

# World Journal of *Hepatology*

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## Disease monitoring of hepatocellular carcinoma through metabolomics

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### Abstract

We elucidate major pathways of hepatocarcinogenesis and accurate diagnostic metabolomic biomarkers of hepatocellular carcinoma (HCC) identified by contemporary HCC metabolomics studies, and delineate a model HCC metabolomics study design. A literature search was carried out on Pubmed for HCC metabolomics articles published in English. All relevant articles were accessed in full text. Major search terms included "HCC", "metabolomics", "metabolomics", "metabonomic" and "biomarkers". We extracted clinical and demographic data on all patients and consolidated the lead candidate biomarkers, pathways, and diagnostic performance of metabolomic expression patterns reported by all studies in tables. Where reported, we also extracted and summarized the metabolites and pathways most highly associated with the development of cirrhosis in table format. Pathways of lysophospholipid, sphingolipid, bile acid, amino acid, and reactive oxygen species metabolism were most consistently associated with HCC in the cited works. Several studies also elucidate metabolic alterations strongly associated with cirrhosis, with  $\gamma$ -glutamyl peptides, bile acids, and dicarboxylic acids exhibiting the highest capacity for stratifying cirrhosis patients from appropriately matched controls. Collectively, global metabolomic profiles of the referenced works exhibit a promising diagnostic capacity for HCC at a capacity greater than that of conventional diagnostic biomarker alpha-fetoprotein. Metabolomics is a powerful strategy for identifying global metabolic signatures that exhibit potential to be leveraged toward the screening, diagnosis, and management of HCC. A streamlined study design and patient matching methodology may improve concordance among metabolomic datasets in future works.

**Key words:** Metabolomics; Hepatocellular carcinoma; Biomarkers; Metabolic profiling; Chromatography/mass spectrometry; Noninvasive biomarkers; Cirrhosis



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**Core tip:** The high-throughput, validated nature of metabolomics makes it an ideal methodology for rapidly identifying the global metabolic alterations associated with hepatocarcinogenesis - alterations that not only enhance our understanding of the metabolic underpinnings of cirrhosis and hepatocellular carcinoma (HCC), but that can be leveraged to improve HCC diagnostic, therapeutic, and disease monitoring efficacy. Indeed, contemporary HCC metabolomics works time and again demonstrate this promise that metabolomics platforms hold in serving as standalone non-invasive HCC diagnostic and disease monitoring modalities.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the world's third most lethal cancer, possessing a five-year survival rate of 10% that results in between 250000 to 1000000 deaths per year<sup>[1,2]</sup>. HCC culminates from a preexisting long-term condition of cirrhosis in 90% of cases<sup>[3]</sup> and cirrhosis patients are among the best characterized individuals at high risk for developing cancer. Notwithstanding the opportunity for clinical surveillance of these patients, the dismal survival rate persists and HCC has now emerged as the fastest rising cause of cancer related death in the United States<sup>[4,5]</sup>. HCC patients in Asia and sub-Saharan Africa face a particularly grim outlook, with > 90% of patients in rural areas of these regions progressing within the first year of HCC onset<sup>[6]</sup>. A major hindrance to successful early diagnosis of HCC stems from the substandard accuracy of the principal HCC diagnostic modalities. Alpha-fetoprotein (AFP) is the principal biomarker for HCC and despite the inexpensive and reproducible nature of the AFP blood test, its sensitivity of 25%-65%<sup>[7,8]</sup> for HCC has exacerbated early detection of this cancer. The low sensitivity of AFP can be explained by the fact that up to 40% of HCC and cirrhosis patients have normal AFP levels, and that AFP is often elevated in patients without HCC<sup>[9-11]</sup>. Moreover, only 10%-20% of patients with early-stage HCC have elevated AFP levels<sup>[12]</sup>. Exclusion of AFP as an HCC diagnostic modality in the AASLD guidelines for HCC surveillance underscores AFP's unreliability to accurately screen for early HCC<sup>[13]</sup>. Efforts to overcome this substandard performance have resulted in the identification and commercialization of novel HCC biomarkers des-gamma-carboxyprothrombin and lectin-bound AFP (AFP-L3). These markers are ineffective when used alone as HCC biomarkers, however,

and even when combined with AFP still demonstrate poor sensitivity for HCC, particularly in the detection of lesions < 3 cm<sup>[14]</sup>. While magnetic resonance imaging and computed tomography offer better accuracy in HCC diagnosis, these sophisticated diagnostic modalities are both economically and logistically incompatible with the resource-poor areas experiencing the brunt of HCC's mortality rate<sup>[15-17]</sup>. Improving early HCC detection and patient outcome globally requires fulfilling of the urgent need for a reproducible, inexpensive, and accurate HCC diagnostic test.

### Metabolomics as an HCC biomarker discovery tool

Numerous genomic and proteomic screening studies have been employed to identify potential biomarkers of HCC<sup>[18-24]</sup> but to date the markers identified in these studies have not been clinically fruitful. Because the liver is the hub of carbohydrate, amino acid, and lipid metabolism<sup>[25,26]</sup>, chronic liver diseases undoubtedly disrupt normal metabolic function. A metabolomics analysis of HCC and cirrhotic tissue can therefore elucidate not just the metabolic pathways most relevant to the hepatocarcinogenic process, thereby identifying metabolites showing promise as HCC biomarkers, but also global metabolic patterns that serve as comprehensive disease signatures and which may therefore be used to stratify cases from controls. Metabolomics is the comprehensive identification of all small metabolites < 2 kD in a tissue sample. Through combined gas or liquid chromatography/mass spectrometry, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI TOF-MS) or nuclear magnetic resonance instrumentation, metabolomics platforms enable investigators to rapidly screen hundreds of metabolites in a large series of biofluid or solid tissue samples and are capable of simultaneously detecting metabolites belonging to a diverse array of pathways including amino acids, lipids, carbohydrates, and nucleotides. Metabolomics platforms are translationally optimal and hold potential for clinical implementation because they reveal global metabolite expression pattern differences among cases and controls in an automated, rapid, high-throughput, quality controlled, and reproducible manner. Metabolomics facilitates rapid identification of diagnostic markers, prognostic markers, and lead drug target pathways and their implementation in drug discovery divisions of pharmaceutical giants underscores their important role in the lead target generation realm<sup>[27]</sup>.

Nearly two dozen HCC metabolomics studies have been reported<sup>[28-46]</sup>. These studies illustrate the key pathways involved in stepwise hepatocarcinogenesis and reveal metabolites that may have utility as biomarkers of HCC and cirrhosis. The findings of these studies are summarized in Tables 1 and 2. These works reveal deregulation of bile acid metabolism, fatty acid  $\beta$ -oxidation by way of the carnitine palmitoyltransferase (CPT) shuttle system, amino acid metabolism, and glycerophospholipid metabolism in HCC vs cirrhosis,



			→ HBV 15% → Alcoholism 29% → NASH 13% → Cryptogenic (8%) → Autoimmune (3%)	TCDCA ↓ Sphingosine 1-phosphate ↑ LPC (16:0) ↑ LPC (17:0) ↑ LPC (18:0) ↑ LPC (15:0) ↑ LPC (22:6) ↑ LPE (22:6) ↑ LPE (20:4) ↑ LPE (20:3) ↑ PS ↑ Glucose ↓ Creatine ↓ PE ↑ Glutamine ↑ Glutamate ↑ PC + GPC ↑ High density lipoproteins Acetate ↑ N-acetyl-glycoproteins ↑ Glutamate ↑ Glutamine ↓	Sphingolipid metabolism LPC metabolism  Glycolysis  LPC metabolism Amino acid metabolism  Bile acid metabolism HDL biosynthesis Ketone body metabolism N-acetyl-glycoprotein Amino acid metabolism  N/A
Yang <i>et al</i> <sup>[28]</sup>	HRMAS 1H NMR	Biopsy (human)	HCC <i>n</i> = 17: → Cirrhosis <i>n</i> = 9 → No cirrhosis <i>n</i> = 8		
Nahon <i>et al</i> <sup>[40]</sup>	NMR	Serum (human)	EtOH cirrhosis		
Budhu <i>et al</i> <sup>[43]</sup>	GC/MS, UPLC/MS-MS	Biopsy samples (human)	HCC <i>n</i> = 356 Training cohort <i>n</i> = 30 Testing cohort <i>n</i> = 217 Validation cohort <i>n</i> = 139	Study reported on markers involved in cancer aggressivity through comparison of stem-like HCC to less benign mature hepatocyte HCC	
Beyoğlu <i>et al</i> <sup>[44]</sup>	GC/MS	Biopsy samples (human)	Six HCC subtypes, liver fibrosis status unknown	Glucose ↓	Glycolysis
Fitian <i>et al</i> <sup>[45]</sup>	UPLC/MS-MS and GC/MS	Serum (human)	HCV cirrhosis-associated HCC <i>n</i> = 30 HCV-cirrhosis <i>n</i> = 27 Healthy volunteers <i>n</i> = 30	Glycerol 3-phosphate ↓ Glycerol 2-phosphate ↓ Malate ↓ Alanine ↓ Myo-inositol ↓ Linoleic acid ↓ Sphingosine ↑  Xanthine ↑ 2-Pyrrolidinone ↑ 2-Hydroxybutyrate ↑ Serine ↑ Glycine ↑ Aspartate ↑ 12-HETE ↑ 15-HETE ↑ Isovalerate ↑ Dihomo-linolenate ↑ Stearic acid	PI3K pathway Prostaglandin biosynthesis Sphingolipid  Oxidative stress metabolism GABA metabolism Oxidative stress metabolism Amino acid  Inflammation pathway  Gut microflora metabolism Inflammation pathway Fatty acid biosynthesis
Gao <i>et al</i> <sup>[46]</sup>	GC-TOF/MS	Serum (human)	HBV cirrhosis-associated HCC <i>n</i> = 39 HBV-cirrhosis ( <i>n</i> = 52)	Heptadecanoic acid Palmitic acid 5-Aminovaleric acid Cholesterol ↑ 3-hydroxybutyric acid ↑ Malic acid ↑ Glutamine ↑ Asparagine ↓ Alanine ↑ Threonine ↓ Leucine ↓ Glutamic acid ↑ β-glutamate ↑ 5-oxoproline ↓ 1,2,4-cyclopropanodicarboxylic acid ↓	Gut microflora metabolism Cholesterol metabolism Ketogenesis TCA metabolism Amino acid  Glutathione metabolism Dicarboxylic acid metabolism

Pathways of importance in the comparison of (1A) HCC *vs* cirrhosis and (2) cirrhosis *vs* healthy controls are shown. Arrows indicate the metabolite's expression in cases *vs* appropriate controls.  $P < 0.05$  was used as the significance level and metabolites reported in table are those which were most significantly upregulated or downregulated in each study. EtOH: Alcohol; TOCSY: Total correlation spectroscopy; HH: Hereditary hemochromatosis; TCA: Tricarboxylic acid; UPLC: Ultrahigh-performance liquid chromatography; QTOF: Quadrupole time of flight; SELDI: Surface-enhanced laser desorption/ionization; HRMAS: High-resolution magic angle spinning; LPE: Lysophosphatidylethanolamine; LPC: Lysophosphatidylcholine; HCC: Hepatocellular carcinoma; MS: Mass spectrometry; TOF: Time-of-flight; GC: Gas chromatography; LC: Liquid chromatography; NMR: Nuclear magnetic resonance; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; FFA: Free fatty acids; PE: Phosphorylethanolamine; GABA: γ-aminobutyric acid.



**Table 2** Significantly altered metabolites in cirrhosis patients *vs* healthy volunteers

Ref.	Platform	Tissue (organism)	Significantly altered metabolites in cirrhosis patients <i>vs</i> healthy volunteers	Main pathways distinguishing cirrhosis from healthy volunteers
Gao <i>et al</i> <sup>[33]</sup>	<sup>1</sup> H NMR	Serum (human)	Isoleucine ↓ Leucine ↓ Valine ↓ Glutamine ↑ Tyrosine ↑ Phenylalanine ↑ 1-methylhistidine ↑ N-acetylglycoproteins ↑ Acetate ↑ Acetoacetate ↓ Pyruvate ↑ α-ketoglutarate ↑ Choline ↓ Taurine ↑ Glycerol ↑	Amino acid metabolism  N-acetylglycoprotein Ketonogenesis  Glycolysis TCA cycle  Bile acid metabolism Amino acid metabolism
Li <i>et al</i> <sup>[42]</sup>	UPLC/QTOF-MS	Serum (mouse)	Leucine ↓ Phenylpyruvic acid ↓ Phenylalanine ↓ Tryptophan ↓ LPE (16:0) ↓ LPE (18:0) ↓ LPC (16:0) ↓ LPC (20:1) ↓ LPC (22:6) ↑ PC (16:0/18:3) ↑ PC (12:1/24:3) ↑ PC (16:0/20:4) ↑ PC (16:0/22:6) ↑ PC (18:0/20:4) ↑ SM (d18:0/16:1) ↓ γ-glutamylalanine ↑ γ-glutamylvaline ↑ γ-glutamylglutamine ↑ γ-glutamylphenyl- γ-glutamylcitrulline ↑ Alanine ↑ Methionine sulfoxide ↑	LPE metabolism  LPC metabolism  Phosphatidylcholine metabolism  Sphingomyelin metabolism  Glutathione metabolism  Amino acid metabolism
Soga <i>et al</i> <sup>[35]</sup>	Capillary electrophoresis/TOF-MS	Serum (human)	LPC-16:0 ↓ LPC-18:0 ↓ 16:0/18:1-PC ↓ 16:0/18:2-PC ↓ 16:0/20:4-PC ↓ 16:0/22:6-PC ↓ 18:0/18:2-PC ↓ Oleamide ↑ Phenylalanine ↑ GCDCA ↑ Canavaninosuccinate ↓ Phenylalanine ↑ GCA ↑ GDCA ↑ Bilirubin ↑ LPE (18:2) ↓ LPC (22:6) ↓ LPC (18:2) ↓ LPC (20:4) ↓ LPC (16:0) ↓ LPC (18:0) ↓ C18:1-CN ↑ Inositol ↓ 2,2-bipyridine ↓ Methionine ↓ Tyrosine ↓ Arginine ↓ Stearic acid ↓ Palmitic acid ↓ Citric acid ↓	LPC metabolism       Fatty acid metabolism  Bile acid metabolism Arginosuccinate synthetase pathway Amino acid metabolism Bile acid metabolism  Hemoglobin metabolism Lysolipid metabolism  CPT shuttle system TCA cycle  Amino acid metabolism  Fatty acid metabolism
Wang <i>et al</i> <sup>[38]</sup>	UPLC/MS-MS; LC/QTOF-MS	Serum (human)		
Zhou <i>et al</i> <sup>[39]</sup>	UPLC/QTOF-MS	Serum (human)		
Chen <i>et al</i> <sup>[30]</sup>	UPLC/QTOF-MS	Serum (human); Urine (human)		

Cao <i>et al</i> <sup>[32]</sup>	UPLC/MS	Fecal (human)	2-piperidine carboxylic acid ↓	
			5-Hydroxy-tryptophan ↓	
			Chenodeoxycholic acid dimeride ↓	Bile acid metabolism
			Urobilin ↓	Hemoglobin metabolism
			Urobilinogen ↓	
Yin <i>et al</i> <sup>[41]</sup>	RPLC/MS	Serum (human)	7-ketolithocholic acid ↓	Microbiome metabolism
			LPC C18:0 ↑	LPC metabolism
			LPC C16:0 ↑	
			Hypoxanthine ↓	Purine synthesis
			Inosine ↓	
			Bilirubin ↑	Hemoglobin metabolism
			GCA ↑	Bile acid metabolism
			GCDCA ↑	
			Taurine ↓	
			LPC C18:2 ↓	LPC metabolism
			LPC C18:3 ↓	
			LPC C16:1 ↓	
			LPC C18:0 ↓	
			LPC C16:1 ↓	
			L-acetylcarnitine ↑	CPT shuttle system
Fitian <i>et al</i> <sup>[45]</sup>	Integrated UPLC/MS-MS and GC/MS	Serum (human)	6-Methylnicotinic acid ↓	Nicotine metabolism
			Glycocholate (GCA) ↑	Bile acid metabolism
			Tauroursodeoxycholate ↑	
			Glychochemodeoxycholate ↑	
			Azelate (nonanedioate) ↑	Dicarboxylic acid metabolism
			Undecanedioate ↑	
			Sebacate (decanedioate) ↑	
			Hexadecanedioate ↑	
			Tetradecanedioate ↑	
			DSGEGDFXAEGGGVR ↑	Fibrinogen cleavage peptide
			ADSGEGDFXAEGGGVR ↑	
			Bilirubin (Z,Z) ↑	Hemoglobin catabolism metabolite
			Biliverdin ↑	
			1,2-propanediol ↑	Ketogenesis
			Succinylcarnitine ↑	CPT shuttle system
Gao <i>et al</i> <sup>[46]</sup>	GC-TOF/MS	Serum (human)	Acetylcarnitine ↑	
			Glutaryl carnitine ↑	
			Palmitic acid ↑	Fatty acid metabolism
			Stearic acid ↑	
			Oleic acid ↑	
			Arachidic acid ↑	Arachidonic acid metabolism
			Aminomalononic acid ↑	Dicarboxylic acid metabolism
			Phenylalanine ↑	Amino acid metabolism
			Cysteine ↑	
			Leucine ↑	
			Citric acid ↑	
			Oxoproline ↑	

EtOH: Alcohol; TOCSY: Total correlation spectroscopy; HH: Hereditary hemochromatosis; TCA: Tricarboxylic acid; UPLC: Ultrahigh-performance liquid chromatography; QTOF: Quadrupole time of flight; SELDI: Surface-enhanced laser desorption/ionization; HRMAS: High-resolution magic angle spinning; LPE: Lysophosphatidylethanolamine; LPC: Lysophosphatidylcholine; MS: Mass spectrometry; TOF: Time-of-flight; NMR: Nuclear magnetic resonance; LC: Liquid chromatography; GC: Gas chromatography; CPT: Carnitine palmitoyltransferase; TCA: Tricarboxylic acid.

and further delineate metabolites with potential utility in stratifying patients with cirrhosis from the healthy population.

