12350]

11 **Cordier S**, Le TB, Verger P, Bard D, Le CD, Larouze B, Dazza MC, Hoang TQ, Abenhaim L. Viral infections and chemical exposures as risk factors for hepatocellular carcinoma in Vietnam. *Int J Cancer* 1993; **55**: 196-201 [PMID: 7690345 DOI: 10.1002/ijc.2910550205]

12 **Nguyen VT**, McLaws ML, Dore GJ. Highly endemic hepatitis B infection in rural Vietnam. *J Gastroenterol Hepatol* 2007; **22**: 2093-2100 [PMID: 17645465 DOI: 10.1111/j.1440-1746.2007.05010.x]

13 **Ishizaki A**, Tran VT, Nguyen CH, Tanimoto T, Hoang HTT, Pham HV, Phan CTT, Bi X, Pham TV, Ichimura H. Discrepancies in prevalence trends for HIV, hepatitis B virus, and hepatitis C virus in Haiphong, Vietnam from 2007 to 2012. *PLoS One* 2017; **12**: e0179616 [PMID: 28662105 DOI: 10.1371/journal.pone.0179616]

14 **Nguyen VT**, Law MG, Dore GJ. An enormous hepatitis B virus-related liver disease burden projected in Vietnam by 2025. *Liver Int* 2008; **28**: 525-531 [PMID: 18266635 DOI: 10.1111/j.1478-3231.2007.01646.x]

15 **Nguyen TH**, Vu MH, Nguyen VC, Nguyen LH, Toda K, Nguyen TN, Dao S, Wannemuehler KA, Hennessey KA. A reduction in chronic hepatitis B virus infection prevalence among children in Vietnam demonstrates the importance of vaccination. *Vaccine* 2014; **32**: 217-222 [PMID: 24284410 DOI: 10.1016/j.vaccine.2013.11.004]

16 **Ngo AD**, Rao C, Hoa NP, Adair T, Chuc NT. Mortality patterns in Vietnam, 2006: Findings from a national verbal autopsy survey. *BMC Res Notes* 2010; **3**: 78 [PMID: 20236551 DOI: 10.1186/1756-0500-3-78]

17 Cho Ray Hospital: main page [cited 2017 Oct 15]. Available from: URL: http://www.choray.vn/

18 **Kokudo N**, Hasegawa K, Akahane M, Igaki H, Izumi N, Ichida T, Uemoto S, Kaneko S, Kawasaki S, Ku Y, Kudo M, Kubo S, Takayama T, Tateishi R, Fukuda T, Matsui O, Matsuyama Y, Murakami T, Arii S, Okazaki M, Makuuchi M. Evidence-based Clinical Practice Guidelines for Hepatocellular Carcinoma: The Japan Society of Hepatology 2013 update (3rd JSH-HCC Guidelines). *Hepatol Res* 2015; **45**: 123-127 [PMID: 25625806 DOI: 10.1111/hepr.12464]

19 **Arii S**, Sata M, Sakamoto M, Shimada M, Kumada T, Shiina S, Yamashita T, Kokudo N, Tanaka M, Takayama T, Kudo M. Management of hepatocellular carcinoma: Report of Consensus Meeting in the 45th Annual Meeting of the Japan Society of Hepatology (2009). *Hepatol Res* 2010; **40**: 667-685 [PMID: 20633193 DOI: 10.1111/j.1872-034X.2010.00673.x]

20 **Kudo M**, Matsui O, Izumi N, Iijima H, Kadoya M, Imai Y, Okusaka T, Miyayama S, Tsuchiya K, Ueshima K, Hiraoka A, Ikeda M, Ogasawara S, Yamashita T, Minami T, Yamakado K; Liver Cancer Study Group of Japan. JSH Consensus-Based Clinical Practice Guidelines for the Management of Hepatocellular Carcinoma: 2014 Update by the Liver Cancer Study Group of Japan. *Liver Cancer* 2014; **3**: 458-468 [PMID: 26280007 DOI: 10.1159/000343875]

21 **Omata M**, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK. Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. *Hepatol Int* 2017; **11**: 317-370 [PMID: 28620797 DOI: 10.1007/s12072-017-9799-9]

22 **Poté N**, Cauchy F, Albuquerque M, Voitot H, Belghiti J, Castera L, Puy H, Bedossa P, Paradis V. Performance of PIVKA-II for early hepatocellular carcinoma diagnosis and prediction of microvascular invasion. *J Hepatol* 2015; **62**: 848-854 [PMID: 25450201 DOI: 10.1016/j.jhep.2014.11.005]

23 **Yu R**, Tan Z, Xiang X, Dan Y, Deng G. Effectiveness of PIVKA-II in the detection of hepatocellular carcinoma based on real-world clinical data. *BMC Cancer* 2017; **17**: 608 [PMID: 28863782 DOI: 10.1186/s12885-017-3609-6]

24 **Seo SI**, Kim HS, Kim WJ, Shin WG, Kim DJ, Kim KH, Jang MK, Lee JH, Kim JS, Kim HY, Kim DJ, Lee MS, Park CK. Diagnostic value of PIVKA-II and alpha-fetoprotein in hepatitis B virus-associated hepatocellular carcinoma. *World J Gastroenterol* 2015; **21**: 3928-3935 [PMID: 25852278 DOI: 10.3748/wjg.v21.i13.3928]

25 2016 Global Burden of Disease 2017 [cited 2017 Oct 15]. Available from: URL: https://vizhub.healthdata.org/gbd-compare/

26 **Yu SJ**. A concise review of updated guidelines regarding the management of hepatocellular carcinoma around the world: 2010-2016. *Clin Mol Hepatol* 2016; **22**: 7-17 [PMID: 27044761 DOI: 10.3350/cmh.2016.22.1.7]

27 **Nguyen TT**, McPhee SJ, Stewart S, Gildengorin G, Zhang L, Wong C, Maxwell AE, Bastani R, Taylor VM, Chen MS Jr. Factors associated with hepatitis B testing among Vietnamese Americans. *J Gen Intern Med* 2010; **25**: 694-700 [PMID: 20306150 DOI: 10.1007/s11606-010-1285-1]

28 **Khue NT**. Diabetes in Vietnam. *Ann Glob Health* 2015; **81**: 870-873 [PMID: 27108154 DOI: 10.1016/j.aogh.2016.01.003]

29 **Tabibian JH**, Lazo M, Durazo FA, Yeh HC, Tong MJ, Clark JM. Nonalcoholic fatty liver disease across ethno-racial groups: do Asian-American adults represent a new at-risk population? *J Gastroenterol Hepatol* 2011; **26**: 501-509 [PMID: 21332546 DOI: 10.1111/j.1440-1746.2010.06443.x]

30 **Wong RJ**, Ahmed A. Obesity and non-alcoholic fatty liver disease: Disparate associations among Asian populations. *World J Hepatol* 2014; **6**: 263-273 [PMID: 24868320 DOI: 10.4254/wjh.v6.i5.263]

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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

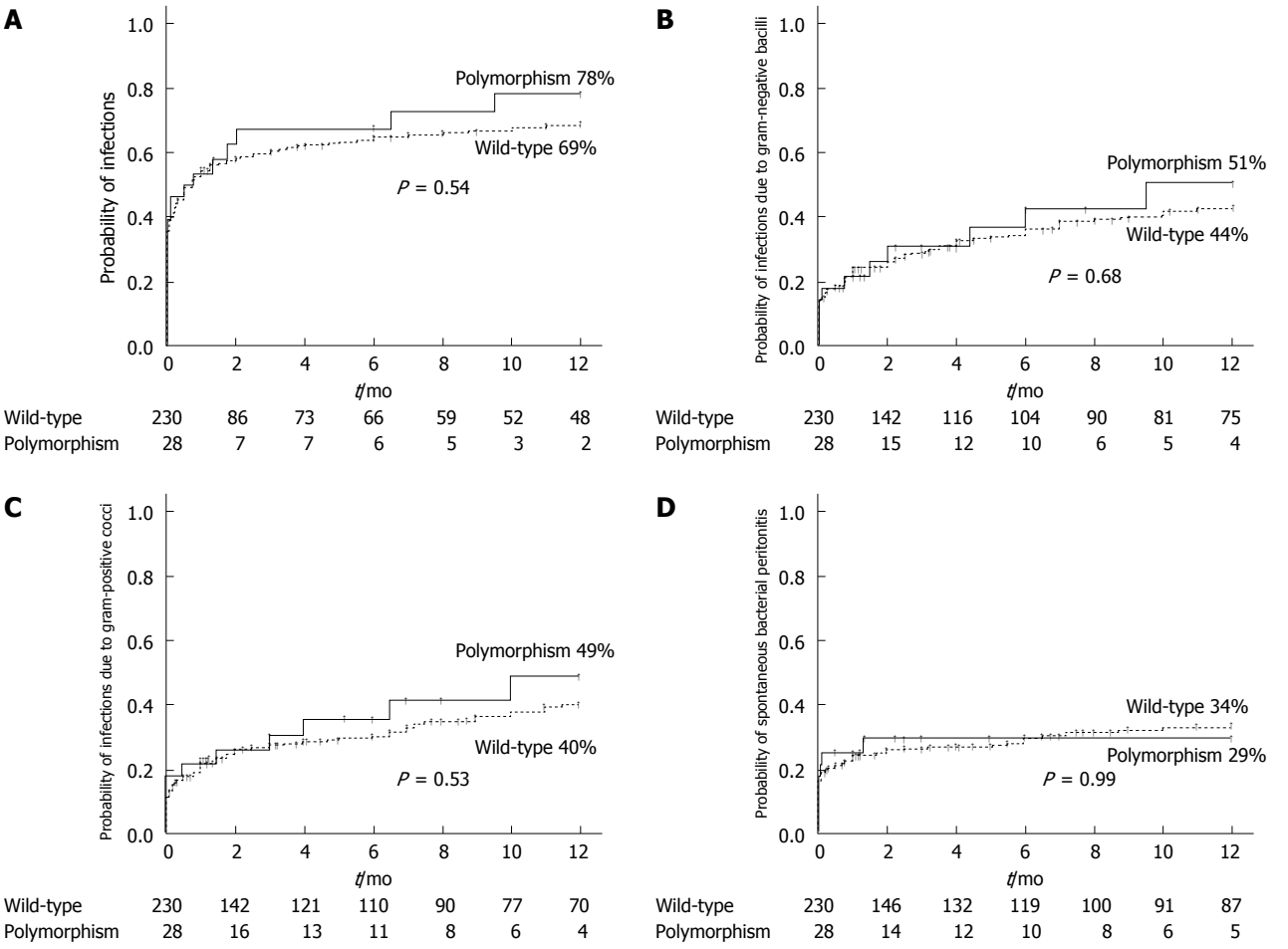

Figure 1 One year-probability of infections in the polymorphism group and in the wild-type group. A: All infections; B: Infections caused by gram-negative bacilli; C: Infections caused by gram-positive cocci; D: Spontaneous bacterial peritonitis.

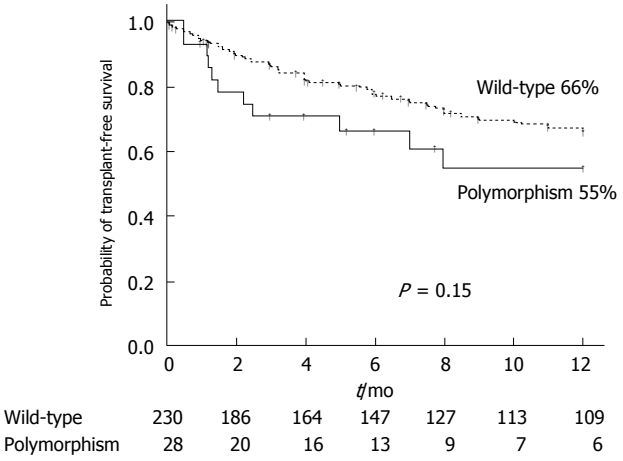

Figure 2 One year-probability of transplant-free survival in the polymorphism group and in the wild-type group.