### Pathways of importance in hepatocarcinogenesis

**Glycerophospholipids:** The liver is the principal organ of lipid metabolism and the presence of cirrhosis and hepatocellular carcinoma results in a dramatic shift in the normal metabolism of fatty acids. Among the first HCC metabolomics studies to elucidate this massive deregulation of lipid metabolism in HCC was the Yang *et al*<sup>[28]</sup> work in 2007 which employed high resolution magic angle-spinning <sup>1</sup>H nuclear magnetic resonance

to delineate the metabolomic profile differences in low-grade HCC, high-grade HCC, and non-involved adjacent cirrhosis patient biopsy specimens. The group reported higher levels of several phospholipids in HCC vs cirrhosis, including glycerophosphocholine, phosphatidylcholine, choline and the phosphorylethanolamine. Increases in phosphatidylcholine were further observed in low-grade HCC vs uninvolved cirrhotic tissue, suggesting possible deregulation of glycerophospholipid metabolism at the early phases of HCC development. These phospholipids also exhibited a direct, positive relationship to tumor burden.

In Yang *et al*<sup>[28]</sup>, the elevation of choline, the head

group of many phospholipids that comprise the plasma membrane, not only reflects increased plasma membrane synthesis demand by the growing tumor but is also consistent with the observed elevations in bile (cholic) acid concentration in HCC vs control tissue.

**Lysophospholipids, free fatty acids, and acylcarnitines:** Significant alterations in the expression of a subtype of glycerophospholipids known as lysophosphatidylcholines (LPC) have routinely been observed in HCC metabolomics studies<sup>[29,36-39]</sup>. Still other classes of lipids observed to be significantly altered in HCC vs cirrhosis include free fatty acids (FFA)<sup>[29,45]</sup>, very long chain fatty acids<sup>[29]</sup>, and acylcarnitines<sup>[37,45]</sup>. Down-regulated LPCs and FFA in HCC vs cirrhosis were among the major metabolomic trends reported by Patterson *et al.*<sup>[29]</sup>, with LPC (14:0), LPC (20:3), LPC (22:6) and very long chain fatty acids FFA (24:0) (lignoceric acid) and FFA (24:1) (nervonic acid) all trending lower in HCC vs cirrhosis. The decreases of lignoceric and nervonic acid in HCC vs cirrhosis were especially patent and may reflect peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) induced enhancement of peroxisomal  $\beta$ -oxidation. Previous reports implicating heightened PPAR- $\alpha$  activity in HCC support this hypothesis<sup>[47,48]</sup>. Decreases in these FFAs may also be related to increased activity of lignoceryl-CoA ligase, the enzyme responsible for very long chain fatty acid catabolism and one acted upon by PPAR- $\alpha$ <sup>[49]</sup>. LPCs are the glycerophospholipid building blocks of cell membranes and elevation of these metabolites may reflect the heightened metabolic needs of growing HCCs. LPCs are also major lipids bound to human albumin<sup>[50]</sup>. Decreased serum albumin is a signature of liver cirrhosis and liver cancer and elevations of systemic LPCs may be attributed to the shortage of appropriate albumin binding sites that results in increased circulating levels of these metabolites.

Further metabolomics work by Xiao *et al.*<sup>[37]</sup> also identified this downregulation of LPCs and LPEs in 40 hepatitis C virus (HCV)-associated HCC patients and 49 cirrhosis controls. Among the novel findings of this investigation included decreased levels of acylcarnitines in HCC vs cirrhosis. Results also showed that as tumor burden worsened, the expression of acylcarnitines and bile acids trended significantly downward. Stage II and III HCC exhibited lower levels of these metabolites in comparison to stage I [staging based on the American Joint Committee on Cancer Tumor Lymph Node Metastatic Disease (TNM) system]. The downregulation of fatty acids, acylcarnitines, and bile acids in HCC vs cirrhosis supports the cancer Warburg effect involving a metabolic shift from tricarboxylic acid (TCA) cycle and mitochondrial  $\beta$ -oxidation to a heightened reliance on glycolysis for energy production. To undergo  $\beta$ -oxidation in the mitochondrial matrix, free fatty acids (fatty acyl-CoA) must link with cytosolic carnitine *via* CPT shuttle system enzymes to form acylcarnitines. Acylcarnitines are capable of penetrating the inner mitochondrial

membrane and once inside the matrix, CPT enzymes liberate fatty acyl-CoA allowing  $\beta$ -oxidation to ensue. Decreased concentration of acylcarnitines in HCC vs cirrhosis suggests impairment of CPT1-mediated formation of these compounds from FFA and carnitine.

**Sphingolipids:** One major lipid expression alteration in HCC reported in HCC metabolomics is a perturbation of sphingosine metabolism, with overexpressed sphingosine-1-phosphate (S1P) and sphingosine reported in HCC vs cirrhosis. The overexpression of LPCs is in agreement with the Patterson study while the upregulation of S1P, a signaling lipid, was a novel finding in HCC metabolomics. S1P has been heavily implicated in promoting the progression of several cancers including HCC<sup>[51,52]</sup> and building the case for this pathway's involvement in HCC development, our HCC metabolomics work identified S1P's precursor sphingosine as one of the most strongly upregulated metabolites in HCC vs cirrhosis<sup>[45]</sup>. Sphingosine is produced *via* acid ceramidase (AC) activity on ceramide. Ceramides are shown to possess apoptotic effects, while sphingosine 1-phosphate is demonstrated as an anti-apoptotic and angiogenic molecule<sup>[53]</sup>. This cell turnover control mechanism is known as the "sphingosine rheostat", and AC is an important modulator of cell death homeostasis. Higher S1P in HCC may also reflect an independent enhancement of sphingosine kinase (SPHK) activity. One study demonstrated the antitumor property of a selective SPHK2 inhibitor in HCC xenografts<sup>[54]</sup>, implicating SPHK as a promoter of HCC progression. Heightened AC activity that results in increased sphingosine may lead to a larger reservoir of S1P *via* sphingosine kinase and may promote the establishment of a microenvironment conducive to HCC initiation.

### Bile acids

Bile acids are synthesized in the liver and aid in fatty acid absorption and digestion. Bile acid elevations in HCC have been reported previously<sup>[55]</sup> and may be explained by HCC invasion and obstruction of the bile duct. Bile duct blockage can impede adequate transfer of bile acids to the small intestine thereby impairing sufficient absorption and digestion of fats and leading to a buildup of both bile acids and cholinergic lipids in the hepatic tumor microenvironment. The majority of studies comparing HCC and cirrhosis metabolomes did not uncover a significant differential expression of bile acids, but where a significant trend was seen<sup>[36-38]</sup>, the metabolites collectively trended downward, in contrast to the aforementioned previous reports<sup>[55,56]</sup>. A significant negative correlation between bile acid levels and tumor burden was also observed<sup>[37]</sup>. Similar to the Yang *et al.*'s work, our metabolomics investigation<sup>[45]</sup> identified a significant elevation of choline in HCC vs cirrhosis, and bile acids in our study were strongly elevated in cirrhosis patients vs healthy subjects. The elevation of choline, a building block of bile acids, is consistent with an impairment of bile acid synthesis. Bile acid down-



regulation in HCC may also reflect a metabolic shift away from  $\beta$ -oxidation and the reduced *de novo* bile acid production caused by the obliteration of healthy hepatocytes during chronic liver disease. Diminished bile acids may also reflect constitutive activation of farnesyl X receptor (FXR), a bile acid-activated nuclear receptor that is also activated by a variety of other lipids including eicosanoids<sup>[57]</sup>. FXR silences Cyp7A1-catalyzed production of bile acids and is implicated in promoting progression of HCC by multiple studies<sup>[58,59]</sup>. In the Fujino study of FXR-induced promotion of HCC progression<sup>[58]</sup>, siR-mediated supplementation of FXR enhanced HepG2, Huh7, and HLE HCC cell line progression while FXR knockdown halted this progression.

### Oxidative stress metabolism

Among the other pathways found to be significantly associated with HCC in metabolomics investigations are pathways of reactive oxygen species metabolism, notable metabolites of which include the  $\gamma$ -glutamyl peptides. A recent capillary electrophoresis-time of flight mass spectrometry analysis involving sera obtained from HCV-associated HCC patients, cirrhosis patients, hepatitis B virus (HBV) and chronic hepatitis C (HCV) patients, and healthy volunteers showed markedly significant variations in  $\gamma$ -glutamyl expression among these groups<sup>[35]</sup>. No differences in  $\gamma$ -glutamyl peptide expression were observed between HCC and cirrhosis controls, but several significant alterations were witnessed in the HCC vs viral hepatitis, HCC vs normal healthy controls (NHC), cirrhosis vs viral hepatitis, and cirrhosis vs NHC comparisons. In general, HCC  $\gamma$ -glutamyl expression was increased in comparison to healthy controls, while  $\gamma$ -glutamyl peptides were decreased in HCC vs viral hepatitis.  $\gamma$ -glutamylglycine,  $\gamma$ -glutamylalanine,  $\gamma$ -glutamylvaline and  $\gamma$ -glutamylserine,  $\gamma$ -glutamyltaurine,  $\gamma$ -glutamylleucine, and  $\gamma$ -glutamyllysine were all strongly ( $0.0001 < P < 0.001$ ) downregulated in HCC vs viral hepatitis B (HBV) and C infection.  $\gamma$ -glutamyl peptides are precursors to glutathione, the chief antioxidant compound primarily synthesized in the liver. The respective elevation of these intermediates in HCC and cirrhosis patients vs NHC suggests that increased oxidative stress contributing to liver dysfunction calls for heightened production of these precursors to combat this deteriorative process. This is consistent with reports implicating oxidative damage as a key pathway in HCC progression and one that increases patient vulnerability for HCC recurrence<sup>[60,61]</sup>.  $\gamma$ -glutamyl peptides are also liberated in free form by gamma-glutamyl transpeptidase (GGT) mediated breakdown of glutathione. GGT is an enzymatic signature of liver disease and a marker routinely used in the clinic to assess the severity of liver dysfunction. Excess breakdown of glutathione may explain the relative elevation of  $\gamma$ -glutamyl peptides in cirrhosis vs NHC and the impaired oxidative stress neutralization commonly witnessed in HCC.

The Wang *et al.*<sup>[38]</sup> study also found an oxidative stress signature in HCC, showed a striking 677-fold elevation

in canavaninosuccinate (CSA) level in HCC patients vs their cirrhosis counterparts ( $P < 0.01$ ). Subsequent receiver operator characteristic (ROC) analysis revealed that the sensitivity and specificity of AFP and CSA for distinguishing the HCC patients from cirrhosis controls were: AFP<sub>20 ng/mL</sub> 74% and 38%; AFP<sub>200 ng/mL</sub> 52% and 90%; CSA 79.3% and 100%; combined CSA and AFP<sub>20 ng/mL</sub> 96.4% and 100%. CSA is the precursor to fumarate, a key metabolite of the TCA cycle, and elevation of CSA may reflect impairment in the formation of TCA intermediates and promotes the above referenced Warburg theory involving a metabolic shift from oxidative to anaerobic energy production in cancer microenvironments, which are often more hypoxic than healthy tissue<sup>[62]</sup>.

In our work<sup>[45]</sup>, a strong oxidative stress signature in HCC was also observed, with xanthine, 2-hydroxybutyrate and several  $\gamma$ -glutamylpeptides trending significantly higher in HCC vs cirrhosis. Still, the work of Gao *et al.*<sup>[46]</sup> uncovered trends suggestive of an opposite phenomenon: Elevations of glutamic acid, lysine and cysteine in HCC compared with healthy controls were observed and are curious in the context of reactive oxygen species (ROS) given that these amino acids are precursors of glutathione (GSH). Increased levels of these amino acids suggest a possible enhancement of GSH production that establishes a microenvironment conducive for tumor survival<sup>[46]</sup>.

### Protein metabolites

The liver is the major organ of protein metabolism and not surprisingly, HCC metabolomics shows that the expression profiles of patients with advanced liver disease exhibit major differences in amino acid expression when compared to metabolic profiles of appropriate diseased or healthy controls. In the Yang *et al.*<sup>[28]</sup> study, significant increases in creatine, glutamine, and glutamate were found in HCC vs cirrhosis, and these trends were juxtaposed by decreases in lactate, alanine, leucine, glutamate, glutamine in HCC vs cirrhosis. The finding of increased amino acids in HCC vs cirrhosis is consistent with numerous studies implicating elevated amino acids and the enzymes responsible for their production in cancer initiation and progression<sup>[63-66]</sup>. In one large scale metabolomics analysis of 60 cancer cell lines, Jain *et al.*<sup>[66]</sup> identified glycine as the most significantly and consistently upregulated metabolite in cancer cells vs healthy lines. Enhanced amino acid production is consistent with the metabolic remodeling hallmark of cancer known as the Warburg effect, which involves a shift from TCA cycle and  $\beta$ -oxidation to a heightened reliance on glycolysis for energy production<sup>[67]</sup>. Amino acids are important glycolytic enzyme activators, and one recent study demonstrated that serine was an activator of pyruvate kinase M2<sup>[68]</sup>, the cancer isoform of glycolytic enzyme pyruvate kinase responsible for conversion of phosphoenolpyruvic acid (PEP) to pyruvate. The group's concomitant observation of elevated lactate in HCC vs cirrhosis is consistent with the Warburg hypothesis.

Gao *et al.*<sup>[46]</sup> propose that their observed elevation of pyruvate, which would seem to counteract the tumor's reliance on the lower-activity PKM2 compared to PKM1, is due to enhanced production of glutamine which is used as an alternative pyruvate precursor. Our study identified a strong amino acid signature associated with HCC with serine, glycine and aspartate being the most significantly upregulated metabolites in HCC vs cirrhosis. These elevations are corroborated by a recent metabolomic analysis of HBV-associated HCC showing a strong upregulation of serine, alanine, glycine, cysteine, aspartic acid, methionine, tyrosine, tryptophan, and phenylalanine in HCC vs healthy controls. Upregulated amino acids in HCC may be explained by the greater protein turnover that transpires in rapidly dividing tumors vs the surrounding non-cancerous tissue along with impaired amino acid utilization in liver.

In addition to very small molecular weight amino acids, metabolomics is also useful for detecting larger protein metabolites weighing near 10 kDa. SELDI TOF-MS metabolomics is a highly versatile methodology for large-scale identification and quantification of these larger metabolites. Wu *et al.*<sup>[31]</sup> tapped this technology to investigate the metabolomic expression profiles of HBV-associated HCC patients, cirrhosis patients, and healthy controls. Their results reveal the upregulation of two proteins, growth related oncogene- $\alpha$  (GRO- $\alpha$ ) and thrombin light chain (TLC), in HCC patients vs cirrhosis controls. To validate these putative markers, serum from an alternative series of HCC, cirrhosis, healthy, and cancer control patients was subjected to Sephadex<sup>TM</sup> Peptide 10/300 GL, Hitrap<sup>TM</sup> CM, and Mono Q 5/50 GL liquid chromatography for protein separation. The separated products were purified using SDS-PAGE and their identities were confirmed using electrospray ionization mass spectrometry. In this alternative series of patients, GRO- $\alpha$  was upregulated four-fold in HCC patients vs cirrhosis controls and its level directly correlated with HCC tumor burden. GRO- $\alpha$  also trended higher in gastric, nasopharyngeal and lung cancers relative to cirrhosis patients, but the magnitude of GRO- $\alpha$  elevation in cancer controls vs cirrhosis was smaller than the elevation witnessed in HCC vs cirrhosis, suggesting that GRO- $\alpha$  may play a more prominent role in HCC progression. Interestingly, TLC was 1.4 times more elevated in HCC patients vs cirrhosis controls but downregulated in the cancer control patients vs cirrhosis, suggesting that TLC is a unique signature of HCC. The combined sensitivity and specificity of GRO- $\alpha$  + TLC + AFP for discriminating HCC patients from cirrhosis and healthy controls was 91.7% and 92.7% respectively. At the 400 ng/mL cutoff for HCC diagnosis, AFP had a sensitivity of 69% and a specificity of 83%.

GRO- $\alpha$  is a chemokine involved in invoking leukocyte cell migration and is associated with pro-inflammatory processes, angiogenesis, and cancer<sup>[69-71]</sup>. Its elevation may be a signature of a viral hepatitis-associated HCC immune deregulation involving heightened monocyte migration and increased inflammation, and a subsequent

increased likelihood for successful host evasion of tumor prophylactic mechanisms<sup>[72]</sup>. TLC is a protein cleavage fragment that is generated from matrix metalloproteinase-associated (MMP) peptide cleavage<sup>[31]</sup>. Its upregulation may be explained by simultaneous E-cadherin loss and MMP activation by Twist1 which has been shown to promote HCC expansion<sup>[73]</sup>. Its utility as a cancer biomarker was also shown in a SELDI TOF-MS study of gastric cancer by Ebert *et al.*<sup>[74]</sup>, where TLC accurately distinguished gastric cancer patients from patients without cancer with a sensitivity of 89.9% and a specificity of 90%.

The destruction of cholinergic receptors in amyloid plaques and neurofibrillary tangles of the brain has also been linked to encephalopathy, a common neurological disorder witnessed in HCC patients<sup>[75]</sup>. The molecular mechanisms linking hepatic encephalopathy (HE) and HCC are not fully understood, but the parallel elevations of choline and glutamine in HCC patients vs cirrhosis controls may partially be associated with a cataclysmic loss in cholinergic receptor concentration and the initial stages of HE onset, respectively<sup>[76]</sup>. A possible neurological metabolic signature was one of the most strongly correlated trends observed in HCC patients vs cirrhosis and vs healthy controls in our study, with 2-pyrrolidinone, a  $\gamma$ -aminobutyric acid (GABA) metabolite, was strongly elevated in HCC when compared to levels in both the cirrhosis controls and the healthy volunteers. GABA is a major inhibitory chemical messenger in the brain, and the sharp elevation of its metabolic byproducts in HCC may reflect heightened production of GABA and may coincide with the neurodegenerative hallmarks of advanced liver disease.

Two previously unidentified fibrinogen cleavage peptides, denoted by amino acid sequence as DSGEGD FXAEGGGVR and ADSGEGDFXAEGGGVR, were observed to be significantly overexpressed in HCC in our study. While they trended higher in HCC vs cirrhosis, their elevation in cirrhosis vs healthy controls was more patent and these metabolites exhibited a better diagnostic utility for cirrhosis. Taken together, the aberrations in protein and amino acid expression in HCC revealed by metabolomics may be harnessed toward the development of clinically fruitful HCC diagnostics.

### Markers of cirrhosis

A number of HCC metabolomics studies report significant alterations between cirrhosis patients and healthy volunteers<sup>[30,32,33,35,38,39,41,42,45,46]</sup> and within these findings are opportunities for development of biomarkers for cirrhosis. Currently, the best cirrhosis diagnostic is liver biopsy, a procedure that by virtue of its invasive nature is not a gold standard diagnostic approach<sup>[77]</sup>. Identification of accurate cirrhosis diagnostic biomarkers can streamline development of a less invasive diagnostic that minimizes patient discomfort and the expenses associated with biopsy while simultaneously enabling early detection of cirrhosis in both industrialized nations and in resource poor areas of the globe. Among the putative markers

of cirrhosis identified in these works include the purine metabolites hypoxanthine<sup>[41,45]</sup> and inosine<sup>[41]</sup>, both significantly downregulated five and six-fold respectively in cirrhosis vs NHC.  $\gamma$ -glutamylalanine,  $\gamma$ -glutamylvaline,  $\gamma$ -glutamylglutamine,  $\gamma$ -glutamylphenylalanine and  $\gamma$ -glutamylcitrulline were also significantly elevated in cirrhosis vs NHCs<sup>[41,45]</sup>. Increases in the  $\gamma$ -glutamyl peptides in cirrhosis vs NHC and HCC vs NHC indicates heightened production of glutathione to combat the oxidative damage process commonly implicated as a promoter of tumor initiation and progression.  $\gamma$ -glutamyl peptides are also liberated in free form by GGT mediated breakdown of glutathione. The elevation of  $\gamma$ -glutamyl peptides in cirrhosis vs NHC may therefore conversely be explained by excessive breakdown of glutathione resulting in impaired oxidative stress neutralization that is commonly witnessed in HCC.

Other promising markers of cirrhosis include bile acids<sup>[45]</sup> and dicarboxylic acids<sup>[45,46]</sup>, with these metabolite classes exhibiting the strongest and most significant fold-differences among all markers significantly altered between cirrhotics and healthy controls in the references metabolomic studies.

### **Etiological metabolomic differences**

Because HCC is linked to a variety of diverse etiologies including viral hepatitis, alcoholic cirrhosis, non-alcoholic fatty liver disease, steatohepatitis, and aflatoxin B1, metabolomics is useful for revealing the variation in metabolism these etiologies cause. The majority of studies have focused on characterizing the metabolomic signatures of single HCC etiologies, or have not accounted for etiology. Studies to date have not queried the metabolomic expression pattern differences between the various etiologies of HCC and as the metabolic underpinnings of each etiology are better characterized, HCC etiology metabolome comparison studies will prove vital in establishing the utility for metabolomics as an accurate diagnostic modality of different HCC subtypes. Only one study, by Zhou *et al.*<sup>[39]</sup>, compared the serum metabolomic expression patterns of HCV cirrhosis-associated HCC and HBV cirrhosis-HCC. The group also looked at whether metabolomic alterations in HCV-HCC and HBV-HCC were significantly different from cirrhosis and healthy control metabolomes. The study revealed a greater magnitude decrease of LPC expression in HBV-infected HCC patients vs cirrhosis controls than the magnitude decrease of LPCs HCV-HCC vs cirrhosis. The analysis also showed significant elevation of bile acids, heme pigmentation compounds bilirubin and biliverdin, upregulation of acylcarnitines and downregulation of glycerophospholipids in cirrhosis patients vs healthy controls, suggesting that these metabolites were signatures of the onset of cirrhosis. Metabolomic profile comparison between HBV-cirrhosis patients vs patients with HBV-only revealed a similar global downregulation of LPCs in the cirrhosis cohort vs viral hepatitis controls. The findings of this study suggest a progressive downregulation of LPCs during the course of progression

from viral hepatitis to cirrhosis and a bottomed out expression occurring with HCC. The downregulation of LPC in HCC may be explained by their well-known anti-tumor roles that include induction of apoptosis, anti-invasive effects, and a direct effect on tumor sensitization to treatment. This trend of diminished LPCs may reflect first the substantial cell death that occurs during cirrhosis resulting in diminished LPC levels, followed by a subsequent massive turnover of residual LPCs by the growing HCC that further exhausts the LPC reservoir. The magnitude of LPC decrease in HBV-HCC vs cirrhosis was greater than HCV-HCC vs cirrhosis, suggesting that HBV exerts a more prominent influence on LPC metabolism than HCV.

### **Integrated “omics” approaches**

As the above studies demonstrate, metabolomics is a powerful strategy for identifying a large panel of metabolites that exhibit promise in accurately diagnosing HCC. What is unclear from these works, however, are the genomic and proteomic synergies that culminate in these metabolic manifestations. Integrating two or more “omics” approaches can unveil the complex genomic-proteomic-metabolomic network galvanizing cancer development. Two recent studies tapped the power of such an approach, coupling metabolomics with transcriptomics to identify the genetic underpinnings of metabolomic disruptions. The first study by Budhu *et al.*<sup>[43]</sup> investigated the network of metabolic pathways and corresponding genes involved in HCC aggressivity. Patient demographic and clinical characteristics, including body mass index, were extensively characterized for 356 HCC cases, which were divided into a training set ( $n = 30$ ), a testing set ( $n = 217$ ), and a validation cohort ( $n = 139$ ). The training cohort consisted of 15 epithelial cell adhesion molecule-positive/AFP-positive (EpCAM+AFP+) HpSC-HCC patients, representing aggressive stem-like HCC, and 15 less aggressive (EpCAM-AFP-) mature hepatocyte MH-HCC patients. Analysis of tumor specimens and uninvolved healthy tissue by principal component analysis revealed clear demarcation between tumor and non-tumor metabolomes. Non-targeted metabolic profiling of tumor and non-tumor specimens identified 48 markers that were significantly altered between HpSC-HCC patients and patients with MH-HCC. This metabolite panel resolved aggressive HCC from the less subtype at a sensitivity of 72% and a specificity of 83% ( $P < 0.05$ ). The group further demonstrated that within the subset of 48 metabolites associated with HCC aggressivity, 28 were significantly associated with overall survival. Subsequent microarray gene expression profiling of the paired tumor specimens identified 169 genes that could significantly distinguish the two HCC subtypes. The group then gauged the correlation between this genetic signature and the panel of 28 metabolites that were associated with both HCC aggressivity and overall survival and found that each of the 28 metabolites were associated with at least one of the 169 genes identified by microarray analysis. To determine the principal metabolite-gene

pairs potentially influencing HCC aggressivity, the group performed correlation analysis with randomization and found 15 metabolites and 121 genes most highly associated with the tumor and stem-like HCC genes. Validation of the genetic signature in an independent testing cohort ( $n = 217$ ) revealed a panel of 273 genes that distinguished HpSC-HCC from MH-HCC with a sensitivity of 72% and a specificity of 91% ( $P < 0.01$ ), and this genetic signature was also strongly ( $P < 0.0001$ ) associated with overall survival. This gene set was also a significant independent predictor of overall survival, progression free survival and recurrence, highlighting its utility as a prognostic panel of aggressive HCC. Many of the genes were associated with fatty acid metabolism and the phosphatidylinositol 3-kinase signaling pathway. Elevated palmitoleate expression in HpSC-HCC tumor vs paired non-tumor specimens reflected overexpression or enhanced activity of stearoyl-CoA dehydrogenase (SCD), an important cell turnover regulatory pathway. Significant downregulation of arachidonate and linoleate, the parent molecules of the eicosanoid signaling cascade, suggested overconsumption of these metabolites from hyperactivity of cyclooxygenase, lipoxygenase, and/or cytochrome P450c in aggressive HCC. SCD, which converts saturated palmitic acid to palmitoleic acid, was among the gene set. To validate the possible role of SCD in cancer aggressivity, *in vitro* and *in vivo* analysis was done and showed that selective SCD inhibitor CGX0168 abrogated Huh7 cell migration and invasion. Supplementation of Huh7 with palmitoleate, the end product of SCD activity, enhanced cell migration and invasion. Xenografted tumors in nude mice showed that SCD-siRNA inhibited tumor migration and colony formation and increased apoptosis, likely owing to the accumulation of pro-apoptotic palmitate. Taken together, this approach showed a strong lipid signature associated with HCC aggressivity. Elevated palmitoleate reflected upregulated SCD, a key regulator of the ratio between saturated and unsaturated fatty acids. A tilt in the delicate balance between saturated and unsaturated fatty acids toward unsaturated has been implicated in cancer aggressiveness.

Remarkably, the integrated metabolomics-transcriptomics study by Beyoğlu *et al.*<sup>[44]</sup> also revealed a consistent role for palmitate, linoleate, and the PI3K pathway in HCC. Microarray analysis found that 11 genes involved in fatty acid were associated with HBV-positive HCC cases. More broadly, the comparison of tumor vs paired non-tumor tissues identified a metabolic shift toward glycolysis reflective of the Warburg effect implicated in other HCC metabolomics works. Levels of glucose, glycerol 3-phosphate, glycerol 2-phosphate, malate, alanine, and myo-inositol were all significantly decreased in tumor vs non-tumor specimens, indicating both impaired mitochondrial respiration and enhanced glycolysis. Myo-inositol is the metabolic precursor of the messenger molecule inositol triphosphate (PI3) required by PI3K-AKT-mTOR, and its downregulation in tumor vs non-tumor specimens is consistent with the hyperactivity of this pathway implicated in numerous cancers. Further-

more, the group showed that 1-stearoylglycerol and 1-palmitoylglycerol were decreased in tumor vs non-tumor. 1-acylglycerols are synthesized by phospholipase activity on LPCs and the routinely reported decrease in LPCs in HCC vs controls may explain the reduced 1-acylglycerols in this study. Through integration of metabolomics and transcriptomics, these groups together show a possible role for SCD in HCC, implicate the PI3K pathway as important to HCC progression, and show a strong lipid signature associated with HCC, in agreement with other HCC metabolomics works. More broadly, these works demonstrate that the achievement of consistent trends in independent, integrated “omics” studies can be achieved and lay important groundwork for future studies of this nature.

### **Sensitivity and specificity of metabolomics in HCC diagnosis**

To evaluate the utility for metabolomic profiles to distinguish between patients with cirrhosis and patients with HCC, these studies primarily employed principal component analysis, orthogonal projection to latent structures, supervised projection to latent structures discriminant analysis, the random forest machine learning algorithm, or ROC curve class prediction analyses. Reported sensitivity/specificity/area under the curve values reflecting the accuracy of metabolomics in distinguishing HCC from cirrhosis are shown in Table 3. In general, metabolomics was a highly accurate diagnostic method and clear demarcation between healthy controls and/or cirrhosis controls vs HCC patients were realized. Where metabolomics profile class prediction was compared with AFP, the metabolomics approach showed greater class prediction power. In general, the focus of these works was the potential standardization of metabolomics as a high-throughput clinical diagnostic platform-big-data biomarkers rather than single metabolite alterations—which may explain the lack of emphasis on the sensitivity and specificity of individual metabolites for diagnosing HCC. Just two studies verified the metabolite expression patterns *in vitro*, and with the exception of the *in vivo* validation performed by Budhu *et al.*<sup>[43]</sup>, no studies used *in vivo* models to validate their metabolomics data. It is therefore recommended that future validation of HCC metabolomics data include preliminary information on metabolite concentrations in HCC cases vs cirrhosis controls as measured *in vitro* or *in vivo* to reveal their utility as potential biomarkers that can be employed in a rapid *in vitro* diagnostic.