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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REFERENCES

1 **Fernández J**, Acevedo J, Castro M, Garcia O, de Lope CR, Roca D, Pavesi M, Sola E, Moreira L, Silva A, Seva-Pereira T, Corradi F, Mensa J, Ginès P, Arroyo V. Prevalence and risk factors of infections by multiresistant bacteria in cirrhosis: a prospective study. *Hepatology* 2012; **55**: 1551-1561 [PMID: 22183941 DOI: 10.1002/hep.25532]

2 **Tandon P**, Garcia-Tsao G. Bacterial infections, sepsis, and multiorgan failure in cirrhosis. *Semin Liver Dis* 2008; **28**: 26-42 [PMID: 18293275 DOI: 10.1055/s-2008-1040319]

3 **Fernández J**, Tandon P, Mensa J, Garcia-Tsao G. Antibiotic prophylaxis in cirrhosis: Good and bad. *Hepatology* 2016; **63**: 2019-2031 [PMID: 26528864 DOI: 10.1002/hep.28330]

4 **Jalan R**, Fernandez J, Wiest R, Schnabl B, Moreau R, Angeli P, Stadlbauer V, Gustot T, Bernardi M, Canton R, Albillos A, Lammert F, Wilmer A, Mookerjee R, Vila J, Garcia-Martinez R, Wendon J, Such J, Cordoba J, Sanyal A, Garcia-Tsao G, Arroyo V, Burroughs A, Ginès P. Bacterial infections in cirrhosis: a position statement based on the EASL Special Conference 2013. *J Hepatol* 2014; **60**: 1310-1324 [PMID: 24530646 DOI: 10.1016/j.jhep.2014.01.024]

5 **Guarner C**, Soriano G. Bacterial translocation and its consequences in patients with cirrhosis. *Eur J Gastroenterol Hepatol* 2005; **17**: 27-31 [PMID: 15647636]

6 **Skevaki C**, Pararas M, Kostelidou K, Tsakris A, Routsias JG. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious diseases. *Clin Exp Immunol* 2015; **180**: 165-177 [PMID: 25560985 DOI: 10.1111/cei.12578]

7 **Allen A**, Obaro S, Bojang K, Awomoyi AA, Greenwood BM, Whittle H, Sirugo G, Newport MJ. Variation in Toll-like receptor 4 and susceptibility to group A meningococcal meningitis in Gambian children. *Pediatr Infect Dis J* 2003; **22**: 1018-1019 [PMID: 14628773]

8 **Lorenz E**, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Arch Intern Med* 2002; **162**: 1028-1032 [PMID: 11996613]

9 **Agnese DM**, Calvano JE, Hahm SJ, Coyle SM, Corbett SA, Calvano SE, Lowry SF. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J Infect Dis* 2002; **186**: 1522-1525 [PMID: 12404174 DOI: 10.1086/344893]

10 **Piñero P**, Juanola O, Caparrós E, Zapater P, Giménez P, González-Navajas JM, Such J, Francés R. Toll-like receptor polymorphisms compromise the inflammatory response against bacterial antigen translocation in cirrhosis. *Sci Rep* 2017; **7**: 46425 [PMID: 28418003 DOI: 10.1038/srep46425]

11 **Schröder NW**, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infect Dis* 2005; **5**: 156-164 [PMID: 15766650 DOI: 10.1016/S1473-3099(05)01308-3]

12 **Nieto JC**, Sánchez E, Román E, Vidal S, Oliva L, Guarner-Argente C, Poca M, Torras X, Juárez C, Guarner C, Soriano G. Cytokine production in patients with cirrhosis and TLR4 polymorphisms. *World J Gastroenterol* 2014; **20**: 17516-17524 [PMID: 25516666 DOI: 10.3748/wjg.v20.i46.17516]

13 **Testro AG**, Gow PJ, Angus PW, Wongseelashote S, Skinner N, Markovska V, Visvanathan K. Effects of antibiotics on expression and function of Toll-like receptors 2 and 4 on mononuclear cells in patients with advanced cirrhosis. *J Hepatol* 2010; **52**: 199-205 [PMID: 20006396 DOI: 10.1016/j.jhep.2009.11.006]

14 **Guarner-Argente C**, Sánchez E, Vidal S, Román E, Concepción M, Poca M, Sánchez D, Juárez C, Soriano G, Guarner C. Toll-like receptor 4 D299G polymorphism and the incidence of infections in cirrhotic patients. *Aliment Pharmacol Ther* 2010; **31**: 1192-1199 [PMID: 20222908 DOI: 10.1111/j.1365-2036.2010.04291.x]

15 **Fernández J**, Gustot T. Management of bacterial infections in cirrhosis. *J Hepatol* 2012; **56** Suppl 1: S1-S12 [PMID: 22300459 DOI: 10.1016/S0168-8278(12)60002-6]

16 **Appenrodt B**, Grünhage F, Gentemann MG, Thyssen L, Sauerbruch T, Lammert F. Nucleotide-binding oligomerization domain containing 2 (NOD2) variants are genetic risk factors for death and spontaneous bacterial peritonitis in liver cirrhosis. *Hepatology* 2010; **51**: 1327-1333 [PMID: 20087966 DOI: 10.1002/hep.23440]

17 **Nischalke HD**, Berger C, Aldenhoff K, Thyssen L, Gentemann M, Grünhage F, Lammert F, Nattermann J, Sauerbruch T, Spengler U, Appenrodt B. Toll-like receptor (TLR) 2 promoter and intron 2 polymorphisms are associated with increased risk for spontaneous bacterial peritonitis in liver cirrhosis. *J Hepatol* 2011; **55**: 1010-1016 [PMID: 21356257 DOI: 10.1016/j.jhep.2011.02.022]

18 **Lutz P**, Krämer B, Kaczmarek DJ, Hübner MP, Langhans B, Appenrodt B, Lammert F, Nattermann J, Hoerauf A, Strassburg CP, Spengler U, Nischalke HD. A variant in the nuclear dot protein 52kDa gene increases the risk for spontaneous bacterial peritonitis in patients with alcoholic liver cirrhosis. *Dig Liver Dis* 2016; **48**: 62-68 [PMID: 26493630 DOI: 10.1016/j.dld.2015.09.011]

19 **Lee SO**, Brown RA, Kang SH, Abdel Massih RC, Razonable RR. Toll-like receptor 4 polymorphisms and the risk of gram-negative bacterial infections after liver transplantation. *Transplantation* 2011; **92**: 690-696 [PMID: 21822168 DOI: 10.1097/TP.0b013e31822b589f]

20 **Ahluwalia V**, Betrapally NS, Hylemon PB, White MB, Gillevet PM, Unser AB, Fagan A, Daita K, Heuman DM, Zhou H, Sikaroodi M, Bajaj JS. Impaired Gut-Liver-Brain Axis in Patients with Cirrhosis. *Sci Rep* 2016; **6**: 26800 [PMID: 27225869 DOI: 10.1038/srep26800]

21 **Córdoba J**, Mínguez B. Hepatic encephalopathy. *Semin Liver Dis* 2008; **28**: 70-80 [PMID: 18293278 DOI: 10.1055/s-2008-1040322]

22 **Guo J**, Loke J, Zheng F, Hong F, Yea S, Fukata M, Tarocchi M, Abar OT, Huang H, Sninsky JJ, Friedman SL. Functional linkage of cirrhosis-predictive single nucleotide polymorphisms of Toll-like receptor 4 to hepatic stellate cell responses. *Hepatology* 2009; **49**: 960-968 [PMID: 19085953 DOI: 10.1002/hep.22697]

23 **D'Amico G**, Garcia-Tsao G, Pagliaro L. Natural history and prognostic indicators of survival in cirrhosis: a systematic review of 118 studies. *J Hepatol* 2006; **44**: 217-231 [PMID: 16298014 DOI: 10.1016/j.jhep.2005.10.013]

24 **Xie YD**, Feng B, Gao Y, Wei L. Effect of abstinence from alcohol on survival of patients with alcoholic cirrhosis: A systematic review and

meta-analysis. *Hepatol Res* 2014; **44**: 436-449 [PMID: 23607793 DOI: 10.1111/hepr.12131]
25 **Hernandez-Gea V**, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology* 2013; **144**: 512-527 [PMID: 23313965 DOI: 10.1053/j.gastro.2013.01.002]
26 **Dong YQ**, Lu CW, Zhang L, Yang J, Hameed W, Chen W. Toll-like receptor 4 signaling promotes invasion of hepatocellular carcinoma cells through MKK4/JNK pathway. *Mol Immunol* 2015; **68**: 671-683 [PMID: 26589455 DOI: 10.1016/j.molimm.2015.10.015]

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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 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.

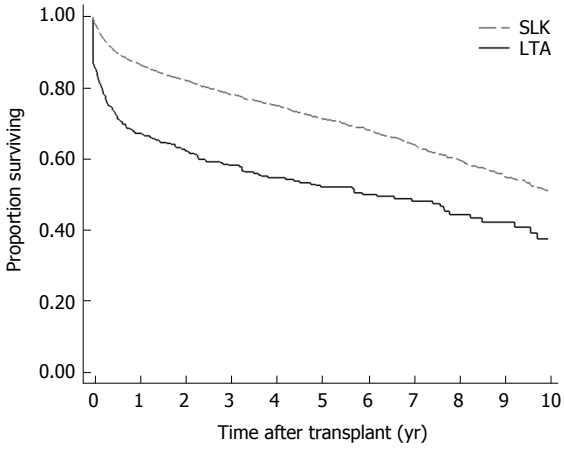

Figure 1 Post-transplant survival according to type of transplant. Kaplan-Meier post-transplant survival curves, according to type of transplant, among patients initially listed for simultaneous liver-kidney transplant. Actuarial 1, 3 and 5 year survival rates among the LTA and SLKT groups were 68% *vs* 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.