## **DISCUSSION**

### **Summary of major findings**

The results of these studies show that aberrations in bile acid, LPC, acylcarnitine, ROS, and protein metabolism may be signatures of HCCs emerging in the setting of cirrhosis. Bile acids trended lower in HCC vs cirrhosis controls in all studies reporting a significant difference in expression of these metabolites<sup>[36-38]</sup>. In general, LPCs were shown



**Table 3** Utility of significantly altered ( $P < 0.05$ ) metabolites in accurately predicting hepatocellular carcinoma (hepatocellular carcinoma cases *vs* patients with cirrhosis)

Ref.	Platform	Comparison	Class prediction methodology	Classification accuracy or sensitivity/specificity	AFP sensitivity /specificity
Patterson <i>et al</i> <sup>[29]</sup>	UPLC/ESI-QTOF-MS	HCC ( $n = 20$ ) <i>vs</i> cirrhosis ( $n = 7$ )	Random forest	96.3	-
Chen <i>et al</i> <sup>[30]</sup>	Integrated GC/QTOF-MS + UPLC/QTOF-MS	HCC ( $n = 82$ ) <i>vs</i> healthy ( $n = 71$ )	OPLS-DA	100.0	-
Wu <i>et al</i> <sup>[31]</sup>	SELDI-TOF MS	HCC ( $n = 48$ ) <i>vs</i> cirrhosis ( $n = 54$ ) or healthy ( $n = 42$ )	GRO- $\alpha$ + thrombin light chain PS20 Protein immunoassay	89.6/89.6	69/83
Cao <i>et al</i> <sup>[32]</sup>	UPLC/QTOF-MS	HCC ( $n = 23$ ) <i>vs</i> cirrhosis ( $n = 22$ )	PLS-DA	67.0	-
Gao <i>et al</i> <sup>[33]</sup>	NMR	HCC ( $n = 39$ ) <i>vs</i> cirrhosis ( $n = 36$ )	PLS-DA	45.7	-
Wu <i>et al</i> <sup>[34]</sup>	GC/MS	HCC ( $n = 20$ ) <i>vs</i> healthy ( $n = 20$ )	PCA with ROC curve analysis	AUC=88.3; AUCAFP = 92.5 when combined with AFP	-
Soga <i>et al</i> <sup>[35]</sup>	LC/MS-MS	HCC ( $n = 32$ ) <i>vs</i> HCV-only ( $n = 35$ ) or cirrhosis ( $n = 18$ )	Multiple logistic regression; ROC curve analysis	88.1	0.760
Wang <i>et al</i> <sup>[38]</sup>	UPLC-MS	HCC (59) <i>vs</i> cirrhosis (20) or NHC (20)	PLS-DA, ROC curve analysis	CSA 79.3/100 CSA + AFP20 96.4/100 UPLC-MS 100/100	AFP20 74/38 AFP200 52/90
Zhou <i>et al</i> <sup>[39]</sup>	UPLC-QTOF-MS	HCC ( $n = 69$ ) <i>vs</i> cirrhosis ( $n = 28$ )	PLS-DA, ROC curve analysis	AEA 88.0 PEA 82.0 AEA + PEA 88.0	-
Nahon <i>et al</i> <sup>[40]</sup>	NMR	Small HCC ( $n = 28$ ) <i>vs</i> cirrhosis ( $n = 93$ ); Large HCC ( $n = 33$ ) <i>vs</i> cirrhosis ( $n = 93$ )	OPLS	Small HCC: 61.0/100.0 Large HCC: 100.0/100.0	-
Yin <i>et al</i> <sup>[41]</sup>	RPLC/QTOF-MS; HILIC/QTOF-MS	HCC ( $n = 25$ ) <i>vs</i> cirrhosis ( $n = 24$ ) or healthy ( $n = 25$ )	OPLS	RPLC: 61.8 HILIC: 57.0 RPLC + HILIC = 63.6	-
Li <i>et al</i> <sup>[42]</sup>	UPLC/QTOF-MS	HCC ( $n = 8$ ) <i>vs</i> cirrhosis ( $n = 6$ ) or healthy ( $n = 6$ ) (murine samples)	OPLS-DA	88.2	-
Budhu <i>et al</i> <sup>[43]</sup>	Training set1: GC/MS + UPLC/MS-MS; Testing set2: Affymetrix GeneChip	Training set: Stem-like aggressive HpSC-HCC ( $n = 15$ ) <i>vs</i> Mature hepatocyte less aggressive MH-HCC ( $n = 15$ ); Testing set: HpSC-HCC and MH-HCC ( $n = 217$ )	Multivariate analysis	172.0/83.0, AUC = 0.830 272.0/91.0, AUC = 0.860	-
Fitian <i>et al</i> <sup>[45]</sup>	UPLC/MS-MS + GC/MS	HCC ( $n = 30$ ) <i>vs</i> HCV-cirrhosis ( $n = 27$ )	Random forest  ROC analysis	72% 12-HETE 73.3/69.2 15-HETE 83.3/59.3 Aspartate 100/51.9 Glycine 83.3/63.0 Serine 73.3/85.2 Phenylalanine 73.3/81.5 Homoserine 70.0/85.2 Sphingosine 58.3/86.7 Xanthine 63.3/88.9 2-Hydroxybutyrate 76.7/77.8	AFP20 63.3/83.6
Gao <i>et al</i> <sup>[46]</sup>	GC-TOF/MS	HCC ( $n = 39$ ) <i>vs</i> HBV-cirrhosis ( $n = 52$ )	Random forest (validation set)  ROC analysis (validation set)  Bayes discriminant function model (validation set)	96.8% in HCC <i>vs</i> HBV-cirrhosis 100% in HBV-cirrhosis <i>vs</i> HBV 100% in HBV <i>vs</i> NHC 100/95.2 HBV <i>vs</i> NC 83.3/100 HBV-cirrhosis <i>vs</i> HBV 76.9/83.3 HCC <i>vs</i> HBV-cirrhosis 76.9% HCC 100% HBV-cirrhosis 94.1% HBV 100% NHC	-

Classification accuracy describes the capacity of the metabolomic classification technique to accurately predict the group of each study subject. UPLC: Ultrahigh-performance liquid chromatography; AEA: Anandamide; OPLS: Orthogonal projection to latent structure; PCA: Principal component analysis; PEA: Palmitylethanolamide; PLS-DA: Partial least squares-discriminant analysis; MS: Mass spectrometry; TOF: Time-of-flight; GC: Gas chromatography; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; GRO- $\alpha$ : Growth related oncogene-alpha; ROC: Receiver operator characteristic; NHC: Normal healthy controls; AFP20: AFP performance at the cutoff of 20 ng/mL; AFP200: AFP performance at the cutoff of 200 ng/mL.



to be downregulated in HCC vs cirrhosis<sup>[37-39,45]</sup>. But the works by Ressom *et al.*<sup>[36]</sup> and Wang *et al.*<sup>[38]</sup> found that LPCs and LPEs were consistently elevated in the HCC vs cirrhosis comparison. Acylcarnitines, which function as liaisons of fatty acid  $\beta$ -oxidation by enabling fatty acid importation to the mitochondrial matrix, were significantly downregulated in HCC vs cirrhosis. Amino acids also trended higher in HCC in all studies reporting significant expression differences between HCC and cirrhosis. The hemoglobin metabolite bilirubin was also consistently elevated in HCC vs cirrhosis<sup>[29,39,41,45]</sup>, consistent with the clinical utility this metabolite possesses for diagnosing advanced liver disease. Biliverdin, another heme catabolite, was significantly upregulated in HCC vs cirrhosis in two studies<sup>[29,45]</sup> and a metabolic derivative of biliverdin downregulated in a third<sup>[37]</sup>.

The elevation of LPCs in HCC vs cirrhosis seen by Ressom *et al.*<sup>[36]</sup> is consistent with increased demand of glycerophospholipids by the growing tumor. LPCs comprise only 3% of the total phospholipids in plasma membranes, however, and a better explanation for their elevation may be that LPCs are major lipids bound to albumin. The loss of albumin commonly witnessed in chronic liver disease and HCC may mean a shortage of docking sites for LPCs, resulting in increased systemic levels of these glycerophospholipids. Diminished bile acids in HCC vs cirrhosis reinforces the Warburg effect commonly witnessed in cancer studies involving a shift in energy production away from oxidative processes like  $\beta$ -oxidation and the TCA cycle toward anaerobic glycolysis, which is more suitable to the hypoxic or anoxic tumor microenvironment. The loss of bile acids in HCC significantly impairs fatty acid absorption and digestion and is consistent with the general trend of increased circulating lipids in HCC vs cirrhosis and cirrhosis vs appropriate controls. The elevation of bile acids has historically served as a clinical indicator of chronic liver disease and specifically cirrhosis and consistent with this function, our metabolomics work showed a global upregulation of bile acids in cirrhosis vs healthy volunteers<sup>[45]</sup>. Decreased acylcarnitines further reflects diminished reliance on  $\beta$ -oxidation, and it may also signify impairment of the mitochondrial carnitine palmitoyltransferase shuttle system.

### **Metabolomic heterogeneity attributed to differences in study design**

While perturbations in phospholipid, bile acid, hemoglobin, acylcarnitine and amino acid metabolism were routinely encountered in these studies, the referenced works did not achieve uniform conclusions regarding the directional shifts of the metabolites' expression in HCC vs cirrhosis, with examples including contradictory patterns of LPC<sup>[36,39]</sup> and amino acid<sup>[28,46]</sup> expression in HCC vs cirrhosis. The heterogeneity among these studies' metabolomes is likely due to differences in study design. Although some studies matched patients by age and gender, the majority of the referenced HCC metabolomics

studies did not extensively characterize HCC and cirrhosis patients by demographic and clinical characteristic parameters. Only four studies reported MELD scores for HCC and cirrhosis patients<sup>[36,37,40,45]</sup>, and just three studies indicated the Child-Pugh status of their HCC or cirrhosis cohorts<sup>[29,40,45]</sup>. This lack of information on whether liver function of the study participants is compensated or decompensated complicates interpretation of metabolomic data. Body mass index (BMI) also went unreported for HCC, cirrhosis, and healthy study subjects in all but two studies<sup>[43,45]</sup> referenced in this review. BMI can have a significant influence on the relative metabolite expression differences between patients, particularly with regard to adiposity. Because these studies did not control for BMI, is likely that the patients recruited for these metabolomics studies had wide-ranging BMIs that may further explain the noticeably different trends of LPC, FFA, and acylcarnitine expression in HCC vs controls. To limit the influence of potential cofounders such as comorbidity, BMI, age or gender, and etiology on HCC metabolomes, patient clinical characteristics should be controlled for more conscientiously in future HCC metabolomics studies.

There was also marked variation in how each group diagnosed and staged their HCC patients. Three studies<sup>[29,43,45]</sup> staged according to the Barcelona Clinic for Liver Cancer staging criteria. The TNM Classification of Malignant Tumors (TNM) was used to diagnose HCC in five studies<sup>[28,30,37,38,43]</sup>, imaging in two, histopathology in one<sup>[36]</sup>, and six studies made no mention of their HCC diagnosis method<sup>[31,33,35,39,41,42]</sup>. Differences in HCC diagnosis and staging among these studies likely contributed to the discordant global metabolomic alterations in HCC vs cirrhosis among these studies and further confounded the interpretation of these metabolomic trends.

### **Lack of emphasis on HCC vs cirrhosis comparison**

The critical metabolomic comparison between HCC patients and cirrhosis controls was reported in just ten out of the twenty studies referenced in this review<sup>[28,29,31,35-39,45,46]</sup>, with half of the cited works instead focusing on the metabolomic profile differences between HCC patients and NHC subjects. While this comparison sheds light on altered pathways during hepatocarcinogenesis, it may not be as applicable from a clinical standpoint as the trends elucidated through a comparison of HCC vs cirrhosis. This owes to the fact that the majority of primary liver cancer cases occur in patients with a preexisting condition of cirrhosis. Therefore, the metabolomic comparison between HCC and cirrhosis is more clinically informative and potentially translational than the comparison of HCC vs NHC. HCC is a complex heterogeneous disease and HCC patients often present with multiple comorbidities. It is therefore likely that marked metabolomic differences will be observed between HCC patients and healthy subjects. Differences between HCC patients and cirrhosis controls are subtler than HCC vs NHC<sup>[45,46]</sup> and it would be expected that conspicuous metabolomic differences

exist between HCC/NHC and cirrhosis/NHC. Given that HCC arose in the background of cirrhosis in all reported HCC metabolomics studies, it is impossible to determine whether the alterations witnessed in HCC vs NHC are related to HCC or cirrhosis, further reinforcing the need for future metabolomic comparisons between HCC and cirrhosis.

## CONCLUSION

Bile acids, acylcarnitines, amino acids, free fatty acids, LPCs, and heme pigmentation molecules exhibited utility in stratifying HCC patients from patients with cirrhosis. Canavaninosuccinate, which showed a striking 680-fold elevation in HCC patients vs cirrhosis controls and outperformed AFP in sensitivity and specificity. Sphingosine 1-phosphate (↑), sphingosine (↑), GRO-α (↑), and thrombin light chain (↑) were other putative HCC biomarkers that had superior predictive utility for HCC than AFP. Bile acids, fibrinogen cleavage byproducts, dicarboxylic fatty acids, and ROS-related γ-glutamyl peptides exhibited strong association with cirrhosis and the further development of these metabolites as diagnostic markers of cirrhosis may be valuable. In addition to individual metabolites, global patient metabolomes exhibited superior sensitivity for diagnosis of HCC vs AFP where a comparison was made<sup>[31,35,38,45]</sup>.

Although these data are preliminary in nature, they reflect the early promise that metabolomics platforms hold in potential clinical implementation for disease diagnosis. Future metabolomics studies with larger, better demographically and clinically characterized patient cohorts may resolve the heterogeneous metabolomic expression patterns in HCC vs cirrhosis.

Given that > 90% of HCCs emerge in the setting of cirrhosis, future HCC metabolomics studies will be most impactful and clinically relevant if they compare the expression patterns of HCC patients vs cirrhosis controls. Moreover, patients with HCC and cirrhosis should be matched by etiology (viral vs non-viral), liver function, and BMI to limit the influence of data confounders. Furthermore, it is paramount the appropriate metabolomic profile comparisons are made, namely HCC vs cirrhosis, cirrhosis vs etiology of cirrhosis (viral or non-viral), and cirrhosis vs NHC. In a recent study, the entire stepwise hepatocarcinogenic process from NHC to HBV, through cirrhosis, and culminating in HCC was analyzed. Appropriately, Gao *et al.*<sup>[46]</sup> focused their expression profile comparisons on HCC vs cirrhosis, and cirrhosis vs HBV/NHC. This approach enables investigators to make adequate conclusions about the interval at which putative biomarkers become relevant in the hepatocarcinogenic process and demonstrates the relevance of the dataset comparisons. Moreover, investigation of the pathways involved in the progression from the initial liver insult to cirrhosis remains a largely untapped realm of biomarker discovery within metabolomics, and may streamline the identification of potential cirrhosis diagnostic markers. More broadly, metabolomics is well-suited for clarifying

the entirety of the metabolic remodeling that occurs throughout hepatocarcinogenesis, and can hence streamline biomarker discovery efforts at each pathological interval.

The findings of these works demonstrate the powerful resource that is metabolomics for identifying potential novel diagnostic biomarkers of HCC. The translational optimality of metabolomics is underscored by its capability to simultaneously process high volumes of patient specimens and interpret metabolic expression profiles through robust, validated and automated software. Still greater, metabolomics holds promise as a novel disease screening and diagnostic modality that, through characterization of a patient's global metabolic profile, can in a more sophisticated and comprehensive fashion accurately predict the presence of disease.

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## PI3K/SHIP2/PTEN pathway in cell polarity and hepatitis C virus pathogenesis

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### Abstract

Hepatitis C virus (HCV) infects hepatocytes, polarized cells

in the liver. Chronic HCV infection often leads to steatosis, fibrosis, cirrhosis and hepatocellular carcinoma, and it has been identified as the leading cause of liver transplantation worldwide. The HCV replication cycle is dependent on lipid metabolism and particularly an accumulation of lipid droplets in host cells. Phosphoinositides (PIs) are minor phospholipids enriched in different membranes and their levels are tightly regulated by specific PI kinases and phosphatases. PIs are implicated in a vast array of cellular responses that are central to morphogenesis, such as cytoskeletal changes, cytokinesis and the recruitment of downstream effectors to govern mechanisms involved in polarization and lumen formation. Important reviews of the literature identified phosphatidylinositol (PtdIns) 4-kinases, and their lipid products PtdIns(4)P, as critical regulators of the HCV life cycle. SH2-containing inositol polyphosphate 5-phosphatase (SHIP2), phosphoinositide 3-kinase (PI3K) and their lipid products PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>, respectively, play an important role in the cell membrane and are key to the establishment of apicobasal polarity and lumen formation. In this review, we will focus on these new functions of PI3K and SHIP2, and their deregulation by HCV, causing a disruption of apicobasal polarity, actin organization and extracellular matrix assembly. Finally we will highlight the involvement of this pathway in the event of insulin resistance and nonalcoholic fatty liver disease related to HCV infection.

**Key words:** Hepatitis C virus; Phosphoinositide 3-kinase; SH2-containing inositol polyphosphate 5-phosphatase; Epithelial cell polarity; Phosphoinositides

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**Core tip:** Chronic hepatitis C virus (HCV) infection leads to liver cirrhosis and cancer. HCV infection modulates the lipid metabolism. Phosphoinositides are minor phospholipids that are also modified by HCV infection. phosphatidylinositol (PtdIns)(3,4,5)P<sub>3</sub> is mainly formed by phosphoinositide 3-kinase (PI3K), and

can be dephosphorylated by SH2-containing inositol polyphosphate 5-phosphatase (SHIP2) to generate PtdIns(3,4)P<sub>2</sub>. In this review, we will discuss the effects of SHIP2 and PI3K on the formation of cell polarity and how their expression and activation are modulated by HCV infection, leading to the disruption of cell polarity. This pathway is also discussed in the event of insulin resistance and nonalcoholic fatty liver disease related to HCV infection.

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## INTRODUCTION

Chronic Hepatitis C virus (HCV) infection leads to cirrhosis that will develop complications such as liver failure and liver cancer<sup>[1]</sup>. The principal target of HCV is hepatocytes, which are highly polarized cells, their plasma membranes being separated by tight junctions into apical (canalicular) and basolateral (sinusoidal) domains<sup>[2,3]</sup>. After it enters the hepatocytes, the life cycle of HCV is very closely linked to cell lipid metabolism. The very low density lipoprotein (VLDL) pathway is required for the assembly and secretion of new viral particles<sup>[4,5]</sup>. However, the effect of lipid droplets (LD) on the replication of HCV is becoming increasingly clear<sup>[6]</sup>. HCV induces an accumulation and change to the cellular distribution of LDs, moving from a dispersed profile in the cytoplasm of non-infected cells to their perinuclear localization in infected cells. This relocation permits the interaction of LDs with viral proteins and genomes<sup>[7]</sup>. While much is known about the role of lipoproteins and LDs in the HCV life cycle, studies are only now emerging on the modulation of phosphoinositides (PI) by HCV infection<sup>[8]</sup>.

PIs are phosphorylated derivatives of phosphatidylinositol (PtdIns). They are minor phospholipids (10%-20%) on the inner surface of the lipid bilayer and an important constituent of the cell membrane. The phosphorylation and dephosphorylation of PIs is achieved by various isoforms of PI kinases and PI phosphatases, distributed in a specific way in the cell, this result in the distribution of different PIs in cell compartments. These complex reactions are mediated by 19 kinases and 28 phosphatases that have been identified in mammals<sup>[9,10]</sup>. Figure 1 illustrates the phosphorylation and dephosphorylation cycles of different monophosphate, diphosphate and triphosphate PIs, as well as the most widely studied kinases and phosphatases. PIs are secondary messengers responsible for transmitting receptor signals to the effectors that induce a cellular response. PIs interact with these effectors *via* specific binding domains that are known to interact with the membrane, either by specific recognition of the membrane components or through attraction by its properties such as charge,

structure, curvature and amphiphilicity<sup>[11]</sup>. As well as acting as precursors of secondary messengers, PIs are spatiotemporal regulators of several target proteins involved in vesicular trafficking [such as PtdIns(4)P and PtdIns(3)P] and cytoskeletal rearrangement [PtdIns(4,5)P<sub>2</sub>], by which they control cell polarity, migration, proliferation and differentiation [PtdIns(3,4)P<sub>2</sub>] and PtdIns(3,4,5)P<sub>3</sub><sup>[12,13]</sup>.

Given the importance of PI metabolism to cellular signaling and trafficking events, numerous intracellular pathogens modulate and exploit PIs in order to ensure their survival and efficient intracellular replication<sup>[14,15]</sup>. A considerable body of literature has addressed the modulation of PIs by HCV<sup>[8]</sup>. Changes to the localization of PtdIns(4)P and activation of PI4KIII $\alpha$  following HCV infection have been identified as being key to membrane network formation and viral replication<sup>[1,16,17]</sup>. In this review we will focus on the roles of phosphoinositide 3-kinase (PI3K), SH2-containing inositol polyphosphate 5-phosphatase (SHIP2) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and their lipid products in the establishment of plasma membrane polarity. We will also discuss how HCV infection modulates these polarity mechanisms to invade host cells and replicate. Finally, we will consider the involvement of the PI3K/PTEN/SHIP2 pathway in insulin resistance and nonalcoholic fatty liver disease (NAFLD) related to HCV infection.

## HCV

HCV is an enveloped virus with linear, single-stranded RNA contained in a capsid protein called core. Following entry of the virus into a host cell and uncoating of the viral genome, the translation of viral RNA produces a polyprotein that will be degraded to form three structural proteins (core, E1 and E2), six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) and p7 protein. The structural and non-structural proteins will mate with viral RNA to form new viral particles. This viral assembly occurs near the endoplasmic reticulum, and then new viral particles are released from the cell through fusion with the cell membrane<sup>[18,19]</sup>. Chronic infection with HCV, especially in cases of cirrhosis or advanced fibrosis, remains the leading cause of hepatocellular carcinoma (HCC) worldwide through the modulation of different pathways such as inflammation, proliferation and differentiation, and DNA damage<sup>[20]</sup>. Effective treatments for HCV have recently been developed, and over 95% of patients can now be cured<sup>[21,22]</sup>, but pathologies such as cancer induced by HCV infection remain a health problem for those already infected. Interactions between viral proteins and host cell mechanisms therefore remain central to understanding the pathogenesis of HCV.

## POLARITY IN THE LIVER AND THE ENTRY OF HCV

The polarity of epithelial cells is a property that is ess-

PI3Ks phosphorylate the hydroxyl group 3-position of the inositol ring of PtdIns. The PI3K family of enzymes contains three different classes ( I , II and III ), based on their substrate specificity and molecular structure<sup>[36]</sup>. PtdIns(3)P can be formed by PI3K- II and PI3K-III, and PtdIns(3,4)P2 is generated by the activity of PI3K- II. The activity of PI3K- I produces PtdIns(3,4,5)P3 which, once produced at the membrane, exercises its vital role of second messenger by recruiting different proteins containing a pleckstrin homology (PH) domain. The protein kinase Akt possesses a PH domain which was the first to be discovered and displays high affinity binding to phosphoinositides. The interaction between Akt and PtdIns(3,4,5)P3 induces a conformational change in

the Akt structure which permits its phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) at threonine 308, and the mammalian target of rapamycin complex 2 (mTORC2) at serine 473 (Figure 2). Activated Akt is responsible for triggering numerous cellular signaling pathways involved in proliferation, survival, apoptosis and autophagy<sup>[12-37]</sup>. PI3K and its lipid product PtdIns(3,4,5)P3 have been identified as key regulators of apicobasal polarity<sup>[38,39]</sup>. Watton and Downward showed that the adhesion of epithelial cells to the extracellular matrix provides protection from apoptosis *via* the activation of PI3K and Akt/PKB. They confirmed the localization of PI3K at the basolateral membrane producing PtdIns(3,4,5)P3<sup>[38]</sup>. Another study also revealed PI3K activation after interaction of the cell with the ECM and cell-cell contact. They showed that E-cadherin, which is responsible for cell-cell junctions, joins the p85 sub-unit of PI3K and activates PI3K/Akt to generate PtdIns(3,4,5)P3 during the early stages of cellular polarization<sup>[40]</sup>. Gassama-Diagne *et al.*<sup>[39]</sup> studied the localization of PtdIns(3,4,5)P3 at the basolateral membrane in madin darby canine kidney (MDCK) polarized cells. This study confirmed that the formation of PtdIns(3,4,5)P3 at the basolateral membrane is essential to the initiation of basolateral polarization through the activation of Rac1. In fact, the experimental addition of exogenous PtdIns(3,4,5)P3 at the apical pole of polarized MDCK cells on transwell filters led in five minutes to the formation of PtdIns(3,4,5)P3 and basolateral protein-rich protrusions above the apical surface, from which apical proteins were excluded<sup>[39]</sup>. In MDCK 3D culture on matrigel, the formation of basolateral protein-rich protrusions was observed after three minutes of treatment with exogenous PtdIns(3,4,5)P3. The location and PI3K activity of these protrusions is important because the action of the PI3K inhibitor LY294002 inhibits the formation of protrusions, even when treating the cells with PtdIns(3,4,5)P3. By testing different ATP-competitive isoform-selective inhibitors of PI3K on the apicobasal polarity of MDCK grown in a 3D culture on Matrigel. Peng *et al.*<sup>[41]</sup> recently showed that treatment with the p110 $\delta$  inhibitors IC87114 and CAL-101 inverted the cell polarity of cysts displaying the apical marker Podocalyxin in contact with the ECM. The treatment of cells with other inhibitors such as PI-103, a multi-targeted inhibitor of p110 $\alpha$ /p110 $\beta$ /p110 $\gamma$ , and AS-605240 which targets p110 $\gamma$ , led to the formation of either multi-lumen or lumen-free cysts. Taken together, these data indicate that the P110 $\delta$  isoform plays a role in establishing the apicobasal polarity axis. Next, the p110 $\delta$  isoform was localized at the basolateral membrane of polarized cysts, and colocalized with the ECM receptor dystroglycan. The depletion of p110 $\delta$  at the basolateral membrane disrupted laminin and type IV collagen assembly by down-regulating  $\beta$ 1-integrin, which is a transmembrane protein with a specific role in ECM assembly and remodeling<sup>[42]</sup>. Overall, these findings revealed the role of epithelial p110 $\delta$  in the orientation of cell polarity and lumen formation by

regulating ECM assembly and interactions<sup>[41]</sup>.

A growing body of evidence in the literature has revealed that PIs and their metabolic enzymes are essential to HCV replication at different stages of the cell cycle<sup>[16,43,44]</sup>. Some studies validated activation of the PI3K/Akt pathway following infection by HCV<sup>[45]</sup>. Epidermal growth factor receptor (EGFR), which activates the PI3K/Akt pathway, was recently shown to be a co-factor for HCV entry in a cell<sup>[46]</sup>. Moreover, Street *et al.*<sup>[47]</sup> demonstrated an interaction between the viral protein NS5A and the p85 subunit of PI3K. This interaction is responsible for activating the p110 subunit of PI3K, which induces the formation of PtdIns(3,4,5)P3. The same study also showed that the NS5A protein induces the phosphorylation of Akt at tyrosine 308, thus causing anti-apoptotic activity. Other than its role in cell proliferation, transcription and migration, PI3K is also responsible for membrane expression of the SR-B1 receptor in HepG2 cells, promoting viral entry into the cells<sup>[48]</sup>. Furthermore, the NS4B protein induces lipogenesis in infected cells by activating the Akt pathway<sup>[49]</sup>. It has been shown that HCV core protein is expressed at the basal membrane of polarized cells, which leads to a deregulation of actin organization and affects focal contacts by increasing the expression of phosphorylated paxillin at the basal membrane<sup>[50]</sup>. The same study showed that the deregulation of actin is due to RhoA inhibition and Rac1 activation in cells expressing HCV core protein. These results also suggest that HCV core disrupts cell adhesion, inducing a reorganization of the actin cytoskeleton and a loss of cell polarity. Is PI3K involved in HCV core expression, thus inducing a disruption of cell adhesion? Many studies have revealed activation of the PI3K/Akt pathway and the formation of PtdIns(3,4,5)P3 following HCV infection<sup>[8,45,51,52]</sup>. This work focused on virus entry and replication and the epithelial to mesenchymal transition, but the effect of PI3K expression on the loss of cell polarity induced by HCV infection was not investigated. Nevertheless, the phenotype of cysts from MDCK cells expressing HCV core protein was of a multi-lumen type<sup>[50]</sup> which differed markedly from that of the inverted polarity cysts obtained from MDCK cells treated with the p100 $\delta$  inhibitors IC87114 and CAL-101, but was similar to those from MDCK cells treated with the p110 $\gamma$  and p110 $\beta$  inhibitors AS-605240 and TGX115, respectively<sup>[41]</sup>. This observation reveals a potential role for p110 $\gamma$  and p110 $\beta$  in the loss of cell polarity induced by HCV infection. Interestingly, Peng *et al.*<sup>[41]</sup> studied an MDCK phenotype involving an over-expression of the PI3K p110 subunit. These cysts displayed a marked ECM assembly (laminin and type IV collagen) at the basal membrane and a loss of cell polarity, and they were flatter than control cells. This result allows us to advance the hypothesis that p110 $\delta$  may be over-expressed in the context of HCV infection, leading to an accumulation of ECM which is responsible for cirrhosis. Taken together, these findings suggest a potential effect of PI3K and PtdIns(3,4,5)P3 on the deregulation of cell polarity induced by HCV infection.



## SHIP2 ACTIVITY, CELL POLARITY AND HCV INFECTION

The level of PtdIns(3,4,5)P3 is maintained through its dephosphorylation by the phosphatases SHIP1/2 and PTEN to produce PtdIns(3,4)P2 and PtdIns(4,5)P2, respectively<sup>[53]</sup> (Figure 3). A recent study highlighted the role of PtdIns(3,4)P2 and SHIP2 as additional determinants of basolateral membrane formation<sup>[50]</sup>. In non-polarized cells, SHIP2 is localized in the perinuclear and cytoplasmic domains. After stimulation with serum, SHIP2 may be localized at focal contacts in the plasma membrane<sup>[53]</sup>. In 3D cultured MDCK cells, Awad *et al.*<sup>[50]</sup> demonstrated a basolateral localization of SHIP2 and its lipid product PtdIns(3,4)P2. The enzymatic activity of SHIP2 gives rise to PtdIns(3,4)P2 and is essential for cell polarization. Indeed, SHIP2 inhibition by siRNA, and exogenous expression of the catalytic mutant of SHIP2 (D607A)<sup>[54,55]</sup> lead to a deregulation of cell polarity and the formation of multi-lumen cysts. Indeed, PtdIns(3,4)P2 is capable of binding Dlg1, the master regulator of basolateral polarity<sup>[56]</sup>. The inhibition of SHIP2 leads to a delocalization of the basolateral polarity proteins  $\beta$ -catenin, Scribble and Dlg1 from cell-cell contacts; moreover, their expression is markedly reduced. Overall, these data suggest that SHIP2 is required for the localization and expression of the basolateral complex proteins Dlg1 and Scribble in order to maintain cell morphogenesis<sup>[50]</sup>. Moreover, the Rho family of GTPases, and particularly RhoA and Rac1 which regulate the formation of stress fibers and lamellipodia, respectively<sup>[57]</sup>, play a pivotal role in cell polarity<sup>[58]</sup>. It has been reported that SHIP2 increases the activation of RhoA in epithelial cells<sup>[50]</sup>. This activation is required for the polarization and migration of glioma cells<sup>[59]</sup>. Awad *et al.*<sup>[50]</sup> confirmed that SHIP2 is an additional target for HCV infection. Their study examined the expression of SHIP2 and PtdIns(3,4)P2 in MDCK cells grown in a 3D culture on Matrigel, and in a 2D culture on transwell filters. MDCK cells expressing HCV core protein displayed a reduction of SHIP2 and PtdIns(3,4)P2 expression at the basal membrane. Interestingly, HCV core protein was localized specifically at the basal membrane in contact with the ECM. Together, these findings indicate that HCV core protein is able to subvert SHIP2 expression in order to disrupt cell membrane morphology<sup>[50]</sup>. In these cells, the down-regulation of SHIP2 and PtdIns(3,4)P2 leads to down-regulation of the expression of Dlg1 and Scribble at the basolateral membrane. These disturbances to the expression of polarity proteins lead accordingly a loss of apicobasal cell polarity and the formation of multi-lumen cysts. HCV core expression also displays a loss of RhoA activation, in the same way as SHIP2 depleted cells. Interestingly, an over-expression of SHIP2 cDNA in HCV core-expressing cells has been seen to restore single lumen formation, RhoA activation and cell polarity. Taken together, these data indicate that HCV core is able to subvert SHIP2 in order to disrupt cell polarity and infected polarized cells<sup>[50]</sup>.

## PTEN AND PTDINS(4,5)P2, CELL POLARITY AND HCV INFECTION

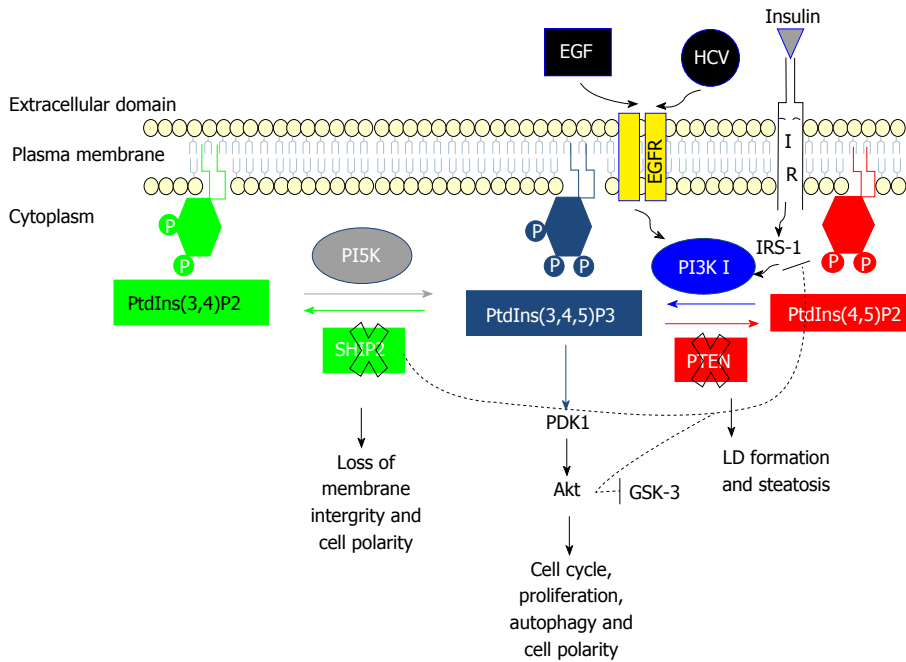
PTEN, the other phosphatase which antagonizes PI3K, is also implicated in cell polarity. Martin-Belmonte *et al.*<sup>[60]</sup> identified PtdIns(4,5)P2 as a key regulator of the apical membrane. During the early stages of cyst formation, PtdIns(3,4,5)P3 and PtdIns(4,5)P2 are co-localized at the plasma membrane of non-polarized cells, while PtdIns(4,5)P2 becomes concentrated at the apical surface of polarized cells. The role of PIP5K in apical membrane trafficking, by which synthesizing PtdIns(4,5)P2, has recently been reported and confirmed the possible production of PtdIns(4,5)P2 at the apical membrane<sup>[61,62]</sup>. Meanwhile, PTEN regulates the apical recruitment of Par3, Par 6, Cdc42 and annexin 2 (Anx2), and is required for lumen development<sup>[63]</sup>. In addition, it has been shown that Par3 membrane targeting is dependent on the binding of its PDZ domain to PtdIns(4,5)P2, the product of PTEN<sup>[64,65]</sup>. The inhibition of PTEN by siRNA, or by a specific inhibitor bpV(pic), prevents the formation of a single central lumen in the cysts and causes a defective segregation of PtdIns(3,4,5)P3 and PtdIns(4,5)P2. This study also identified the fact that PTEN binds Anx2, which is responsible for the recruitment of Cdc42 and hence of apical aPKC, causing polarization of the apical membrane<sup>[60]</sup>.