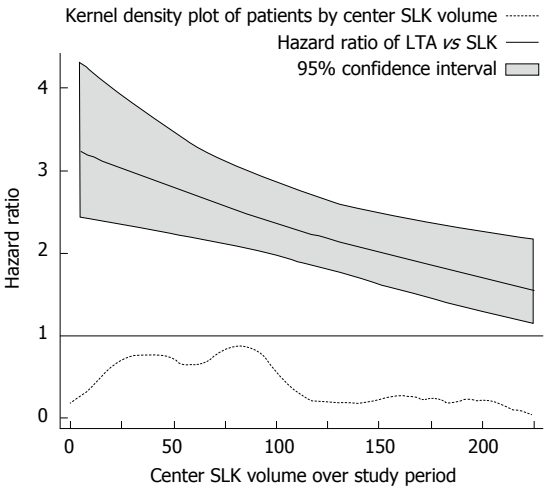

Figure 2 Post-transplant survival according to center volume of simultaneous liver-kidney transplants. Estimated hazard ratios for post-transplant survival, comparing liver transplant alone to simultaneous liver-kidney transplant among patients initially listed for simultaneous liver-kidney transplant, 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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REFERENCES
1 **Saxena V**, Lai JC. Kidney Failure and Liver Allocation: Current Practices and Potential Improvements. *Adv Chronic Kidney Dis* 2015; **22**: 391-398 [PMID: 26311601 DOI: 10.1053/j.ackd.2015.05.002]
2 **Davis CL**, Feng S, Sung R, Wong F, Goodrich NP, Melton LB, Reddy KR, Guidinger MK, Wilkinson A, Lake J. Simultaneous liver-kidney transplantation: evaluation to decision making. *Am J Transplant* 2007; **7**: 1702-1709 [PMID: 17532752 DOI: 10.1111/j.1600-6143.2007.01856.x]
3 **Eason JD**, Gonwa TA, Davis CL, Sung RS, Gerber D, Bloom RD. Proceedings of Consensus Conference on Simultaneous Liver Kidney Transplantation (SLK). *Am J Transplant* 2008; **8**: 2243-2251 [PMID: 18808402 DOI: 10.1111/j.1600-6143.2008.02416.x]
4 **Nadim MK**, Sung RS, Davis CL, Andreoni KA, Biggins SW, Danovitch GM, Feng S, Friedewald JJ, Hong JC, Kellum JA, Kim WR, Lake JR, Melton LB, Pomfret EA, Saab S, Genyk YS. Simultaneous liver-kidney transplantation summit: current state and future directions. *Am J Transplant* 2012; **12**: 2901-2908 [PMID: 22822723 DOI: 10.1111/j.1600-6143.2012.04190.x]
5 **Nadim MK**, Davis CL, Sung R, Kellum JA, Genyk YS. Simultaneous liver-kidney transplantation: a survey of US transplant centers. *Am J Transplant* 2012; **12**: 3119-3127 [PMID: 22759208 DOI: 10.1111/j.1600-6143.2012.04176.x]
6 **Locke JE**, Warren DS, Singer AL, Segev DL, Simpkins CE, Maley WR, Montgomery RA, Danovitch G, Cameron AM. Declining outcomes in simultaneous liver-kidney transplantation in the MELD era: ineffective usage of renal allografts. *Transplantation* 2008; **85**: 935-942 [PMID: 18408571 DOI: 10.1097/TP.0b013e318168476d]
7 **Fong TL**, Khemichian S, Shah T, Hutchinson IV, Cho YW. Combined liver-kidney transplantation is preferable to liver transplant alone for cirrhotic patients with renal failure. *Transplantation* 2012; **94**: 411-416 [PMID: 22805440 DOI: 10.1097/TP.0b013e3182590d6b]
8 **Jeyarajah DR**, Gonwa TA, McBride M, Testa G, Abbasoglu O, Husberg BS, Levy MF, Goldstein RM, Klintmalm GB. Hepatorenal syndrome: combined liver kidney transplants versus isolated liver transplant. *Transplantation* 1997; **64**: 1760-1765 [PMID: 9422417 DOI: 10.1097/00007890-199712270-00024]
9 **Martin EF**, Huang J, Xiang Q, Klein JP, Bajaj J, Saeian K. Recipient survival and graft survival are not diminished by simultaneous liver-kidney transplantation: an analysis of the united network for organ sharing database. *Liver Transpl* 2012; **18**: 914-929 [PMID: 22467623 DOI: 10.1002/lt.23440]
10 **Hmoud B**, Kuo YF, Wiesner RH, Singal AK. Outcomes of liver transplantation alone after listing for simultaneous kidney: comparison to simultaneous liver kidney transplantation. *Transplantation* 2015; **99**: 823-828 [PMID: 25250648 DOI: 10.1097/tp.0000000000000438]
11 **Sharma P**, Shu X, Schaubel DE, Sung RS, Magee JC. Propensity score-based survival benefit of simultaneous liver-kidney transplant over liver transplant alone for recipients with pretransplant renal dysfunction. *Liver Transpl* 2016; **22**: 71-79 [PMID: 26069168 DOI: 10.1002/lt.24189]
12 **Catalano G**, Tandoi F, Mazza E, Simonato F, Tognarelli G, Biancone L, Lupo F, Romagnoli R, Salizzoni M. Simultaneous Liver-Kidney Transplantation in Adults: A Single-center Experience Comparing Results With Isolated Liver Transplantation. *Transplant Proc* 2015; **47**: 2156-2158 [PMID: 26361666 DOI: 10.1016/j.transproceed.2014.11.073]
13 **Ruiz R**, Kunitake H, Wilkinson AH, Danovitch GM, Farmer DG, Ghobrial RM, Yersiz H, Hiatt JR, Busuttil RW. Long-term analysis of combined liver and kidney transplantation at a single center. *Arch Surg* 2006; **141**: 735-741; discussion 741-742 [PMID: 16924080 DOI: 10.1001/archsurg.141.8.735]
14 **Mehrabi A**, Fonouni H, Ayoub E, Rahbari NN, Müller SA, Morath Ch, Seckinger J, Sadeghi M, Golriz M, Esmaeilzadeh M, Hillebrand N, Weitz J, Zeier M, Büchler MW, Schmidt J, Schmied BM. A single center experience of combined liver kidney transplantation. *Clin Transplant* 2009; **23** Suppl 21: 102-114 [PMID: 19930323 DOI: 10.1111/j.1399-0012.2009.01146.x]
15 **Doyle MB**, Subramanian V, Vachharajani N, Maynard E, Shenoy S, Wellen JR, Lin Y, Chapman WC. Results of Simultaneous Liver and Kidney Transplantation: A Single-Center Review. *J Am Coll Surg* 2016; **223**: 193-201 [PMID: 27103549 DOI: 10.1016/j.jamcollsurg.2016.04.005]
16 **Ozhathil DK**, Li YF, Smith JK, Tseng JF, Saidi RF, Bozorgzadeh A, Shah SA. Impact of center volume on outcomes of increased-risk liver transplants. *Liver Transpl* 2011; **17**: 1191-1199 [PMID: 21604357 DOI: 10.1002/lt.22343]
17 **Macomber CW,** Shaw JJ, Santry H, Saidi RF, Jabbour N, Tseng JF, Bozorgzadeh A, Shah SA. Centre volume and resource consumption in liver transplantation. *HPB* (Oxford) 2012; **14**: 554-559 [PMID: 22762404 DOI: 10.1111/j.1477-2574.2012.00503.x]
18 **U.S. Department of Human and Health Services**. United network for organ sharing / organ procurement and transplantation network standard transplant analysis and research database; 2016

19 **Hayes D**, Hartwig MG, Tobias JD, Tumin D. Lung Transplant Center Volume Ameliorates Adverse Influence of Prolonged Ischemic Time on Mortality. *Am J Transplant* 2017; **17**: 218-226 [PMID: 27278264 DOI: 10.1111/ajt.13916]
20 **Sharma P**, Goodrich NP, Zhang M, Guidinger MK, Schaubel DE, Merion RM. Short-term pretransplant renal replacement therapy and renal nonrecovery after liver transplantation alone. *Clin J Am Soc Nephrol* 2013; **8**: 1135-1142 [PMID: 23449770 DOI: 10.2215/cjn.09600912]
21 **Chang Y**, Gallon L, Shetty K, Chang Y, Jay C, Levitsky J, Ho B, Baker T, Ladner D, Friedewald J, Abecassis M, Hazen G, Skaro AI. Simulation modeling of the impact of proposed new simultaneous liver and kidney transplantation policies. *Transplantation* 2015; **99**: 424-430 [PMID: 25099700 DOI: 10.1097/tp.0000000000000270]
22 **Cheng X SM,** Kim W, Tan J. Utility in treating renal failure in end-stage liver disease with simultaneous liver-kidney transplantation. *Transplantation* 2016 [DOI: 10.1097/TP.0000000000001491]
23 **Unos/optn kidney transplantation committee**. Simultaneous liver kidney (slk) allocation policy; 2015
24 **Formica RN**, Aeder M, Boyle G, Kucheryavaya A, Stewart D, Hirose R, Mulligan D. Simultaneous Liver-Kidney Allocation Policy: A Proposal to Optimize Appropriate Utilization of Scarce Resources. *Am J Transplant* 2016; **16**: 758-766 [PMID: 26603142 DOI: 10.1111/ajt.13631]
25 **Wadei HM**, Gonwa TA, Taner CB. Simultaneous Liver Kidney Transplant (SLK) Allocation Policy Change Proposal: Is It Really a Smart Move? *Am J Transplant* 2016; **16**: 2763-2764 [PMID: 27129113 DOI: 10.1111/ajt.13844]

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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 with

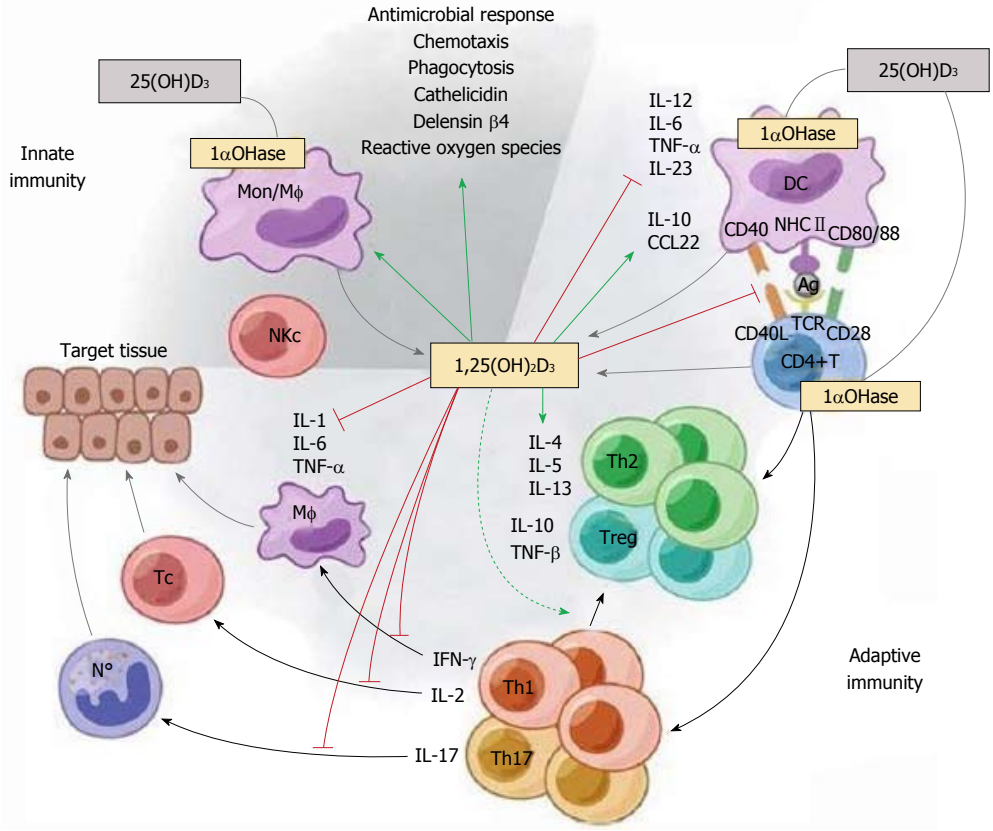

Figure 1 The immunomodulatory effects of 1,25(OH)$_2$D$_3$. 1,25(OH)$_2$D$_3$ targets different players of the innate and adaptive immune compartment. 1,25(OH)$_2$D$_3$ stimulates innate immune responses by enhancing the chemotactic and phagocytotic responses of macrophages, as well as the production 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].

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, METAVIR 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]. There was substantial heterogeneity between studies as 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 this finding, 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

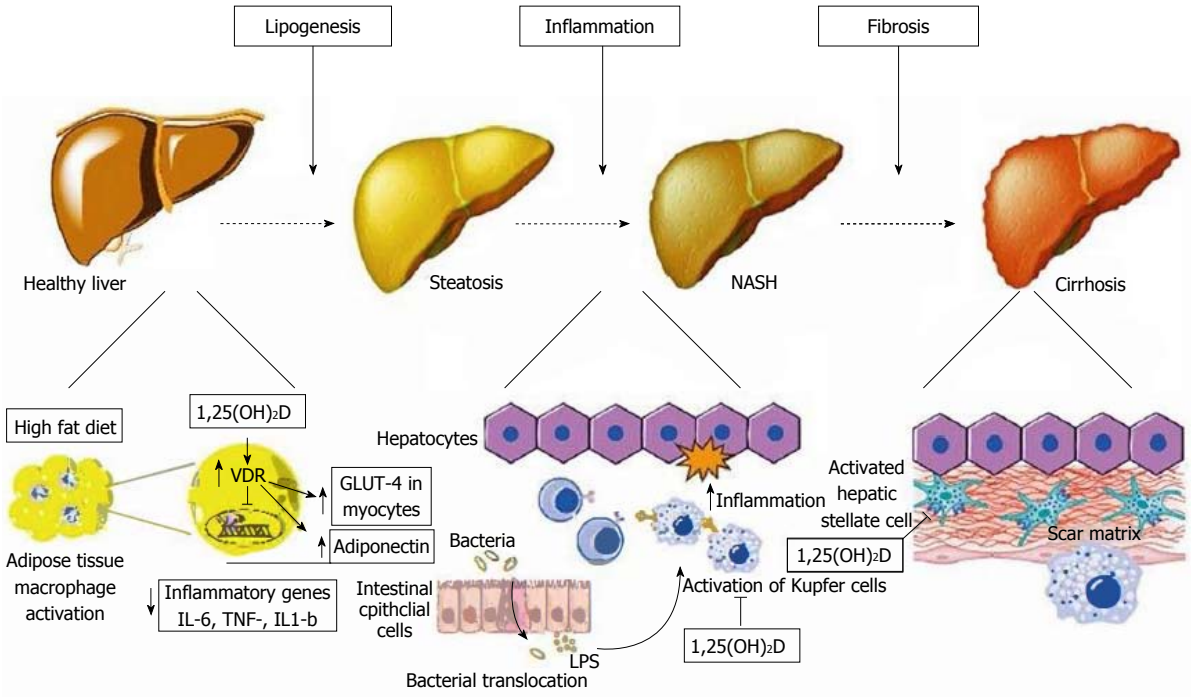

Figure 2 Schematic representation of metabolic, anti-inflammatory, and anti-fibrotic effects of vitamin D on hepatocytes and non-parenchymal hepatic cells (hepatic stellate cells, Kupffer cells) in non-alcoholic fatty liver disease. Left: At the initial stage of lipogenesis, 1,25(OH)D acts on adipocytes 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].

degree of hepatic fibrosis. Based on the METAVIR[34] system of histopathological staging in patients with NAFLD, we performed a systematic review and meta-analysis.

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

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	226

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 methodologies utilized by the authors to assess the severity of fibrosis by METAVIR 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.