HCV replication is dependent on PtdIns(4,5)P2<sup>[66]</sup> the lipid product of PTEN. HCV infection leads to a down-regulation of PTEN, triggering an acceleration of lipid droplet formation and insulin resistance<sup>[67,68]</sup>. The down-regulation of PTEN causes a malformation of the apical domain in an MDCK 3D culture<sup>[60]</sup>, suggesting that the multi-lumen phenotype in MDCK cells expressing HCV core protein may be caused not only by RhoA down-regulation<sup>[50]</sup> but also by modifying PTEN expression. It has been shown that the core protein of HCV genotype 3a Core decreases the expression of PTEN by blocking the translation of messenger RNA and causing an accumulation of lipid droplets in the cells. In addition, PTEN over-expression in these cells is capable of reducing the accumulation of lipid droplets. This study therefore suggests that this down-regulation of PTEN by HCV infection is a critical mechanism leading to steatosis and its progression toward fibrosis and hepatocellular carcinoma<sup>[67]</sup>.

## INSULIN RESISTANCE AND HCV INFECTION

Type II diabetes, and more generally insulin resistance, is very common in the context of chronic HCV infection, as has been established by several recent epidemiological studies<sup>[69-71]</sup>. Other studies have also shown that HCV infection can cause insulin resistance by the phosphorylation of IRS-1 at a serine residue (Ser307) followed by a decreased phosphorylation of Akt Thr(308), FoxO1 Ser(256) and GSK3 $\beta$  Ser(9), downstream players





**Figure 3 PI3K/Akt pathway and its activation by hepatitis C virus infection.** When EGF binds to EGFR this will activate PI3K I. PI3K activation will increase the level of PtdIns(3,4,5)P3 to phosphorylate Akt and induce cell proliferation and cell polarity. Hepatitis C virus (HCV) is able to bind EGFR at its entry into a cell, thus increasing PI3K activation and the level of PtdIns(3,4,5)P3, the latter being sustained by the down-regulation of SHIP2 and PTEN. The down-regulation of SHIP2 by HCV induces a loss of membrane integrity and cell polarity, while the down regulation of PTEN induces lipid droplet (LD) formation and steatosis. PtdIns: Phosphatidylinositol; PI3K: Phosphoinositide 3-kinase; SHIP2: SH2-containing inositol polyphosphate 5-phosphatase; PTEN: Phosphatase and tensin homologue deleted on chromosome 10; EGFR: Epidermal growth factor receptor.

in the insulin signaling pathway<sup>[72,73]</sup>. These data raise the question whether insulin resistance is the cause of liver steatosis in patients with chronic HCV infection, or the consequence of viral molecular expression. Indeed, Shintani *et al.*<sup>[74]</sup> showed that insulin resistance preceded the onset of steatosis in transgenic mice expressing HCV core protein, suggesting that insulin resistance was not a consequence of hepatic steatosis in these animals. Another study confirmed that the pathophysiology of fatty liver-associated chronic hepatitis C differed in patients infected with genotypes 1 and 3, showing that insulin resistance in genotype 1 patients is the cause rather than the consequence of hepatic steatosis and fibrosis, and suggesting that elevated circulating insulin levels are a risk factor for fibrosis through steatosis induced by insulin resistance. In genotype 3-infected patients, steatosis was related to HCV viral load<sup>[75]</sup>. These findings suggest that antiviral therapy in genotype 1-infected patients will not be sufficient. But does an improvement in metabolic syndrome increase the success rates of antiviral therapy? Walsh *et al.*<sup>[76]</sup> confirmed that in patients with chronic HCV viral genotype 1, an increased expression of factors inhibiting interferon signaling could be a mechanism by which obesity reduces the biological response to IFN- $\alpha$ . In 2006, Tarantino *et al.*<sup>[77]</sup> also confirmed that by improving metabolic syndrome, a lowering of the body mass index could play a key role in reducing the importance of metabolic co-factors and improving the foundations for a good antiviral response. For this reason, insulin sensitizers such as metformin are known to improve the response to HCV treatment and have been associated

with a lower risk of developing HCC. Very recently, a clinical trial was initiated by the Ottawa Hospital Research Institute to evaluate the effects of metformin on liver fibrosis in HCV-HIV co-infected and HCV mono-infected patients suffering from insulin resistance. If metformin proves to be effective in reducing liver fibrosis in this patient population, it will represent a well-tolerated, easy-to-administer, inexpensive therapy that could protect against negative HCV outcomes. This study will also provide an opportunity to evaluate the impact of insulin resistance and hyperglycemia on viral clearance in HCV-infected patients treated with interferon-free regimens<sup>[78]</sup>.

## PI3K/SHIP2/PTEN PATHWAY AND INSULIN RESISTANCE

Akt activity is essential for the translocation and fusion of glucose transporter 4 (GLUT4) to the plasma membrane of cells in the skeletal muscle and adipose tissue. In turn, GLUT4 plays a crucial role in the absorption of glucose. Akt induced by insulin signaling is also critical to the regulation of gluconeogenesis and glycolysis in the liver. When binding insulin to its receptor, SHIP2 also binds to the cell membrane and negatively regulates insulin signaling. An over-expression of SHIP2 in adipocyte 3T3-L1 cells inhibits insulin signaling, and expression of the catalytic mutant SHIP2 enhances the activity of Akt induced by insulin and thus generates glucose uptake and glycogen synthesis<sup>[79]</sup>. In 2001, Clément *et al.*<sup>[80]</sup> developed transgenic mice deficient in SHIP2.

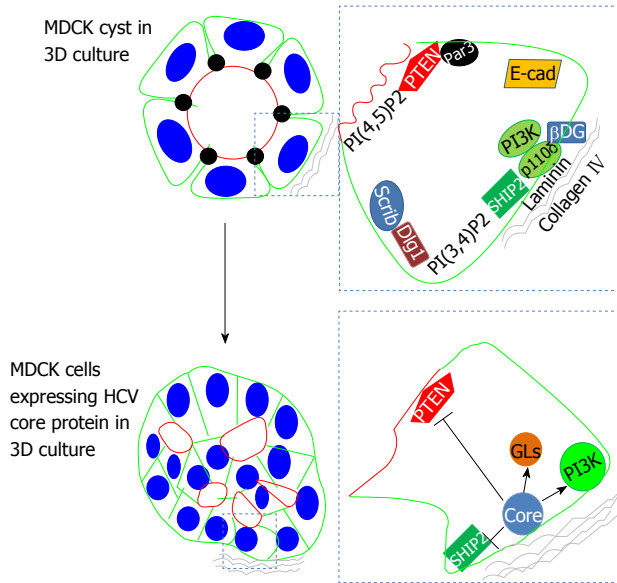
They showed that adult mice with the heterozygous SHIP2 mutation increased their glucose tolerance and insulin sensitivity, which was also associated with an increase in recruitment of the glucose transporter GLUT4. Homozygous mice with a SHIP2 deficiency experienced severe neonatal hypoglycemia and died within three days. These results show that SHIP2 is a potent negative regulator of insulin signaling and insulin sensitivity *in vivo*. A second SHIP2 deficient mouse model was studied by Sleeman *et al.*<sup>[81]</sup> in 2005. They found that SHIP2 deficient mice were viable, with normal glucose levels and normal tolerance to insulin. However, they were very resistant to putting on weight when fed a high-fat diet<sup>[81]</sup>. Although these two models had different phenotypes, the results suggest that SHIP2 is a key regulator of glucose, and its inhibition would be useful regarding efforts to improve diet-induced obesity. Furthermore, transgenic mice expressing catalytically-inactive SHIP2 have displayed altered lipid metabolism and insulin secretion<sup>[82]</sup>. In addition transgenic mice over-expressing SHIP2 WT are obese and suffer from hepatic insulin resistance<sup>[83]</sup>. These results show that the inhibition of SHIP2 may influence lipid metabolism and insulin signaling. For these reasons, the use of antisense oligonucleotides against SHIP2 in model diabetic rats produced a rapid improvement in insulin sensitivity<sup>[84]</sup>. Taken together, these *in vivo* studies suggest that an inhibition of SHIP2 expression may be effective in the treatment of type 2 diabetes. The relationship between SHIP2 and insulin resistance has also been studied in patients with type II diabetes whose SHIP2 gene (*INPPL1*) had a deletion of the 3' extremity. This mutation enhanced the expression of SHIP2 in the adipose tissue and skeletal muscles of diabetic patients, causing insulin resistance<sup>[85]</sup>. Overall, these findings suggest that SHIP2 is a key regulator of glucose homeostasis and could be targeted when treating diseases that affect insulin metabolism such as diabetes type II. This central role of SHIP2 as a negative regulator of insulin signaling encouraged Sumie *et al.*<sup>[86]</sup> to investigate changes to SHIP2 expression in HCC patients with HCV infection. They showed that the cumulative survival rate was significantly lower in the glucose intolerance group than that in the normal glucose tolerance group, and that the level of SHIP2 expression fell in a context of HCC when compared to that seen in non-tumor tissues. This study therefore indicated a prognostic role for glucose tolerance and SHIP2 expression in HCC patients with HCV infection.

PTEN is the second phosphoinositide phosphatase that negatively regulates insulin signaling<sup>[83-87]</sup>. Studies using 3T3-L1 adipocytes clearly demonstrated that PTEN over-expression inhibited the production of insulin-induced PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>, the activation of Akt/PKB, the translocation of GLUT4 to the cell membrane and glucose uptake<sup>[88,89]</sup>. By contrast, the down-regulation of PTEN by small interfering RNAs enhanced Akt/PKB activation and glucose uptake in response to insulin<sup>[90]</sup>. Furthermore, an over-expression of catalytically-inactive or dominant-negative PTEN mutants also indicated

that it is the lipid phosphatase activity of PTEN which is necessary to down-regulate Akt/PKB signaling and glucose uptake in response to insulin<sup>[89-91]</sup>. Finally, all these studies showed that the PI3K/Akt pathway offers a target to improve steatosis and insulin resistance during the development of NAFLD. Different treatments such as Silibinin and FAM3A (cytokine-like gene family) activate PI3K p110 $\alpha$ /Akt signaling in order to ameliorate hepatic gluconeogenesis and lipogenesis<sup>[92,93]</sup>. Flanovol Quercetin is another treatment with favorable effects on the progression of NAFLD, acting *via* the PI3K/Akt pathway. Treatment with quercetin has been shown to reduce oxidative/nitrosative stress and inflammation, and genes related to lipid metabolism displayed a tendency to normalize in both *in vivo* and *in vitro* models<sup>[94]</sup>.

### PI3K/SHIP2/PTEN PATHWAY AND NAFLD

NAFLD is often associated with HCV infection. NAFLD is frequently described as encompassing a histological spectrum from nonalcoholic fatty liver to simple hepatic steatosis (SHS) plus a characteristic pattern of steatohepatitis [nonalcoholic steatohepatitis (NASH)]. HCV infection also gives rise to liver steatosis. So is the PI3K/SHIP2/PTEN pathway implicated in NAFLD in a context of HCV infection? In the liver, insulin controls lipid metabolism through its cell surface receptor and intracellular mediators such as PI3K and serine-threonine kinase Akt. It has been shown that insulin inhibits apoB100 secretion through the activation of PI3K. And insulin signaling *via* PI3K inhibited the maturation of VLDL lipoprotein particles by preventing lipidation of the VLDL precursor<sup>[95]</sup>. For this reason, a disruption of phospholipid metabolism is present in NAFLD. Indeed, a recent study demonstrated that plasma phospholipids differed between liver biopsies from NAFLD patients and healthy subjects. Phosphatidylinositol levels were higher in SHS and NASH patients compared with healthy controls<sup>[96]</sup>. Another study also identified the role of dietary phosphatidylinositol (and particularly phosphatidylcholine and phosphatidylserine) in preventing NAFLD in a rat model of metabolic syndrome<sup>[97]</sup>. Furthermore, in transgenic mice with hepatic steatosis and developing a tumor, alongside an abnormal accumulation of fatty acids, the study demonstrated activation of the Akt/mTOR pathway, and a reduction in the expression of tumor suppressor genes, including *Pten*. This confirmed that an accumulation of fatty acids may have a role in promoting *in vivo* hepatic tumorigenesis under constitutive activation of the PI3K pathway<sup>[98]</sup>. Another study confirmed that high unsaturated fatty acid levels significantly decreased PTEN mRNA expression in hepatic cells by means of a mechanism involving the sequential activation of mTOR and NFB, which were found to form a complex in cultured cells<sup>[99]</sup> which led to a significant alteration of PTEN expression. It is important to remember that inflammatory cytokines such as transforming growth factor  $\beta$ , tumor necrosis factor  $\alpha$ , interleukin-6



**Figure 4** Effects of hepatitis C virus infection on cell polarity. In a 3D culture, MDCK form a cyst comprising a monolayer of cells around a lumen (the basolateral membrane is indicated in green, the apical membrane in red and the tight junctions in black). The right-hand column shows a zoom of a polarized cell. PI3K is activated by E-cadherin. The p110 $\delta$  subunit is activated by ECM (Laminin and collagen IV). SHIP2 and PI(3,4)P2 at the basal membrane are responsible for the Dlg1/Scribble complex at the basolateral membrane. PTEN interacts with Par3 at the tight junctions and its lipid product PtdIns(4,5)P2 is localized at the apical membrane. The bottom column represents the multi-lumen phenotype of cysts expressing HCV core protein. The presence of this core protein at the basolateral membrane activates PI3K and inhibits SHIP2 and PTEN, leading to a loss of cell polarity and an accumulation of lipid droplets (LDs). HCV: Hepatitis C virus; PtdIns: Phosphatidylinositol; PI3K: Phosphoinositide 3-kinase; SHIP2: SH2-containing inositol polyphosphate 5-phosphatase; PTEN: Phosphatase and tensin homologue deleted on chromosome 10; MDCK: Madin darby canine kidney; DG: Dystroglycan.

and interleukin-1, which are produced in the course of NAFLD, also significantly alter PTEN expression, as has been shown in non-liver cells. These studies offer an interesting link between insulin resistance and steatosis, which may also explain (at least in part) the high risk of developing HCC associated with diabetes and obesity<sup>[100]</sup>. PTEN deregulation has also been demonstrated during *in vivo* studies. First, a study of heterozygous PTEN deletion was confirmed as inducing atypical adenomatous liver hyperplasia<sup>[101]</sup>. Subsequently, a genetic inhibition of PTEN expression, specifically in the liver of rodents, was shown to trigger liver steatosis, insulin hypersensitivity and HCC<sup>[102]</sup>. Because of the lack of PTEN activity, there may be an increase in fatty acid uptake by hepatocytes, and in fatty acid synthesis<sup>[103]</sup>. Hepatocyte-specific PTEN deficient mice display similar histological features to human NASH patients. These hepatocytes display enhanced lipid accumulation, inflammatory changes and hyperoxidation, and also develop into HCC<sup>[104]</sup>. Therefore, an impairment of PI3K/PTEN signaling could be involved in some NASH/HCC cases in humans. These results are very compatible with the down-regulation of PTEN in HCV infection, which leads to an acceleration of lipid droplet formation and insulin resistance<sup>[67,68]</sup>. Taken

together, these studies have suggested a role for PTEN in regulating lipogenesis in liver cells; however, less information is available on the effects of another lipid phosphatase, SHIP2, on lipid and lipoprotein metabolism in the liver. A very recent study found a molecular link between SHIP2 expression and metabolic dyslipidemia using the over-expression or suppression of the *SHIP2* gene in HepG2 cells. SHIP2 over-expression led to higher lipid production and secretion *via* apoB100 secretion and *de novo* lipogenesis<sup>[105]</sup>. Another study confirmed that PBX-regulating protein 1 enhances *Ship2* transcription, leading to hepatic lipogenesis and steatohepatitis in mice. However, Prep1 hypomorphic heterozygous [Prep1 (i/+)] mice displayed lower SHIP2 levels, and significantly decreased serum triacylglycerol levels and the liver expression of fatty acid synthase<sup>[106]</sup>. We have discussed above the down-regulation of SHIP2 in HCV core-expressing cells, so is this down-regulation of SHIP2 the cause of lipid droplet accumulation in these cells? Overall, these findings confirm that SHIP2 is responsible for hepatic lipogenesis and secretion. To conclude, the PI3K/SHIP2/PTEN pathway, which is markedly deregulated in the context of HCV infection, activates Akt causing an over-expression of fatty acids, leading subsequently to liver steatosis, insulin hypersensitivity and HCC.

## CONCLUSION

PI3K and SHIP2 have been widely studied for their roles in intracellular signaling and membrane trafficking. However, their membrane segregation and the effects of their enzymatic activity on the establishment of cell polarity are only now starting to be investigated. Recent studies have defined the manipulation of these PI enzymes and their lipid products by the hepatitis C virus, so that it can enter and replicate in epithelial host cells (Figure 4). The PI3K/PTEN/SHIP2 pathway is now better understood in the context of HCV infection, inasmuch as it induces changes to cell polarity and lipid metabolism which can generate several pathologies such as insulin resistance, liver steatosis, NAFLD and HCC.

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## Drug-induced liver injury: Towards early prediction and risk stratification

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### Abstract

Drug-induced liver injury (DILI) is a hot topic for clinicians, academia, drug companies and regulators, as shown by the steadily increasing number of publications and agents listed as causing liver damage ([\[livertox.nih.gov/\]\(http://livertox.nih.gov/\)\). As it was the case in the past decade with drug-induced QT prolongation/arrhythmia, there is an urgent unmet clinical need to develop tools for risk assessment and stratification in clinical practice and, in parallel, to improve prediction of pre-clinical models to support regulatory steps and facilitate early detection of liver-specific adverse drug events. Although drug discontinuation and therapy reconciliation still remain the mainstay in patient management to minimize occurrence of DILI, especially acute liver failure events, different multidisciplinary attempts have been proposed in 2016 to predict and assess drug-related risk in individual patients; these promising, albeit preliminary, results strongly support the need to pursue this innovative pathway.](http://</a></p>
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**Key words:** Hepatotoxicity; Predictivity; Risk assessment; Safety

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**Core tip:** The interest in drug-induced liver injury (DILI) is growing, especially in 2015-2016, with pioneering studies addressing DILI annotation, *i.e.*, risk stratification of drugs capable of causing liver damage. The latest experiences from worldwide consortia provided promising data, although there is still room for improvement before reaching an algorithm capable of discriminating hepatotoxic from non-hepatotoxic compounds, or at least of classifying high, intermediate and low risk drugs within the same therapeutic class. We should take advantage of integration of real-world data (*i.e.*, registries, healthcare databases, spontaneous reporting systems, literature) with cheminformatics to provide a comprehensive DILI risk score.

Raschi E, De Ponti F. Drug-induced liver injury: Towards early prediction and risk stratification. *World J Hepatol* 2017; 9(1): 30-37 Available from: URL: <http://www.wjgnet.com/1948-5182/>

## INTRODUCTION

The year 2015 witnessed an outstanding scientific production of studies dealing with drug-induced liver injury (DILI) and the list of drugs capable of causing liver dysfunction needs constant update, thus making DILI an emerging safety issue requiring attention by academia, regulators, drug companies and clinicians, both in specialty and general practice<sup>[1,2]</sup>.

A search in MEDLINE using the strategy "DILI or drug-induced liver injury or drug-induced liver damage or herb-induced liver injury or herb-induced liver damage or hepatotoxicity" yielded 2196 publications in 2015 (performed on June 7<sup>th</sup>, 2016) (Figure 1), with more than 2000 studies per year published in the past 4 years. The proportion among the different types of studies has not substantially changed over time, with pre-clinical investigations representing the majority of publications (more than 60% of total studies in 2015). This body of evidence has generated concern within the scientific community, especially among clinicians, who are not fully aware that a number of drugs are likely to affect liver function and must be therefore considered among the differential diagnoses in patients presenting with elevated transaminases.

DILI has tremendous impact on medical prescribing attitudes: The latest data confirmed that hepatotoxicity was the most commonly reported adverse drug reaction leading to drug withdrawal worldwide (81 cases; 18%)<sup>[3]</sup>. Several global registries (in United States, Latin America, Europe and China) have continued to update case series and implement completeness and accuracy of data<sup>[4]</sup>. It is interesting to note that antineoplastic/antimicrobials are the most frequently implicated drugs in DILI reports across all data registries and population-based studies, with herbal and dietary supplements being an emerging concern especially in United States<sup>[5-7]</sup>.

While population-based studies are useful to estimate DILI incidence (despite suffering the inability to account for genetic backgrounds), prospective registries across various DILI consortia allow careful case adjudication. It is worth mentioning that registries consistently enrolled sicker patients as compared to epidemiological studies, with 70% of the patients jaundiced at presentation and half of them requiring hospitalization, thus the proportion of non-"true" DILI cases is probably negligible. This selection bias, probably related to the fact that DILI patients are mainly recruited in hospital units, is useful to appreciate phenotypes of liver damage (hepatocellular, cholestatic and mixed) and investigate specific features or drug signatures: Female sex, hepatocellular type of damage and high bilirubin levels emerged as risk factors for fulminant liver failure and death<sup>[8]</sup>, with higher mortality risk in patients with preexisting liver disease<sup>[9]</sup>.

In this minireview, we highlight advances in DILI re-

search, focusing on recent studies that, in our opinion, provide key contribution towards an unmet clinical need: Risk stratification of drugs capable of causing liver damage, also known as DILI annotation.

## SUSPECTING AND DIAGNOSING DILI: A CURRENT DILEMMA

### *The contribution of drugs in DILI occurrence*

Different drugs have been convincingly documented to cause liver injury in numerous case reports and case series<sup>[10]</sup>. Paracetamol has been consistently reported as a leading cause of acute liver failure, whereas chlorpromazine, halothane, sulpiride and amoxicillin-clavulanate such as found to be the most common drugs leading to hepatotoxicity in all prospective studies<sup>[11]</sup>. Apart from antibiotics, the list of top 10 drugs implicated in DILI cases (in terms of frequency) comprises statins (only rarely severe liver injury was likely to be associated with statins), antitumor necrosis factor antagonists (with infliximab being the most common implicated agent, with autoimmune features), and herbal and dietary supplements (with weight loss and bodybuilding products being the most frequent causes of serious hepatotoxicity)<sup>[12]</sup>.

A risk of DILI greater than 100 per 100000 users was found for chlorpromazine and sulpiride. Drugs with an intermediate risk were amoxicillin-clavulanic acid and emerged with a risk of 10 per 100000 users<sup>[13]</sup>. All other drugs were found to be less than 10 per 100000 users.

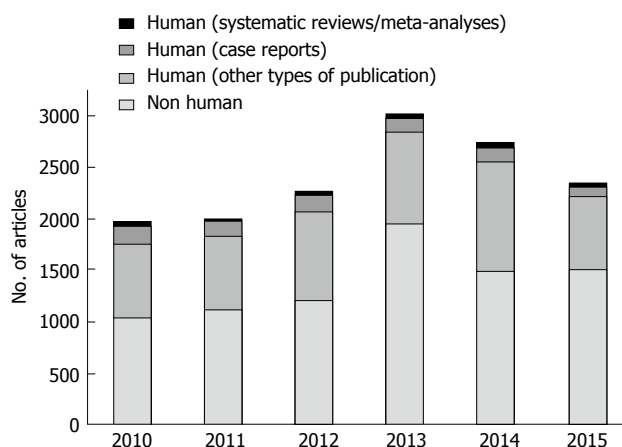
Unfortunately, in most of the cases, DILI is unpredictable because of its idiosyncratic nature; in fact, only rarely have the precise underlying mechanisms been identified (*e.g.*, mitochondrial injury, reactive metabolites, biliary transport inhibition, and immune responses). Paracetamol is a well-known example of drug causing dose-dependent DILI.

### *Obtaining evidence-based data to support DILI diagnosis*

DILI is a diagnosis of exclusion, thus strengthening the importance of anamnesis and clinical experience. Apart from ruling out competing causes (*e.g.*, viral infections), it is crucial in the clinician's mind to have information on the notoriety, *i.e.*, whether the drug is known or has the potential to cause hepatotoxicity. However, these evidence-based data are not always easily accessible<sup>[11]</sup>.

The first aid is represented by the product information or summary of the product characteristics (in United States and Europe, respectively), which however is variable in terms of details and may also substantially differ in the labeling of liver risk<sup>[14]</sup>. The key information to be checked is the existence of contraindications in patients with pre-existing liver diseases and the presence of specific warnings on the risk of liver damage, with relevant precautions in appropriate monitoring and management. It must also be kept in mind that the wording of these documents follows rules that are not always patient- and physician-friendly. Other sources of information are therefore highly needed.





**Figure 1** Trend in publication of articles on drug-induced liver injury, classified in terms of types of evidence. The search was performed in MEDLINE on June 7<sup>th</sup>, 2016, through automatic filters and keywords.

Ascertainment of the literature is the second step, which is a more challenging and time-consuming task. While some drugs have been convincingly documented to cause liver injury and clinical signatures have been demonstrated (e.g., isoniazid, amoxicillin-clavulanic acid), for some agents only a few case reports are available and, most importantly, only in a minority critical clinical data are provided to ascertain the causative role of drugs<sup>[15]</sup>.

The third source of data is represented by LiverTox<sup>®</sup> (<http://livertox.nih.gov/>), a public website set up to provide up-to-date, accurate, and easily accessible information on the diagnosis, causes, frequency and patterns of liver injury attributable to both prescription and nonprescription medications. Although LiverTox<sup>®</sup> is based on a thorough literature analysis, the quality of the published reports and the causality of the suspected liver injury reported are not provided.

Specific algorithms, such as the Roussel Uclaf Causality Assessment Method scale, have been proposed and validated to assess causality, although it should be recognized that these scores are particularly useful for regulatory and research purposes, *i.e.*, to verify a posteriori the likelihood of the association rather than to support a prospective diagnosis<sup>[16]</sup>. During the preapproval development process, Hy's Law (*i.e.*, ALT/AST > 3 ULN in combination with total bilirubin > 2 ULN in the absence of cholestatic injury - alkaline phosphatase < 2 ULN) is an essential part of the stopping rules to prevent hepatotoxicity, although it was never specifically validated in a clinical trial. Different research group have recently attempted to optimize the definition of Hy's Law and develop models for predicting acute liver failure in DILI, in combination with other biomarkers such as total bilirubin and platelet count<sup>[8,17]</sup>. However, whether such revised definitions can become part of clinical practice is yet to be determined.

### **Risk stratification of DILI in clinical practice: A dream or a reality?**

Current expectations regard the development and im-

plementation of risk stratification tools to assign a certain liver risk to a given drug. In other words, clinical research is trying to establish the so-called DILI annotation, a global score reflecting the frequency, causal role and severity of DILI for each drug<sup>[18]</sup>. This scenario recalls what occurred in the past decade with drug-induced QT prolongation and Torsade de Pointes (DITdP), which has been a largely debated regulatory issue for the past 20 years with still suboptimal tools for risk stratification in clinical practice<sup>[19]</sup>. With this experience in mind, we should immediately understand the importance of coordinating and harmonizing the various ongoing projects and the need to set up a global response to efficiently assess drug-related hazards. A parallel between DILI and DITdP is presented in Table 1.

Identification of baseline risk is the first step towards final risk stratification. DILI has a multifactorial nature with both environment- drug- and patient-related risk factors that may coexist and increase the likelihood of DILI occurrence.

Apart from age and sex, genetics plays a role, at least for some drugs. A recent genome-wide association study involving 620 European cases of DILI and 10588 population controls, the DRB1\*16:01-DQB1\*05:02 haplotype was identified as a risk factor for flupirtine-induced liver damage<sup>[20]</sup>. Although the inclusion of genetic tests in causality assessment may improve consistency and precision of DILI diagnosis as well as appropriateness of drug administration, there is only initial positive experience in clinical application of N-acetyltransferase 2 genotyping to determine the appropriate dose of isoniazid<sup>[21]</sup>.

A current area of research deals with the identification of biomarkers, keeping in mind the aim of detecting patient's susceptibility to DILI prior to and during drug exposure, predicting the course of DILI once it occurs and differentiate DILI from other causes of liver injury. Among others, miR-122 expression was demonstrated to be a liver specific biomarker of paracetamol hepatotoxicity; high levels of High Mobility Group Box-1 with circulating colony stimulating factor-1 were correlated to poor prognosis and outcome in patients with established acute liver injury following paracetamol overdose; likewise, the prognostic utility of Keratin-18 has been proposed; notably, up-regulation of Kidney Injury Molecule-1, a marker of renal proximal tubular epithelia, could be a determinant of mortality in patients with paracetamol overdose and secondary kidney damage; finally, Glutamate Dehydrogenase might indicate hepatocellular necrosis, although lacking specificity in discriminating benign transaminases elevation from severe DILI occurrence. All these biomarkers, however, still require formal qualification before being considered for routine clinical use<sup>[22]</sup>.

Among drug-related features, oral medications with high lipophilicity (*i.e.*, logP ≥ 3) administered at daily doses of ≥ 100 mg (known as the concept of the "Rule-of-2") have been associated with higher risk of DILI<sup>[23]</sup>. Bile salt export pump and multidrug resistance-associated protein 4 inhibitions have been also identified

**Table 1** Similarities and differences between drug-induced torsade de pointes and drug-induced liver injury

	DITdP	DILI
Endpoint/biomarker	Surrogate, but well defined biomarker of risk (QT prolongation with specific thresholds)	Surrogate, but well defined biomarker of risk (transaminase elevation with specific thresholds)
Key mechanism	Largely described (dose-dependent hERG K <sup>+</sup> channel inhibition)	Only partially understood (different hypotheses)
Dose-response relationship	Dose dependent (with only a few exceptions)	Idiosyncratic, although dose-dependence exists
Regulatory impact	Pre-clinical and clinical guidelines (pre-marketing)	Clinical guideline (pre-marketing)
Clinical impact	Significant (a leading cause of drug withdrawal worldwide)	Significant (a leading cause of drug withdrawal worldwide)
Predictivity of pre-clinical assays	Reasonably good (new models under investigation)	Sub-optimal (especially for <i>in vivo</i> models)
Predictivity of clinical studies	Good (thorough QT study), albeit imperfect	Good (Hy's law), albeit imperfect
Role of genetics	Important (long QT syndrome)	Partially defined (only for some drugs)
Awareness (clinicians, regulators, drug developers, researchers)	Significant at all levels	Significant at some levels (drug developers, researchers)
Risk assessment tools (clinical)	Drug- and patient-related risk factors are well recognized (www.crediblemeds.org); CDSSs are under implementation	Drug- and patient-related risk factors are only partially recognized (www.livertox.nih.gov)
Causality assessment tools (clinical)	Not present, but the majority of TdP cases are drug induced (the so-called designated medical event); phenotype standardized	Specific, but challenging (several differential diagnoses)
Therapy	Magnesium sulphate, electrical cardioversion or isoproterenol (isoprenaline) or transvenous pacing (refractory TdP cases); removal or correction of precipitants, including drugs	No specific treatment other than drug discontinuation; liver transplantation may be required in acute liver failure cases

For details on DITdP<sup>[50-53]</sup>. CDSSs: Clinical decision support systems; DILI: Drug-induced liver injury; DITdP: Drug-induced torsade de pointes.

as important determinants of cholestatic DILI risk in humans<sup>[24,25]</sup>. However, the contents and the extent of information of these transporters in the summaries of the product characteristics may vary considerably between United States and Europe, especially for novel drugs<sup>[26]</sup>.

Therefore, the recent literature attempted to annotate DILI risk through different approaches, all of which rely on the assessment of already available data. Among the various experiences, risk categories were created based on the information extracted from drug compendium, such as Physicians Desk Reference, and case reports (alone or integrated with literature and drug labeling)<sup>[18,27-32]</sup>. However, the validity of these published annotations is still a matter of debate because all methods present limitations and a gold standard to define DILI risk is lacking<sup>[33]</sup>. This is an unresolved concern, common to all drug-related safety issues.