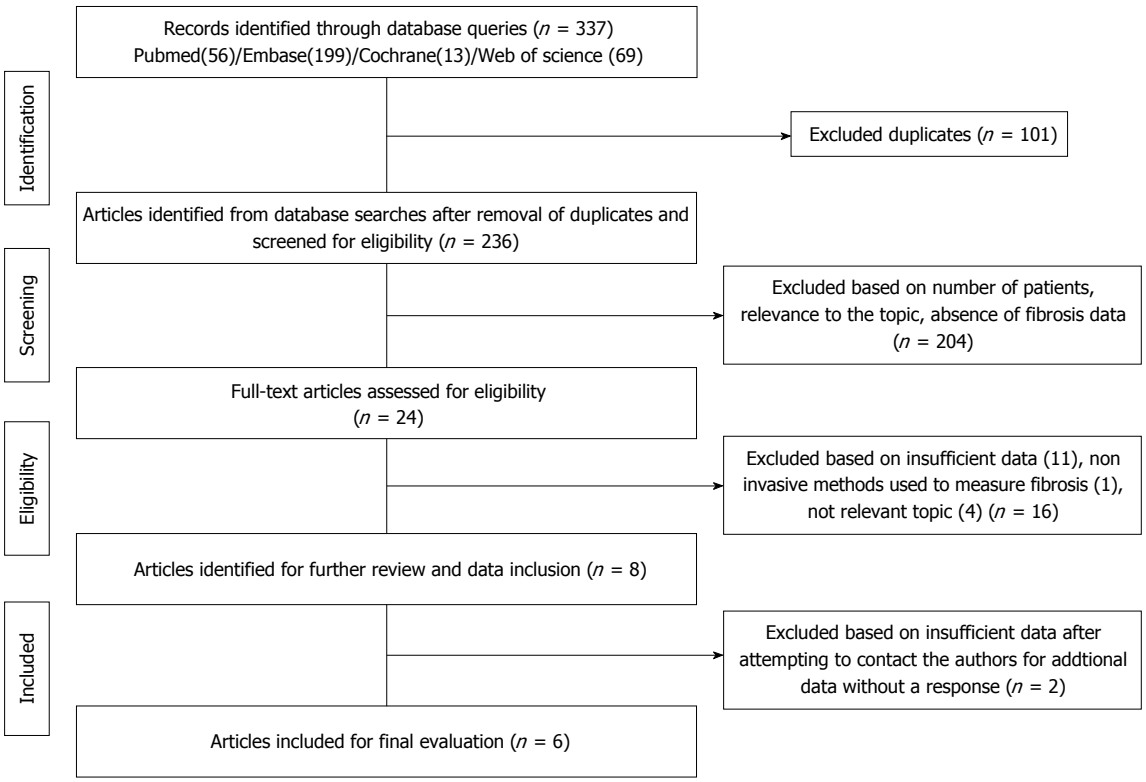

Figure 3 Flowchart illustrating the process for the selection of the included articles. Three hundred and thirty-seven articles were identified using PubMed (*n* = 56)/EMBASE (*n* = 199)/Cochrane (*n* = 13)/Web of Science (*n* = 69) search engines. A detailed evaluation of the articles by at least two 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.

RESULTS

Study selection

The search strategy utilized medical subject headings (MeSH) terms used to identify articles that evaluated 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

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*[30]	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.

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 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

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.

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-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

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

conducted a meta-regression to determine whether there was an association between serum vitamin D levels and METAVIR stage of liver fibrosis (F0-F4) in NAFLD. As shown in Table 4, there are conflicting reports with three studies demonstrating significance (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

Study or subgroup	Low fibrosis score Mean	SD	Total	High fibrosis score Mean	SD	Total	Weight	Mean difference IV, Random, 95%CI
Anty *et al*[27], 2016	26.9	10.7	172	29.4	15.4	72	23.9%	-2.50 [-6.40, 1.40]
Barchetta *et al*[28], 2012	16.6	10	33	6	10.8	6	9.4%	10.60 [1.31, 19.89]
Bril *et al*[29], 2015	19.5	9.1	381	17.7	10	17	20.3%	1.80 [-3.04. 6.64]
Luger *et al*[30], 2016	15.2	5.6	40	18.6	6.4	6	18.3%	-3.40 [-8.81, 2.01]
Patel *et al*[32], 2016	22.3	13.2	158	24.7	11.5	27	20.4%	-2.40 [-7.20, 2.40]
Targher *et al*[26], 2007	20.2	11.07	7	26.7	14.2	18	7.8%	-6.50 [-17.00, 4.00]
Total (95%CI)			791			146	100.0%	-0.86 [-4.17, 2.46]

Heterogeneity: Tau^2 = 8.00; χ^2 = 9.95, df = 5 (P = 0.08); I^2 = 50%
Test for overall effect: Z = 0.51 (P = 0.61)

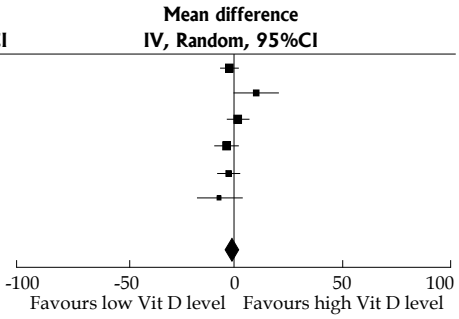

Figure 4 Random effects pooled the mean difference of 25-hydroxyvitamin D levels in nonalcoholic fatty liver disease patients with high and low fibrosis scores. A meta-analysis of the pooled data of the six included studies according to METAVIR fibrosis scores of low F0-2 *vs* high F3-4. Figure 4 illustrates 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 METAVIR low *vs* high score in NAFLD patients from the six included studies.

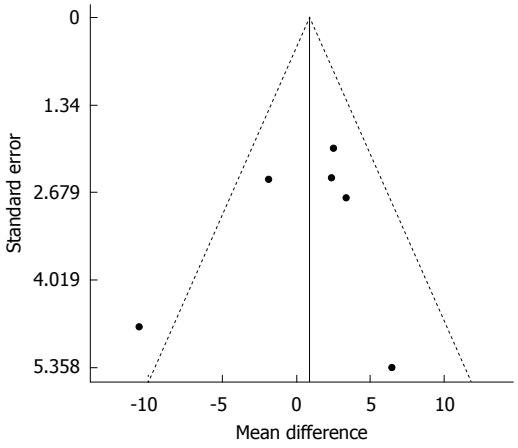

Figure 5 Funnel plot of standard error by differences in Means for 25(OH)D. We analyzed the data for a possible publication bias. The circles represent observed published studies. The funnel plot was asymmetric, thereby suggesting a possible publication bias.

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 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.

REFERENCES

1 **Sayiner M**, Koenig A, Henry L, Younossi ZM. Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis in the United States and the Rest of the World. *Clin Liver Dis* 2016; **20**: 205-214 [PMID: 27063264 DOI: 10.1016/j.cld.2015.10.001]

2 **Younossi ZM**, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* 2016; **64**: 73-84 [PMID: 26707365 DOI: 10.1002/hep.28431]

3 **Do A,** Lim JK. Epidemiology of nonalcoholic fatty liver disease: A primer. *Clinical Liver Disease* 2016 [DOI: 10.1002/cld.547]

4 **Vernon G**, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; **34**: 274-285 [PMID: 21623852 DOI: 10.1111/j.1365-2036.2011.04724.x]

5 **Rinella ME**. Nonalcoholic fatty liver disease: a systematic review. *JAMA* 2015; **313**: 2263-2273 [PMID: 26057287 DOI: 10.1001/jama.2015.5370]

6 **Wong RJ**, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, Ahmed A. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. *Gastroenterology* 2015; **148**: 547-555 [PMID: 25461851 DOI: 10.1053/j.gastro.2014.11.039]

7 **Holick MF**. Vitamin D deficiency. *N Engl J Med* 2007; **357**: 266-281 [PMID: 17634462 DOI: 10.1056/NEJMra070553]

8 **Iruzubieta P**, Terán Á, Crespo J, Fábrega E. Vitamin D deficiency in chronic liver disease. *World J Hepatol* 2014; **6**: 901-915 [PMID: 25544877 DOI: 10.4254/wjh.v6.i12.901]

9 **Eliades M**, Spyrou E. Vitamin D: a new player in non-alcoholic fatty liver disease? *World J Gastroenterol* 2015; **21**: 1718-1727 [PMID: 25684936 DOI: 10.3748/wjg.v21.i6.1718]

10 **Cantorna MT**. Mechanisms underlying the effect of vitamin D on the immune system. *Proc Nutr Soc* 2010; **69**: 286-289 [PMID: 20515520 DOI: 10.1017/S0029665110001722]

11 **Mocanu V**, Oboroceanu T, Zugun-Eloae F. Current status in vitamin D and regulatory T cells--immunological implications. *Rev Med Chir Soc Med Nat Iasi* 2013; **117**: 965-973 [PMID: 24502077]

12 **Alkharfy KM**, Al-Daghri NM, Yakout SM, Hussain T, Mohammed AK, Krishnaswamy S. Influence of vitamin D treatment on transcriptional regulation of insulin-sensitive genes. *Metab Syndr Relat Disord* 2013; **11**: 283-288 [PMID: 23621113 DOI: 10.1089/met.2012.0068]

13 **Adolph TE**, Grander C, Grabherr F, Tilg H. Adipokines and Non-Alcoholic Fatty Liver Disease: Multiple Interactions. *Int J Mol Sci* 2017; **18**: pii: E1649 [PMID: 28758929 DOI: 10.3390/ijms18081649]

14 **Carlberg C**. Genome-wide (over)view on the actions of vitamin D. *Front Physiol* 2014; **5**: 167 [PMID: 24808867 DOI: 10.3389/fphys.2014.00167]

15 **Banerjee A**, Khemka VK, Roy D, Poddar J, Roy TKS, Karnam SA. Role of Serum Adiponectin and Vitamin D in Prediabetes and Diabetes Mellitus. *Can J Diabetes* 2017; **41**: 259-265 [PMID: 28236525 DOI: 10.1016/j.jcjd.2016.10.006]

16 **Abramovitch S**, Sharvit E, Weisman Y, Bentov A, Brazowski E, Cohen G, Volovelsky O, Reif S. Vitamin D inhibits development of liver fibrosis in an animal model but cannot ameliorate established cirrhosis. *Am J Physiol Gastrointest Liver Physiol* 2015; **308**: G112-G120 [PMID: 25214398 DOI: 10.1152/ajpgi.00132.2013]

17 **Arteh J**, Narra S, Nair S. Prevalence of vitamin D deficiency in chronic liver disease. *Dig Dis Sci* 2010; **55**: 2624-2628 [PMID: 19960254 DOI: 10.1007/s10620-009-1069-9]

18 **Terrier B**, Carrat F, Geri G, Pol S, Piroth L, Halfon P, Poynard T, Souberbielle JC, Cacoub P. Low 25-OH vitamin D serum levels correlate with severe fibrosis in HIV-HCV co-infected patients with chronic hepatitis. *J Hepatol* 2011; **55**: 756-761 [PMID: 21334402 DOI: 10.1016/j.jhep.2011.01.041]

19 **Farnik H**, Bojunga J, Berger A, Allwinn R, Waidmann O, Kronenberger B, Keppler OT, Zeuzem S, Sarrazin C, Lange CM. Low vitamin D serum concentration is associated with high levels of hepatitis B virus replication in chronically infected patients. *Hepatology* 2013; **58**: 1270-1276 [PMID: 23703797 DOI: 10.1002/hep.26488]

20 **Efe C**, Kav T, Aydin C, Cengiz M, Imga NN, Purnak T, Smyk DS, Torgutalp M, Turhan T, Ozenirler S, Ozaslan E, Bogdanos DP. Low serum vitamin D levels are associated with severe histological features and poor response to therapy in patients with autoimmune hepatitis. *Dig Dis Sci* 2014; **59**: 3035-3042 [PMID: 25002309 DOI: 10.1007/s10620-014-3267-3]

21 **Franco AS**, Freitas TQ, Bernardo WM, Pereira RMR. Vitamin D supplementation and disease activity in patients with immune-mediated rheumatic diseases: A systematic review and meta-analysis. *Medicine* (Baltimore) 2017; **96**: e7024 [PMID: 28591033 DOI: 10.1097/MD.0000000000007024]

22 **Dadabhai AS**, Saberi B, Lobner K, Shinohara RT, Mullin GE. Influence of vitamin D on liver fibrosis in chronic hepatitis C: A systematic review and meta-analysis of the pooled clinical trials data. *World J Hepatol* 2017; **9**: 278-287 [PMID: 28261385 DOI: 10.4254/wjh.v9.i5.278]

23 **Wang X**, Li W, Zhang Y, Yang Y, Qin G. Association between vitamin D and non-alcoholic fatty liver disease/non-alcoholic steatohepatitis: results from a meta-analysis. *Int J Clin Exp Med* 2015; **8**: 17221-17234 [PMID: 26770315]

24 **Dasarathy J**, Periyalwar P, Allampati S, Bhinder V, Hawkins C, Brandt P, Khiyami A, McCullough AJ, Dasarathy S. Hypovitaminosis D is associated with increased whole body fat mass and greater severity of non-alcoholic fatty liver disease. *Liver Int* 2014; **34**: e118-e127 [PMID: 24118743 DOI: 10.1111/liv.12312]

25 **Liangpunsakul S**, Chalasani N. Serum vitamin D concentrations and unexplained elevation in ALT among US adults. *Dig Dis Sci*

2011; **56**: 2124-2129 [PMID: 21503677 DOI: 10.1007/s10620-011-1707-x]
26 **Targher G**, Bertolini L, Scala L, Cigolini M, Zenari L, Falezza G, Arcaro G. Associations between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis* 2007; **17**: 517-524 [PMID: 16928437 DOI: 10.1016/j.numecd.2006.04.002]
27 **Anty R**, Hastier A, Canivet CM, Patouraux S, Schneck AS, Ferrari-Panaia P, Ben-Amor I, Saint-Paul MC, Gugenheim J, Gual P, Iannelli A, Tran A. Severe Vitamin D Deficiency Is Not Associated with Liver Damage in Morbidly Obese Patients. *Obes Surg* 2016; **26**: 2138-2143 [PMID: 26787197 DOI: 10.1007/s11695-016-2070-y]
28 **Barchetta I**, Carotti S, Labbadia G, Gentilucci UV, Muda AO, Angelico F, Silecchia G, Leonetti F, Fraioli A, Picardi A, Morini S, Cavallo MG. Liver vitamin D receptor, CYP2R1, and CYP27A1 expression: relationship with liver histology and vitamin D3 levels in patients with nonalcoholic steatohepatitis or hepatitis C virus. *Hepatology* 2012; **56**: 2180-2187 [PMID: 22753133 DOI: 10.1002/hep.25930]
29 **Bril F**, Maximos M, Portillo-Sanchez P, Biernacki D, Lomonaco R, Subbarayan S, Correa M, Lo M, Suman A, Cusi K. Relationship of vitamin D with insulin resistance and disease severity in non-alcoholic steatohepatitis. *J Hepatol* 2015; **62**: 405-411 [PMID: 25195551 DOI: 10.1016/j.jhep.2014.08.040]
30 **Luger M**, Kruschitz R, Kienbacher C, Traussnigg S, Langer FB, Schindler K, Würger T, Wrba F, Trauner M, Prager G, Ludvik B. Prevalence of Liver Fibrosis and its Association with Non-invasive Fibrosis and Metabolic Markers in Morbidly Obese Patients with Vitamin D Deficiency. *Obes Surg* 2016; **26**: 2425-2432 [PMID: 26989059 DOI: 10.1007/s11695-016-2123-2]
31 **Nelson JE**, Roth CL, Wilson LA, Yates KP, Aouizerat B, Morgan-Stevenson V, Whalen E, Hoofnagle A, Mason M, Gersuk V, Yeh MM, Kowdley KV. Vitamin D Deficiency Is Associated With Increased Risk of Non-alcoholic Steatohepatitis in Adults With Non-alcoholic Fatty Liver Disease: Possible Role for MAPK and NF-κB? *Am J Gastroenterol* 2016; **111**: 852-863 [PMID: 27002799 DOI: 10.1038/ajg.2016.51]
32 **Patel YA**, Henao R, Moylan CA, Guy CD, Piercy DL, Diehl AM, Abdelmalek MF. Vitamin D is Not Associated With Severity in NAFLD: Results of a Paired Clinical and Gene Expression Profile Analysis. *Am J Gastroenterol* 2016; **111**: 1591-1598 [PMID: 27644736 DOI: 10.1038/ajg.2016.406]
33 **Jaruvongvanich V**, Ahuja W, Sanguankeo A, Wijarnpreecha K, Upala S. Vitamin D and histologic severity of nonalcoholic fatty liver disease: A systematic review and meta-analysis. *Dig Liver Dis* 2017; **49**: 618-622 [PMID: 28274829 DOI: 10.1016/j.dld.2017.02.003]
34 **Mohamadnejad M**, Tavangar SM, Sotoudeh M, Kosari F, Khosravi M, Geramizadeh B, Montazeri G, Estakhri A, Mirnasseri MM, Fazlollahi A, Zamani F, Malekzadeh R. Histopathological Study of Chronic Hepatitis B: A Comparative Study of Ishak and METAVIR Scoring Systems. *Int J Organ Transplant Med* 2010; **1**: 171-176 [PMID: 25013582]
35 **Knobloch K**, Yoon U, Vogt PM. Preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement and publication bias. *J Craniomaxillofac Surg* 2011; **39**: 91-92 [PMID: 21145753 DOI: 10.1016/j.jcms.2010.11.001]
36 **R Foundation for Statistical Computing.** A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2014
37 **Thacher TD**, Clarke BL. Vitamin D insufficiency. *Mayo Clin Proc* 2011; **86**: 50-60 [PMID: 21193656 DOI: 10.4065/mcp.2010.0567]
38 **Satapathy SK**, Sanyal AJ. Epidemiology and Natural History of Nonalcoholic Fatty Liver Disease. *Semin Liver Dis* 2015; **35**: 221-235 [PMID: 26378640 DOI: 10.1055/s-0035-1562943]
39 **Singh S**, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. *Clin Gastroenterol Hepatol* 2015; **13**: 643-654. e1-9; quiz e39-40 [PMID: 24768810 DOI: 10.1016/j.cgh.2014.04.014]
40 **Chun RF**, Liu PT, Modlin RL, Adams JS, Hewison M. Impact of vitamin D on immune function: lessons learned from genome-wide analysis. *Front Physiol* 2014; **5**: 151 [PMID: 24795646 DOI: 10.3389/fphys.2014.00151]
41 **Pittas AG**, Lau J, Hu FB, Dawson-Hughes B. The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. *J Clin Endocrinol Metab* 2007; **92**: 2017-2029 [PMID: 17389701 DOI: 10.1210/jc.2007-0298]
42 **Friedman SL**. Liver fibrosis -- from bench to bedside. *J Hepatol* 2003; **38** Suppl 1: S38-S53 [PMID: 12591185 DOI: 10.1016/S0168-8278(02)00429-4]
43 **Ding N**, Liddle C, Evans RM, Downes M. Hepatic actions of vitamin D receptor ligands: a sunshine option for chronic liver disease? *Expert Rev Clin Pharmacol* 2013; **6**: 597-599 [PMID: 24164608 DOI: 10.1586/17512433.2013.841078]
44 **Gascon-Barré M**, Demers C, Mirshahi A, Néron S, Zalzal S, Nanci A. The normal liver harbors the vitamin D nuclear receptor in nonparenchymal and biliary epithelial cells. *Hepatology* 2003; **37**: 1034-1042 [PMID: 12717384 DOI: 10.1053/jhep.2003.50176]
45 **Ding N**, Yu RT, Subramaniam N, Sherman MH, Wilson C, Rao R, Leblanc M, Coulter S, He M, Scott C, Lau SL, Atkins AR, Barish GD, Gunton JE, Liddle C, Downes M, Evans RM. A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 2013; **153**: 601-613 [PMID: 23622244 DOI: 10.1016/j.cell.2013.03.028]
46 **Autier P**, Boniol M, Pizot C, Mullie P. Vitamin D status and ill health: a systematic review. *Lancet Diabetes Endocrinol* 2014; **2**: 76-89 [PMID: 24622671 DOI: 10.1016/S2213-8587(13)70165-7]
47 **Sharifi N**, Amani R, Hajiani E, Cheraghian B. Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial. *Endocrine* 2014; **47**: 70-80 [PMID: 24968737 DOI: 10.1007/s12020-014-0336-5]
48 **Lorvand Amiri H**, Agah S, Mousavi SN, Hosseini AF, Shidfar F. Regression of Non-Alcoholic Fatty Liver by Vitamin D Supplement: A Double-Blind Randomized Controlled Clinical Trial. *Arch Iran Med* 2016; **19**: 631-638 [PMID: 27631178 DOI: 10.0161909/AIM.006:]
49 **Barchetta I**, Del Ben M, Angelico F, Di Martino M, Fraioli A, La Torre G, Saulle R, Perri L, Morini S, Tiberti C, Bertoccini L, Cimini FA, Panimolle F, Catalano C, Baroni MG, Cavallo MG. No effects of oral vitamin D supplementation on non-alcoholic fatty liver disease in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled trial. *BMC Med* 2016; **14**: 92 [PMID: 27353492 DOI: 10.1186/s12916-016-0638-y]
50 **Della Corte C**, Carpino G, De Vito R, De Stefanis C, Alisi A, Cianfarani S, Overi D, Mosca A, Stronati L, Cucchiara S, Raponi M, Gaudio E, Byrne CD, Nobili V. Docosahexanoic Acid Plus Vitamin D Treatment Improves Features of NAFLD in Children with Serum Vitamin D Deficiency: Results from a Single Centre Trial. *PLoS One* 2016; **11**: e0168216 [PMID: 27977757 DOI: 10.1371/journal.pone.0168216]
51 **Dasarathy J**, Varghese R, Feldman A, Khiyami A, McCullough AJ, Dasarathy S. Patients with Nonalcoholic Fatty Liver Disease Have a Low Response Rate to Vitamin D Supplementation. *J Nutr* 2017; **147**: 1938-1946 [PMID: 28814531 DOI: 10.3945/jn.117.254292]
52 **Mathieu C.** Vitamin D and the immune system: Getting it right. I. *IBMS BoneKEy* 2011; **8**: 178-186 [DOI: 10.1138/20110505]
53 **Kleiner DE**, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321 [PMID: 15915461 DOI: 10.1002/hep.20701]
54 **Brunt EM**, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**:

2467-2474 [PMID: 10484010 DOI: 10.1111/j.1572-0241.1999.01377.x]

55 **Klingberg E**, Oleröd G, Konar J, Petzold M, Hammarsten O. Seasonal variations in serum 25-hydroxy vitamin D levels in a Swedish cohort. *Endocrine* 2015; **49**: 800-808 [PMID: 25681052 DOI: 10.1007/s12020-015-0548-3]

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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.

Bhat V, Srinathan S, Pasini E, Angeli M, Chen E, Baciu C, Bhat M. Epigenetic basis of hepatocellular carcinoma: A network-based integrative meta-analysis. *World J Hepatol* 2018; 10(1): 155-165 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i1/155.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i1.155

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

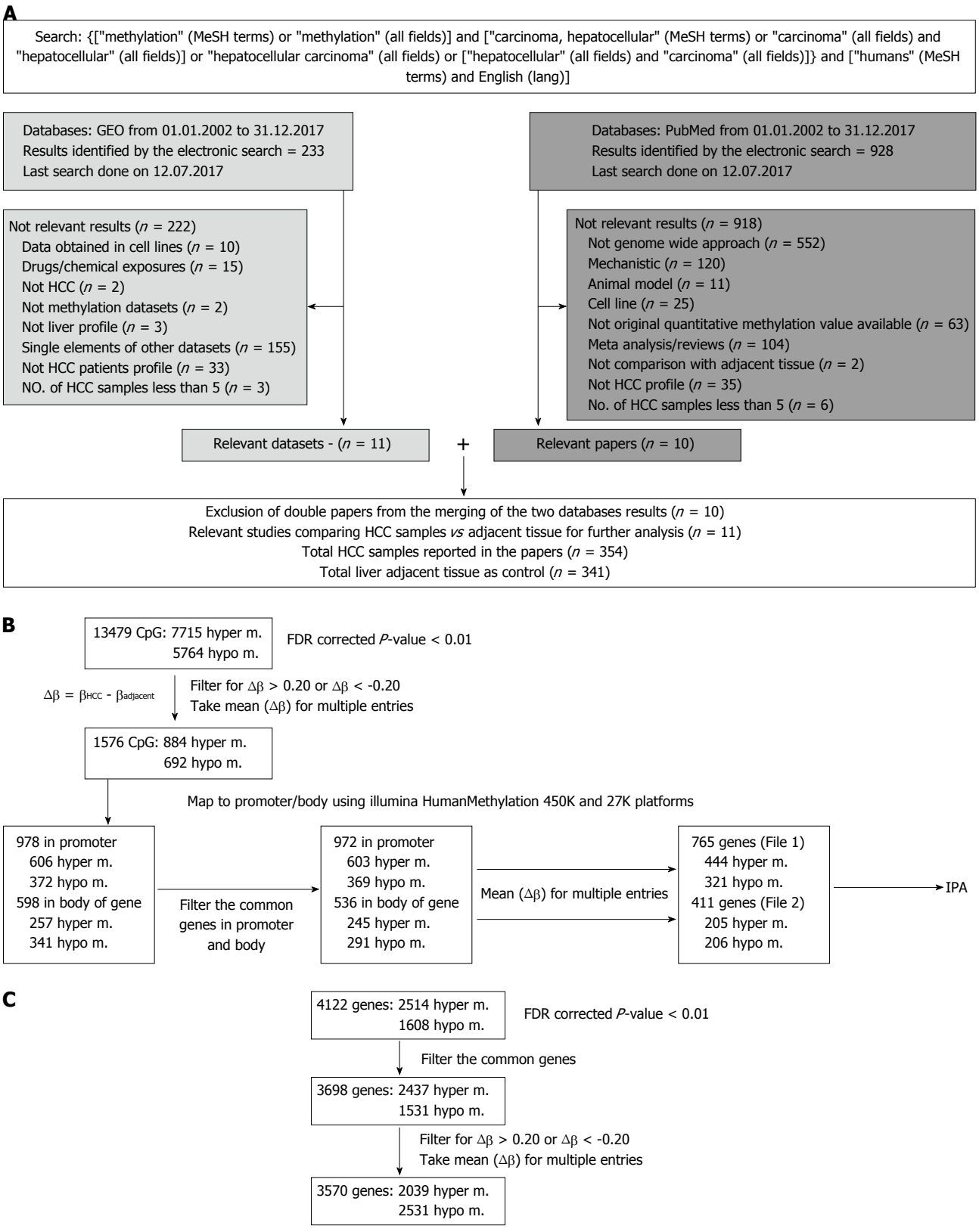

Figure 1 Flow chart. A: Workflow of Data collection, analysis and database compiling; B: Bioinformatics flow for selecting differentially methylated CpG sites from HCC meta-analysis; C: Bioinformatics flow for selecting differentially methylated genes from HCC meta-analysis. HCC: Hepatocellular carcinoma.

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

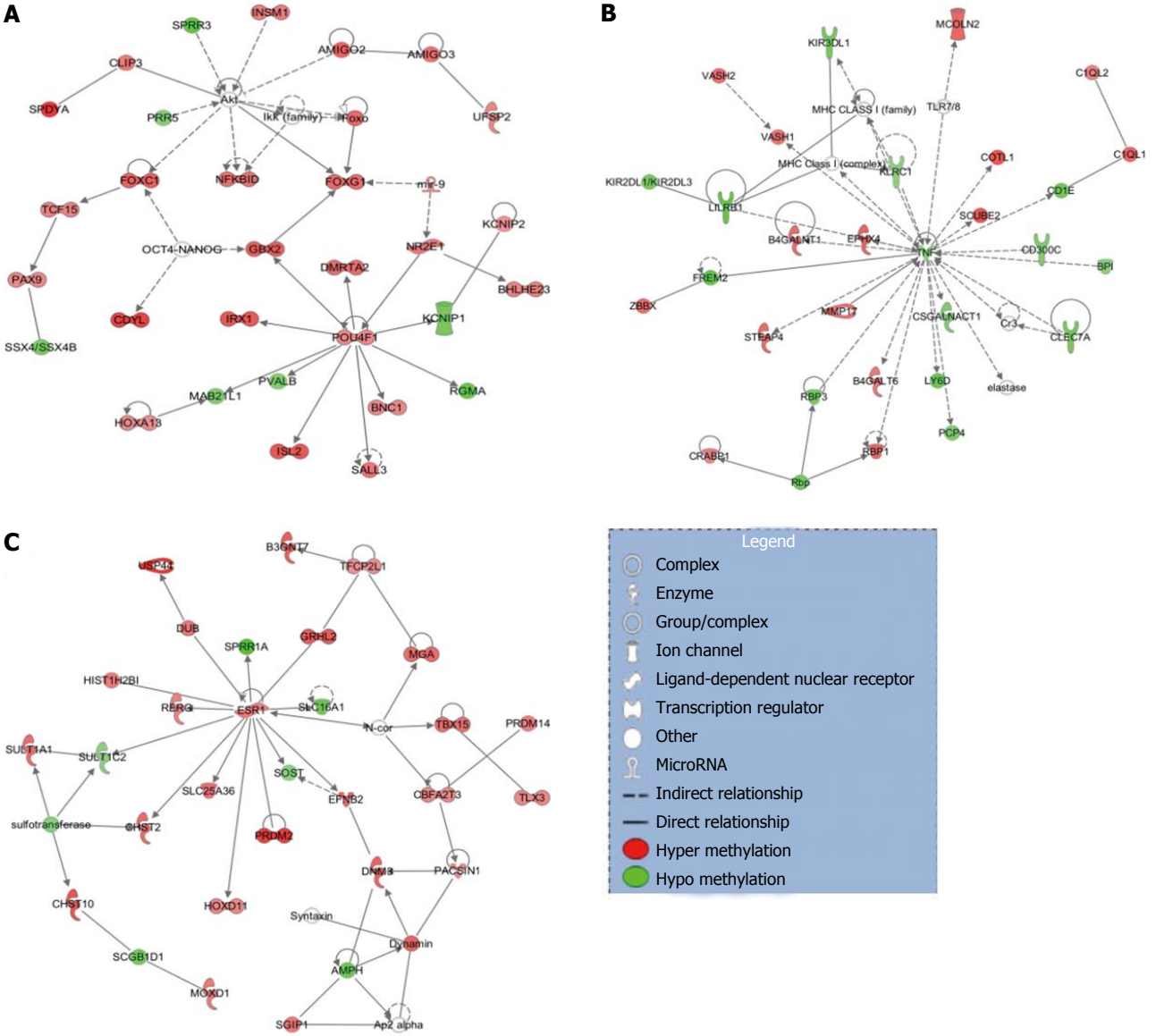

Figure 2 Networks associated with differentially methylated CpG sites in the promoter regions of genes. A: Organismal development, organismal injury and abnormalities, cellular development; B: Lipid metabolism, small molecule biochemistry, cell death and survival; C: Cell-to-cell signaling and interaction, 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

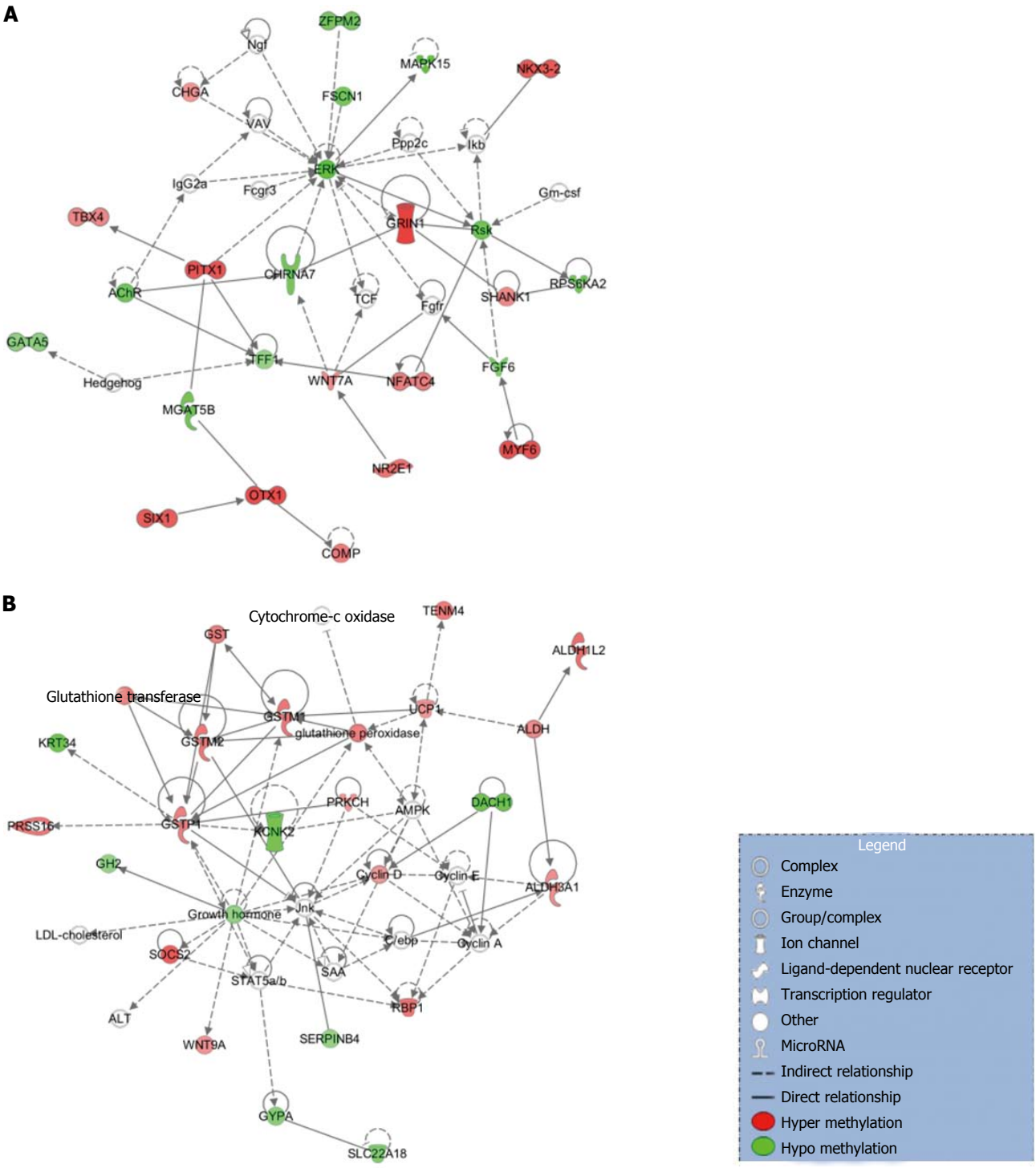

Figure 3 Networks associated with differentially methylated CpG sites in body of the gene. A: Cellular assembly and organization, cellular function and maintenance; B: Drug metabolism, glutathione depletion in liver, small molecule biochemistry.

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

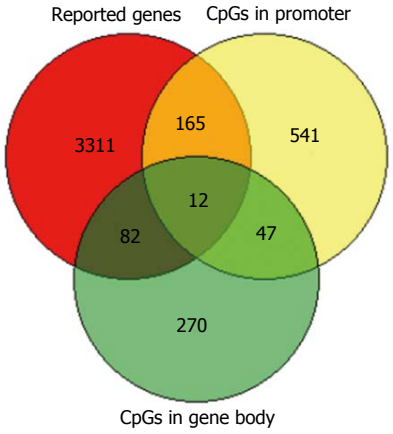

Figure 4 Venn diagram intersecting three lists: (1) reported differentially methylated genes in HCC from studies not providing information on the corresponding CpG sites; (2) identified differentially methylated genes in HCC corresponding to CpG sites in promoter; and (3) identified differentially methylated 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.

REFERENCES

1 **El-Serag HB**, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576 [PMID: 17570226 DOI: 10.1053/j.gastro.2007.04.061]

2 **Bruix J**, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]

3 **Llovet JM**, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol* 2015; **12**: 436 [PMID: 26099984 DOI: 10.1038/nrclinonc.2015.121]

4 **Egger G**, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004; **429**: 457-463 [PMID: 15164071 DOI: 10.1038/nature02625]

5 **Jones PA**, Takai D. The role of DNA methylation in mammalian epigenetics. *Science* 2001; **293**: 1068-1070 [PMID: 11498573 DOI: 10.1126/science.1063852]

6 **Karpf AR**, Jones DA. Reactivating the expression of methylation silenced genes in human cancer. *Oncogene* 2002; **21**: 5496-5503 [PMID: 12154410 DOI: 10.1038/sj.onc.1205602]

7 **Das PM**, Singal R. DNA methylation and cancer. *J Clin Oncol* 2004; **22**: 4632-4642 [PMID: 15542813 DOI: 10.1200/JCO.2004.07.151]

8 **Yang B**, Guo M, Herman JG, Clark DP. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. *Am J Pathol* 2003; **163**: 1101-1107 [PMID: 12937151 DOI: 10.1016/S0002-9440(10)63469-4]

9 **Ma L**, Chua MS, Andrisani O, So S. Epigenetics in hepatocellular carcinoma: an update and future therapy perspectives. *World J Gastroenterol* 2014; **20**: 333-345 [PMID: 24574704 DOI: 10.3748/wjg.v20.i2.333]

10 **Matsuda Y**, Ichida T, Matsuzawa J, Sugimura K, Asakura H. p16(INK4) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. *Gastroenterology* 1999; **116**: 394-400 [PMID: 9922321]

11 **Yeo W**, Wong N, Wong WL, Lai PB, Zhong S, Johnson PJ. High frequency of promoter hypermethylation of RASSF1A in tumor and plasma of patients with hepatocellular carcinoma. *Liver Int* 2005; **25**: 266-272 [PMID: 15780049 DOI: 10.1111/j.1478-3231.2005.01084.x]

12 **Zhang YJ**, Ahsan H, Chen Y, Lunn RM, Wang LY, Chen SY, Lee PH, Chen CJ, Santella RM. High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. *Mol Carcinog* 2002; **35**: 85-92 [PMID: 12325038 DOI: 10.1002/mc.10076]

13 **Zhang YJ**, Rossner P Jr, Chen Y, Agrawal M, Wang Q, Wang L, Ahsan H, Yu MW, Lee PH, Santella RM. Aflatoxin B1 and polycyclic aromatic hydrocarbon adducts, p53 mutations and p16 methylation in liver tissue and plasma of hepatocellular carcinoma patients. *Int J Cancer* 2006; **119**: 985-991 [PMID: 16570275 DOI: 10.1002/ijc.21699]

14 **Zhong S**, Tang MW, Yeo W, Liu C, Lo YM, Johnson PJ. Silencing of GSTP1 gene by CpG island DNA hypermethylation in HBV-associated hepatocellular carcinomas. *Clin Cancer Res* 2002; **8**: 1087-1092 [PMID: 11948118]

15 **Zhang YJ**, Chen Y, Ahsan H, Lunn RM, Lee PH, Chen CJ, Santella RM. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation and its relationship to aflatoxin B1-DNA adducts and p53 mutation in hepatocellular carcinoma. *Int J Cancer* 2003; **103**: 440-444 [PMID: 12478658 DOI: 10.1002/ijc.10852]

16 **Song MA**, Tiirikainen M, Kwee S, Okimoto G, Yu H, Wong LL. Elucidating the landscape of aberrant DNA methylation in hepatocellular carcinoma. *PLoS One* 2013; **8**: e55761 [PMID: 23437062 DOI: 10.1371/journal.pone.0055761]

17 **Shangguan H**, Tan SY, Zhang JR. Bioinformatics analysis of gene expression profiles in hepatocellular carcinoma. *Eur Rev Med Pharmacol Sci* 2015; **19**: 2054-2061 [PMID: 26125269]

18 **Fortney K**, Griesman J, Kotlyar M, Pastrello C, Angeli M, Sound-Tsao M, Jurisica I. Prioritizing therapeutics for lung cancer: an integrative meta-analysis of cancer gene signatures and chemogenomic data. *PLoS Comput Biol* 2015; **11**: e1004068 [PMID: 25786242 DOI: 10.1371/journal.pcbi.1004068]

19 **Stefanska B**, Huang J, Bhattacharyya B, Suderman M, Hallett M, Han ZG, Szyf M. Definition of the landscape of promoter DNA hypomethylation in liver cancer. *Cancer Res* 2011; **71**: 5891-5903 [PMID: 21747116 DOI: 10.1158/0008-5472.CAN-10-3823]

20 **Archer KJ**, Mas VR, Maluf DG, Fisher RA. High-throughput assessment of CpG site methylation for distinguishing between HCV-cirrhosis and HCV-associated hepatocellular carcinoma. *Mol Genet Genomics* 2010; **283**: 341-349 [PMID: 20165882 DOI: 10.1007/s00438-010-0522-y]

21 **Shen J**, Wang S, Zhang YJ, Kappil M, Wu HC, Kibriya MG, Wang Q, Jasmine F, Ahsan H, Lee PH, Yu MW, Chen CJ, Santella RM. Genome-wide DNA methylation profiles in hepatocellular carcinoma. *Hepatology* 2012; **55**: 1799-1808 [PMID: 22234943 DOI: 10.1002/hep.25569]

22 **Revill K**, Wang T, Lachenmayer A, Kojima K, Harrington A, Li J, Hoshida Y, Llovet JM, Powers S. Genome-wide methylation analysis and epigenetic unmasking identify tumor suppressor genes in hepatocellular carcinoma. *Gastroenterology* 2013; **145**: 1424-35.e1-25 [PMID: 24012984 DOI: 10.1053/j.gastro.2013.08.055]

23 **Shen J**, Wang S, Zhang YJ, Wu HC, Kibriya MG, Jasmine F, Ahsan H, Wu DP, Siegel AB, Remotti H, Santella RM. Exploring genome-wide DNA methylation profiles altered in hepatocellular carcinoma using Infinium HumanMethylation 450 BeadChips. *Epigenetics* 2013; **8**: 34-43 [PMID: 23208076 DOI: 10.4161/epi.23062]

24 **Mah WC**, Thurnherr T, Chow PK, Chung AY, Ooi LL, Toh HC, Teh BT, Sauntharajah Y, Lee CG. Methylation profiles reveal distinct subgroup of hepatocellular carcinoma patients with poor prognosis. *PLoS One* 2014; **9**: e104158 [PMID: 25093504 DOI: 10.1371/journal.pone.0104158]

25 **Hlady RA**, Tiedemann RL, Puszyk W, Zendejas I, Roberts LR, Choi JH, Liu C, Robertson KD. Epigenetic signatures of alcohol abuse and hepatitis infection during human hepatocarcinogenesis. *Oncotarget* 2014; **5**: 9425-9443 [PMID: 25294808 DOI: 10.18632/oncotarget.2444]

26 **Ammerpohl O**, Pratschke J, Schafmayer C, Haake A, Faber W, von Kampen O, Brosch M, Sipos B, von Schönfels W, Balschun K, Röcken C, Arlt A, Schniewind B, Grauholm J, Kalthoff H, Neuhaus P, Stickel F, Schreiber S, Becker T, Siebert R, Hampe J. Distinct DNA methylation patterns in cirrhotic liver and hepatocellular carcinoma. *Int J Cancer* 2012; **130**: 1319-1328 [PMID: 21500188 DOI: 10.1002/ijc.26136]

27 **Udali S**, Guarini P, Ruzzenente A, Ferrarini A, Guglielmi A, Lotto V, Tononi P, Pattini P, Moruzzi S, Campagnaro T, Conci S, Olivieri O, Corrocher R, Delledonne M, Choi SW, Friso S. DNA methylation and gene expression profiles show novel regulatory pathways in hepatocellular carcinoma. *Clin Epigenetics* 2015; **7**: 43 [PMID: 25945129 DOI: 10.1186/s13148-015-0077-1]

28 **Hou X**, Peng JX, Hao XY, Cai JP, Liang LJ, Zhai JM, Zhang KS, Lai JM, Yin XY. DNA methylation profiling identifies EYA4 gene as a prognostic molecular marker in hepatocellular carcinoma. *Ann Surg Oncol* 2014; **21**: 3891-3899 [PMID: 24306662 DOI: 10.1245/s10434-013-3401-z]

29 **Nishida N**, Chishina H, Arizumi T, Takita M, Kitai S, Yada N, Hagiwara S, Inoue T, Minami Y, Ueshima K, Sakurai T, Kudo M. Identification of epigenetically inactivated genes in human hepatocellular carcinoma by integrative analyses of methylation profiling and pharmacological unmasking. *Dig Dis* 2014; **32**: 740-746 [PMID: 25376292 DOI: 10.1159/000368015]

30 **Woo HG**, Choi JH, Yoon S, Jee BA, Cho EJ, Lee JH, Yu SJ, Yoon JH, Yi NJ, Lee KW, Suh KS, Kim YJ. Integrative analysis of genomic and epigenomic regulation of the transcriptome in liver cancer. *Nat Commun* 2017; **8**: 839 [PMID: 29018224 DOI: 10.1038/s41467-017-00991-w]

31 **R Development Core Team**. R: A Language and Environment

for Statistical Computing. R Foundation for Statistical Computing 2008
32 **Benjamini Y,** Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B* (Methodological) 1995; **57**: 125-133
33 **Fisher RA**. On the Interpretation of χ2 from Contingency Tables, and the Calculation of P. *J Royal Stat Soc* 1922; **85**: 87-94 [DOI: 10.2307/2340521]
34 **Llovet JM**, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol* 2015; **12**: 408-424 [PMID: 26054909 DOI: 10.1038/nrclinonc.2015.103]
35 **Heimbach JK**, Kulik LM, Finn R, Sirlin CB, Abecassis M, Roberts LR, Zhu A, Murad MH, Marrero J. Aasld guidelines for the treatment of hepatocellular carcinoma. *Hepatology* 2017; **67**: 358-380 [PMID: 28130846 DOI: 10.1002/hep.29086]
36 **Wahid B**, Ali A, Rafique S, Idrees M. New Insights into the Epigenetics of Hepatocellular Carcinoma. *Biomed Res Int* 2017; **2017**: 1609575 [PMID: 28401148 DOI: 10.1155/2017/1609575]
37 **Barabási AL**, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. *Nat Rev Genet* 2011; **12**: 56-68 [PMID: 21164525 DOI: 10.1038/nrg2918]
38 **Roadmap Epigenomics Consortium**, Kundaje A, Meuleman W, Ernst J, Bilenky M, Yen A, Heravi-Moussavi A, Kheradpour P, Zhang Z, Wang J, Ziller MJ, Amin V, Whitaker JW, Schultz MD, Ward LD, Sarkar A, Quon G, Sandstrom RS, Eaton ML, Wu YC, Pfenning AR, Wang X, Claussnitzer M, Liu Y, Coarfa C, Harris RA, Shoresh N, Epstein CB, Gjoneska E, Leung D, Xie W, Hawkins RD, Lister R, Hong C, Gascard P, Mungall AJ, Moore R, Chuah E, Tam A, Canfield TK, Hansen RS, Kaul R, Sabo PJ, Bansal MS, Carles A, Dixon JR, Farh KH, Feizi S, Karlic R, Kim AR, Kulkarni A, Li D, Lowdon R, Elliott G, Mercer TR, Neph SJ, Onuchic V, Polak P, Rajagopal N, Ray P, Sallari RC, Siebenthall KT, Sinnott-Armstrong NA, Stevens M, Thurman RE, Wu J, Zhang B, Zhou X, Beaudet AE, Boyer LA, De Jager PL, Farnham PJ, Fisher SJ, Haussler D, Jones SJ, Li W, Marra MA, McManus MT, Sunyaev S, Thomson JA, Tlsty TD, Tsai LH, Wang W, Waterland RA, Zhang MQ, Chadwick LH, Bernstein BE, Costello JF, Ecker JR, Hirst M, Meissner A, Milosavljevic A, Ren B, Stamatoyannopoulos JA, Wang T, Kellis M. Integrative analysis of 111 reference human epigenomes. *Nature* 2015; **518**: 317-330 [PMID: 25693563 DOI: 10.1038/nature14248]
39 **Stransky N**, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortés ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareño C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA, Grandis JR. The mutational landscape of head and neck squamous cell carcinoma. *Science* 2011; **333**: 1157-1160 [PMID: 21798893 DOI: 10.1126/science.1208130]
40 **Li CW**, Chen BS. Investigating core genetic-and-epigenetic cell cycle networks for stemness and carcinogenic mechanisms, and cancer drug design using big database mining and genome-wide next-generation sequencing data. *Cell Cycle* 2016; **15**: 2593-2607 [PMID: 27295129 DOI: 10.1080/15384101.2016.1198862]
41 **Mani S**, Herceg Z. DNA demethylating agents and epigenetic therapy of cancer. *Adv Genet* 2010; **70**: 327-340 [PMID: 20920754 DOI: 10.1016/B978-0-12-380866-0.60012-5]

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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

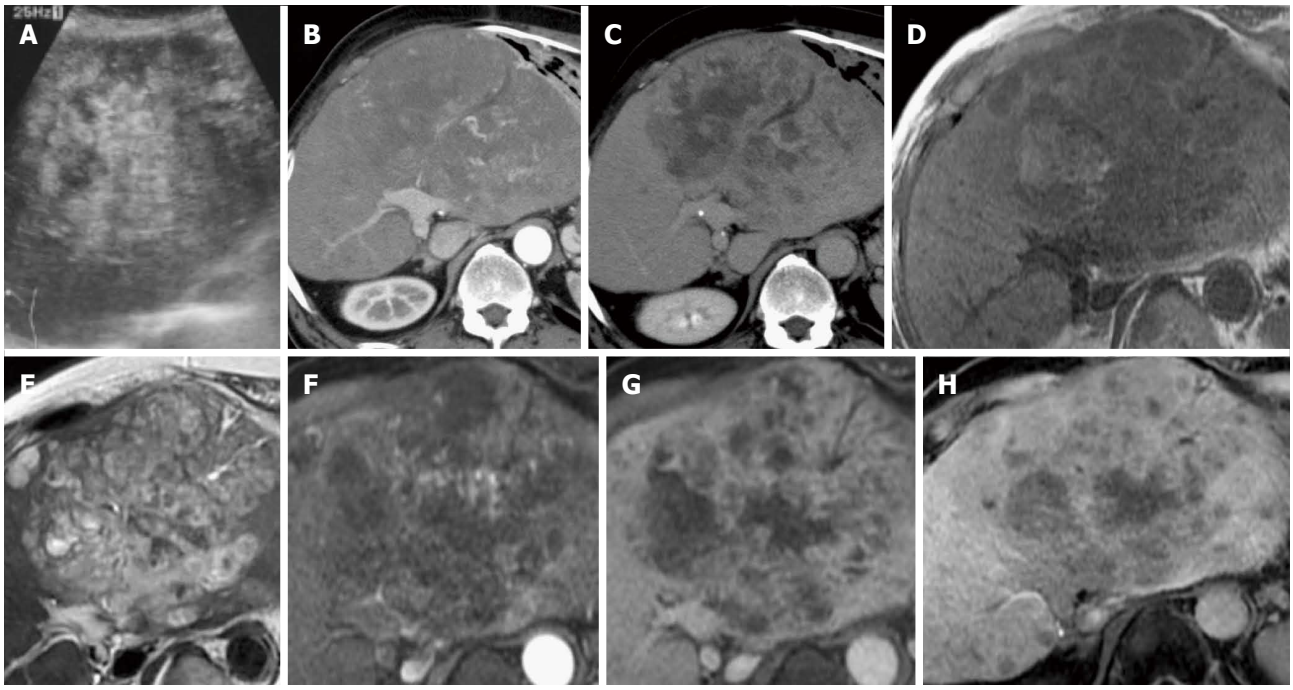

Figure 1 Abdominal ultrasonography. A: B-mode ultrasonography; B: Arterial phase; C: Venous phase images obtained using contrast-enhanced computed tomography; D: T1-weighted phase; E: T2-weighted phase; F: Arterial phase; G: Venous phase; H: Hepatobiliary phase images obtained using gadolinium ethoxybenzyl-diethylenetriamine-enhanced 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.

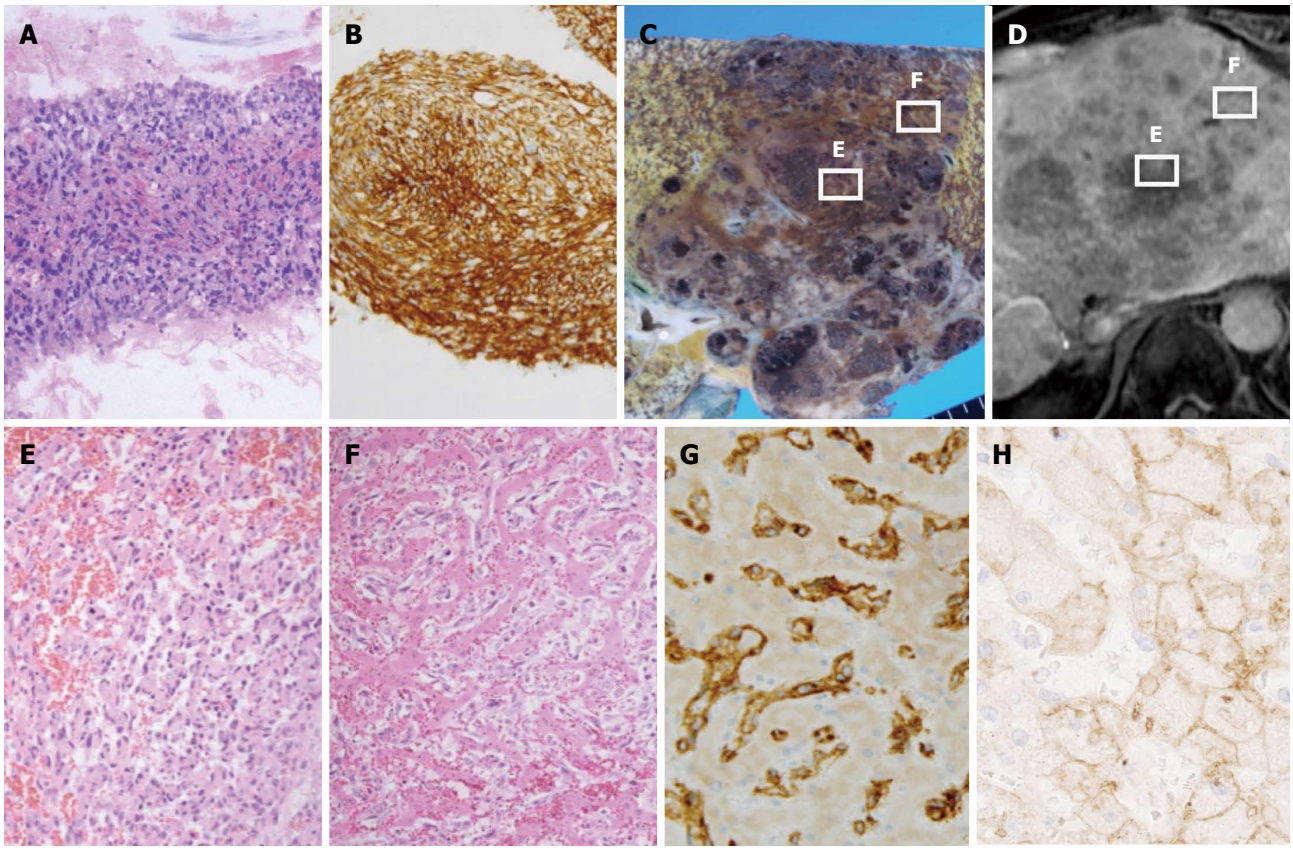

Figure 2 Histological findings from a liver tumor biopsy revealed the solid proliferation of spindle cells. A: Hematoxylin and eosin (HE) staining, × 20; B: CD34 staining, × 20; C: Macroscopically, a large tumor and many satellite nodules were observed in the liver; D: A hepatobiliary phase image obtained 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.

REFERENCES

1 **Alrenga DP**. Primary angiosarcoma of the liver. Review article. *Int Surg* 1975; **60**: 198-203 [PMID: 1091575]
2 **Ishak K**, Peters R, editors. Mesenchymal tumor of the liver. Hepatocellular carcinoma. New York: Wiley, 1976: 247-308
3 **Locker GY**, Doroshow JH, Zwelling LA, Chabner BA. The clinical features of hepatic angiosarcoma: a report of four cases and a review of the English literature. *Medicine* (Baltimore) 1979; **58**: 48-64 [PMID: 368508]
4 **Buetow PC**, Buck JL, Ros PR, Goodman ZD. Malignant vascular tumors of the liver: radiologic-pathologic correlation. *Radiographics* 1994; **14**: 153-166; quiz 167-168 [PMID: 8128048 DOI: 10.1148/radiographics.14.1.8128048]
5 **Pickhardt PJ**, Kitchin D, Lubner MG, Ganeshan DM, Bhalla S, Covey AM. Primary hepatic angiosarcoma: multi-institutional comprehensive cancer centre review of multiphasic CT and MR imaging in 35 patients. *Eur Radiol* 2015; **25**: 315-322 [PMID: 25278246 DOI: 10.1007/s00330-014-3442-0]
6 **Kamatani T**, Iguchi H, Okada T, Yamazaki H, Tsunoda H, Watanabe M, Oda M, Ohbu M, Yokomori H. Co-registered positron emission tomography/computed tomography and gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid magnetic resonance imaging features of multiple angiosarcoma of the liver. *Hepatol Res* 2014; **44**: E297-E303 [PMID: 24147907 DOI: 10.1111/hepr.12261]
7 **Koyama T**, Fletcher JG, Johnson CD, Kuo MS, Notohara K, Burgart LJ. Primary hepatic angiosarcoma: findings at CT and MR imaging. *Radiology* 2002; **222**: 667-673 [PMID: 11867783 DOI: 10.1148/radiol.2223010877]
8 **Bota S**, Piscaglia F, Marinelli S, Pecorelli A, Terzi E, Bolondi L. Comparison of international guidelines for noninvasive diagnosis of hepatocellular carcinoma. *Liver Cancer* 2012; **1**: 190-200 [PMID: 24159584 DOI: 10.1159/000343833]
9 **Lee JH**, Lee JM, Kim SJ, Baek JH, Yun SH, Kim KW, Han JK, Choi BI. Enhancement patterns of hepatocellular carcinomas on multiphasicmultidetector row CT: comparison with pathological

differentiation. *Br J Radiol* 2012; **85**: e573-e583 [PMID: 22919011 DOI: 10.1259/bjr/86767895]
10 **Ichikawa T**, Sano K, Morisaka H. Diagnosis of Pathologically Early HCC with EOB-MRI: Experiences and Current Consensus. *Liver Cancer* 2014; **3**: 97-107 [PMID: 24945000 DOI: 10.1159/000343865]
11 **Park HJ**, Choi BI, Lee ES, Park SB, Lee JB. How to Differentiate Borderline Hepatic Nodules in Hepatocarcinogenesis: Emphasis on Imaging Diagnosis. *Liver Cancer* 2017; **6**: 189-203 [PMID: 28626731 DOI: 10.1159/000455949]
12 **Narita M**, Hatano E, Arizono S, Miyagawa-Hayashino A, Isoda H, Kitamura K, Taura K, Yasuchika K, Nitta T, Ikai I, Uemoto S. Expression of OATP1B3 determines uptake of Gd-EOB-DTPA in hepatocellular carcinoma. *J Gastroenterol* 2009; **44**: 793-798 [PMID: 19404564 DOI: 10.1007/s00535-009-0056-4]
13 **Kitao A**, Zen Y, Matsui O, Gabata T, Kobayashi S, Koda W, Kozaka K, Yoneda N, Yamashita T, Kaneko S, Nakanuma Y. Hepatocellular carcinoma: signal intensity at gadoxetic acid-enhanced MR Imaging--correlation with molecular transporters and histopathologic features. *Radiology* 2010; **256**: 817-826 [PMID: 20663969 DOI: 10.1148/radiol.10092214]
14 **Osame A**, Fujimitsu R, Ida M, Majima S, Takeshita M, Yoshimitsu K. Multinodular pseudolymphoma of the liver: computed tomography and magnetic resonance imaging findings. *Jpn J Radiol* 2011; **29**: 524-527 [PMID: 21882097 DOI: 10.1007/s11604-011-0581-y]
15 **Weitz J**, Klimstra DS, Cymes K, Jarnagin WR, D'Angelica M, La Quaglia MP, Fong Y, Brennan MF, Blumgart LH, Dematteo RP. Management of primary liver sarcomas. *Cancer* 2007; **109**: 1391-1396 [PMID: 17315167 DOI: 10.1002/cncr.22530]
16 **Kim HR**, Rha SY, Cheon SH, Roh JK, Park YN, Yoo NC. Clinical features and treatment outcomes of advanced stage primary hepatic angiosarcoma. *Ann Oncol* 2009; **20**: 780-787 [PMID: 19179547 DOI: 10.1093/annonc/mdn702]
17 **Kojiro M**, Nakashima T, Ito Y, Ikezaki H, Mori T, Kido C. Thorium dioxide-related angiosarcoma of the liver. Pathomorphologic study of 29 autopsy cases. *Arch Pathol Lab Med* 1985; **109**: 853-857 [PMID: 3927870]
18 **Orlando G**, Adam R, Mirza D, Soderdahl G, Porte RJ, Paul A, Burroughs AK, Seiler CA, Colledan M, Graziadei I, Garcia Valdecasas JC, Pruvot FR, Karam V, Lerut J. Hepatic hemangiosarcoma: an absolute contraindication to liver transplantation--the European Liver Transplant Registry experience. *Transplantation* 2013; **95**: 872-877 [PMID: 23354302 DOI: 10.1097/TP.0b013e318281b902]
19 **Timaran CH**, Grandas OH, Bell JL. Hepatic angiosarcoma: long-term survival after complete surgical removal. *Am Surg* 2000; **66**: 1153-1157 [PMID: 11149588]
20 **Ozden I**, Bilge O, Erkan M, Cevikbaş U, Acarli K. Five years and 4 months of recurrence-free survival in hepatic angiosarcoma. *J Hepatobiliary Pancreat Surg* 2003; **10**: 250-252 [PMID: 14605984 DOI: 10.1007/s00534-003-0849-4]
21 **Yamaguchi R**, Nakashima O, Yano H, Kutami R, Kusaba A, Kojiro M. Hepatocellular carcinoma with sarcomatous change. *Oncol Rep* 1997; **4**: 525-529 [PMID: 21590091]

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