Very recently, two different approaches stimulate interest in annotating DILI risk. Chen *et al.*<sup>[34]</sup> combined the rule-of-two with the capacity to produce reactive metabolites and implemented a model to assess the risk of DILI onset and severity. Both dose-based and C<sub>max</sub> based-scores were calculated. Initial validation of this score indicated that half (19/38) of DILI cases with a dose-based DILI score  $\geq 7$  were associated with severe clinical outcome (e.g., hepatic failure or death), while none of the cases with a DILI score  $< 3$  were linked to severe liver injury. Statistical analysis revealed that a DILI score  $\geq 7$  and  $< 3$  was significantly associated with higher or lower risk for severe hepatic outcome.

Conversely, Björnsson *et al.*<sup>[35]</sup> classified drugs listed in LiverTox<sup>®</sup> website. Specifically, drugs were categorized based on the number of case reports (Category A  $\geq 50$  published reports, B = 12-50, C = 4-12, and D = 1-3)

and another category, T, was added for agents leading to hepatotoxicity mainly in higher-than-therapeutic doses. In this study, fewer drugs than expected emerged with a documented hepatotoxicity. Among 671 drugs available for analysis, 353 (53%) had published convincing case reports of hepatotoxicity. Thus, overall, 47% of the drugs listed in LiverTox actually do not have evidence of hepatotoxicity. However, the main limitation of this analysis is that new drugs approved within the last five years were not included. Therefore, old drugs with consolidated clinical use are likely to result in higher risk. In fact, drugs in categories A and B were more likely than those in C and D to have been marketed for a long time, and both were more likely to have at least one fatal case of liver injury and reported cases of positive rechallenge. While there is little doubt that the majority drugs in category A and B are hepatotoxic, it is still unclear whether agents listed in C and D are really liver offenders.

## A CRITICAL ANALYSIS OF THE DILI RISK SCORE: THE CASE OF DIRECT-ACTING ORAL ANTICOAGULANTS

Liver safety of direct-acting oral anticoagulants (DOACs) was highly debated in 2014-2015, when several publications highlighted possible occurrence of liver damage (including acute liver failure) during DOAC administration<sup>[36-39]</sup>. The majority of data are derived from case reports/series, which emphasized the relatively rapid time-to-onset and the concomitant reporting of drug that are implicated in liver damage or have the potential to result in drug interactions<sup>[39]</sup>. In particular, the time-to-onset from published case reports suggests

**Table 2** Chemical and pharmacological properties of direct-acting anticoagulants likely to be associated with drug-induced liver injury risk in humans

	Dabigatran etexilate	Rivaroxaban	Apixaban	Edoxaban
Max daily dose (indication) <sup>1</sup>	220 (DVT prophylaxis) - 300 (NVAf)	5 (post ACS <sup>2</sup> ) - 10 (DVT prophylaxis) - 20 (NVAf) - 30 (treatment of DVT/PE)	5 (DVT prophylaxis) - 20 (acute treatment of DVT/PE)	60 (NVAf and DVT)
Bioavailability <sup>1</sup>	6.50%	80%-100%	50%	62%
Protein binding	35%	> 90%	87%	55%
Cmax (ng/mL)	697 (at steady state after 400 mg/3 die) <sup>[54]</sup>	450 (multiple dose 30 mg/die) <sup>[55]</sup>	469 (single 20 mg dose) <sup>[56]</sup>	424 (90 mg daily at day 10) <sup>[57]</sup>
Lipophilicity (LogP) <sup>5</sup>	5.17	1.74	2.22	1.61
Biotransformation <sup>1</sup>	Conjugation forming 4 pharmacologically active acylglucuronides	Oxidative degradation of the morpholinone moiety and hydrolysis of the amide bonds	O-demethylation and hydroxylation at the 3-oxopiperidiny moiety	Hydrolysis (mediated by carboxylesterase 1), conjugation or oxidation by CYP3A4/5 (< 10%)
Hepatic metabolism <sup>1</sup>	Only the prodrug is a substrate of P-gp; no induction/inhibition of principal isoenzymes of cytochrome P450	CYP3A4, CYP2J2 and CYP-independent mechanisms. Substrate of P-gp and BCRP	CYP3A4/5. Substrate of P-gp and BCRP	Substrate of P-gp
Structural alerts associated with RM formation	NO (aniline motif) <sup>[58,59]</sup>	NO (chlorothiophene and bis-anilide motifs) <sup>[42,58]</sup>	NO (para-methoxyaniline and bis-anilide motifs) <sup>[41,58]</sup>	ND (no published data in the literature)
Dose-based DILI Risk Score <sup>3</sup>	2.68	1.29	1.29	1.45 <sup>4</sup>
Cmax-based DILI Risk Score <sup>3</sup>	2.98	1.87	2.02	1.82 <sup>4</sup>

<sup>1</sup>From official European Summary of Product Characteristics; <sup>2</sup>Only in EU; <sup>3</sup>Calculated based on formulas reported by Chen *et al*<sup>[34]</sup>; <sup>4</sup>Calculated based on formulas reported by Chen *et al*<sup>[34]</sup> and assuming no RM formation; <sup>5</sup>Data obtained from Drug Bank (www.drugbank.ca; source: ALOGPS). ACS: Acute coronary syndrome; BCRP: Breast cancer resistance protein; DVT: Deep vein thrombosis; NVAf: Non valvular atrial fibrillation; ND: Not determined; RM: Reactive metabolites; DITdP: Drug-induced torsade de pointes.

that early evaluation of hepatic enzymes (*i.e.*, within the first month) may be considered at least in patients under complex treatment regimen with comorbidities; subsequently, liver function can be monitored on a yearly basis<sup>[40]</sup>. This is especially the case of rivaroxaban, for which a probable but unquantified association is likely to exist. Notably, rivaroxaban is the only DOAC reported in the list provided by Björnsson *et al*<sup>[35]</sup> and classified in category B.

Therefore, we applied the score developed by Chen *et al*<sup>[34]</sup> to DOACs and found intriguing data (Table 2). Based on these results, different issues emerge: (1) no DOAC appears to be associated with risk of severe liver damage (they all received a score well below the threshold of 7); (2) the highest score emerged for dabigatran; (3) the risk does not appear to be strongly influenced by dose or Cmax (there is only a small increase in Cmax-based score), or chemical motifs; (4) DOACs pose a lower risk as compared to warfarin (the dose-based risk score is 4.67, according to Chen *et al*<sup>[34]</sup>).

However, among DOACs, it is difficult to discriminate the agent with the highest risk, keeping in mind that post-marketing data have reported rivaroxaban to be most likely associated with DILI<sup>[40]</sup>. Therefore, these data suggested that current performance of this risk stratification tool is still suboptimal. In fact, this algorithm is based on pharmacokinetics characteristics and chemical features. Based on published data, apixaban, rivaroxaban and dabigatran contain structural moieties

that suggest some alerts (para-methoxyaniline and bis-anilide motifs in apixaban; chlorothiophene and bis-anilide motifs in rivaroxaban; bis-anilide motifs in dabigatran), which, however, do not seem to undergo metabolism and/or generate reactive metabolites<sup>[41,42]</sup>. In the case of rivaroxaban, the pendant chlorothiophene motif is also essential for pharmacology and cannot be replaced. The aniline structural moiety is also present in the oral direct thrombin inhibitor dabigatran, which, however is not subject to oxidative metabolism by CYP enzymes in humans<sup>[43]</sup>. In summary, only partially may these peculiarities explain the risk observed in humans for rivaroxaban. This is also emphasized by the case of ximelagatran, which does not possess structural moieties implicated in liver toxicity (dose-based risk score = 2.55; Cmax-based risk score = 1.90, according to Chen *et al*<sup>[34]</sup>), thus suggesting that additional mechanisms are likely to be implicated in DILI occurrence in humans.

Therefore, our hypothesis is that there should be additional aspects that may modify the likelihood of DILI occurrence in DOAC users. Apart from host-related factors (which are not modifiable), we propose that: (1) concomitant drug with hepatotoxic and/or interacting potential may cause a subclinical liver damage that can results in symptomatic injury in susceptible patients (a concept similar to the repolarization reserve postulated for DITdP<sup>[44]</sup>); and (2) the underlying disease for which the DOAC is prescribed may contribute in increasing the likelihood of DILI with unknown mechanisms. In fact, the

majority of published case reports occurred in surgical patients with venous thromboembolism rather than with atrial fibrillation.

This calls for monitoring of liver safety when making treatment changes (addition of drugs with recognized hepatotoxicity potential, especially for long-term use) considering the different therapeutic indications of DOACs, where their role is still incompletely defined (e.g., heparin-induced thrombocytopenia, cancer, triple therapy, coronary diseases, heart failure)<sup>[45]</sup>. In the meantime, chemists, pharmacologists and clinicians should join efforts to understand drug signature subtending the mechanistic basis of DILI and establish causality.

## CONCLUSION AND PERSPECTIVE

Early detection, prediction and accurate risk stratification represent an urgent need for clinicians, basic scientists, regulators and drug companies. As compared to DITdP, predictivity of pre-clinical assays for DILI is still suboptimal. The role of animal studies remains questionable, mainly because of the incomplete understanding of the mechanisms underlying DILI, as well as marked species differences in response to, and in the metabolism of, xenobiotics.

As a result, there is currently no universally accepted animal model. It seems unlikely that a single *in vitro* system will be able to mimic the complex interactions in the human liver. Three-dimensional multicellular systems together with toxicogenomics-based methodologies and next-generation sequencing technologies are promising tools to develop predictive models in the near future<sup>[46]</sup>. In particular, pluripotent stem cells, which include embryonic and induced pluripotent stem cells, are being investigated to replace human primary hepatocytes (the current gold standard for preclinical toxicological screening), because they provide a stable source of hepatocytes and can be exploited for multiple applications, including early preclinical hepatotoxicity screening<sup>[47]</sup>.

Risk stratification in humans is even more challenging, especially for herbals/food supplements as well as biotechnological products, because of their unpredictable kinetics and sometimes variable content.

Case reports are of course of great importance for timely detection of safety signals, although they cannot be formally used *per se* for a reliable risk assessment and stratification, but should be integrated with other data sources such as clinical trials, cohort and case-control analyses.

The importance of this global approach in the overall assessment of drug-related toxicities is recommended by the recent Pharmacovigilance legislation, which calls for integrated risk/benefit assessment based on an integrated view of all pieces of evidence<sup>[48]</sup>. This was the case of pancreatitis with incretin-based drugs: While the signal emerged from case reports, the actual existence and the magnitude of a true association was later investigated through multiple data sources, including a recent systematic review with meta-analysis of both

clinical trials and observational studies, which suggested that the incidence of pancreatitis in users of incretin-based therapy is low and that the drugs do not increase the risk of pancreatitis<sup>[49-59]</sup>.

In conclusion, existing consortia should pursue a joint effort along this innovative pathway aiming to develop algorithms capable not only of discriminating hepatotoxic from non-hepatotoxic compounds, but also to differentiate the risk among agents belonging to the same therapeutic class. In particular, in the era of big data, it is important to integrate real-world information (i.e., registries, healthcare databases, spontaneous reporting systems, literature) with cheminformatics in order to provide a comprehensive DILI risk score and fulfill clinicians' and patients' expectations about "primum non nocere".

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Basic Study

## Ultrasound shear wave elastography and liver fibrosis: A Prospective Multicenter Study

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**Institutional review board statement:** Aga Khan University Faculty of Health Sciences Research and Ethics Committee reviewed the proposal and related documentation submitted and approved the study based on core scientific and ethical standards which were fully instituted in the protocol.

**Informed consent statement:** Informed voluntary consent was acquired from all the study participants.

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**Data sharing statement:** Technical appendix, statistical code and data set available from the corresponding author at [sande.joyce@gmail.com](mailto:sande.joyce@gmail.com). Presented data are anonymised and risk of identification is nil.

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### Abstract

#### AIM

To assess the accuracy of shear wave elastography (SWE) alone and in combination with aminotransferase platelet ratio index (APRI) score in the staging of liver fibrosis.

#### METHODS

A multicenter prospective study was conducted to assess the accuracy of SWE (medians) and APRI to predict biopsy results. The analysis focused on distinguishing the different stages of liver disease, namely, F0 from F1-4, F0-1 from F2-4, F0-2 from F3-4 and F0-3 from F4; F0-F1 from F2-F4 being of primary interest. The area under the receiver operating characteristic (AUROC) curve was computed using logistic regression model. The role of age, gender and steatosis was also assessed.

#### RESULTS

SWE alone accurately distinguished F0-1 from F2-4 with a high probability. The AUROC using SWE alone was 0.91 compared to 0.78 for using the APRI score alone.

The APRI score, when used in conjunction with SWE, did not make a significant contribution to the AUROC. SWE and steatosis were the only significant predictors that differentiated F0-1 from F2-4 with an AUROC of 0.944.

### CONCLUSION

Our study validates the use of SWE in the diagnosis and staging of liver fibrosis. Furthermore, the probability of a correct diagnosis is significantly enhanced with the addition of steatosis as a prognostic factor.

**Key words:** Shear wave elastography; Aminotransferase platelet ratio; Liver fibrosis; Liver biopsy

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**Core tip:** The gold standard in the diagnosis and staging of liver fibrosis is an invasive liver biopsy. The accuracy of non-invasive tools such as ultrasound shear wave elastography either alone or in combination with the use of the aspartate transaminase platelet ratio index score compared to histology to guide management of liver fibrosis is not known. We addressed this question in a multicenter trial in patients with chronic progressive liver disease in a low to middle income country.

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### INTRODUCTION

Liver fibrosis is a progressive condition that if diagnosed early and staged accurately, allows early clinical intervention that may arrest or slow down progression to end stage decompensated cirrhosis. The spectrum of chronic liver disease and fibrosis that leads to end stage decompensated cirrhosis, is an important cause of morbidity and mortality in the world<sup>[1]</sup>. Early diagnosis, accurate staging and re-evaluation of liver fibrosis is aimed at avoiding the progression from normal to minimal to significant fibrosis and timely management of patients with advanced disease.

There are several chronic progressive liver diseases that lead to liver fibrosis. Non-alcoholic fatty liver disease (NAFLD) is one of the most common. NAFLD is closely associated with obesity and insulin resistance. Pathological changes in the biochemical profile of the liver that lead to liver fibrosis also occur due to chronic metabolic conditions such as diabetes and degenerative conditions like atherosclerosis<sup>[2,3]</sup>. Other common causes of liver fibrosis include infections such as chronic viral hepatitis and human immunodeficiency virus. Drugs and other toxins also play an important role. This list

is not exhaustive but this paper focuses on the last four causes. Liver fibrosis is characterized by excessive accumulation of extracellular matrix due to the release of inflammatory mediators and free radicals to cause oxidative stress and liver fibrogenesis. During this process hepatic stellate cells activation occurs. Platelet derived growth factor, tumor necrosis factor  $\alpha$ , transforming growth factor  $\beta$  or reactive oxygen species play a role in the progression to liver fibrosis. Several phenotypic alterations occur with the end result being irreversible<sup>[4]</sup>.

The current gold standard in the diagnosis and staging of liver fibrosis is liver biopsy. Liver biopsy and more recently ultrasound guided liver biopsy only evaluates 1/50000 of the liver parenchyma. It is invasive, has a complication rate (albeit small,) and is subject to intra and inter-observer variability<sup>[5]</sup>. Because of the imperfect nature of liver biopsies, over the last several years there has been a growing trend to validate non-invasive tools to diagnose and stage liver fibrosis. Alkaline aminotransferase platelet ratio index (APRI) is a laboratory marker that has been shown to have some value but is inferior to liver biopsy. Ultrasound and magnetic resonance have been used for elasticity imaging. Magnetic resonance elastography, even though promising, has some disadvantages. Aside from the significant cost of the study, it cannot be performed in a liver with iron overload because of signal-to-noise limitations; has longer examination times compared to ultrasound elastography, and is subject to respiratory artifact<sup>[6]</sup>. Ultrasound elastography has been validated and has been shown in many studies to have similar sensitivity and specificity to liver biopsies<sup>[5,7]</sup>.

Ultrasound elastography measures the liver stiffness/elasticity by assessing at least 100 times the proportion of the liver that a biopsy does. Transient elastography (TE) has been validated in multiple studies<sup>[8]</sup> but shear wave elastography (SWE) may be preferred because unlike transient elastography, which consists of a vibrator producing shear waves, the latter can perform a conventional ultrasound at the same time. The technique is integrated into an ultrasound system. The principle behind the interpretation of shear wave elastography is that shear waves produced by a focused ultrasound beam are directly related to the stiffness of the liver from where they are generated<sup>[5,7,8]</sup>. SWE is also reportedly more accurate than TE in assessing significant fibrosis ( $\geq F2$ )<sup>[8,9]</sup>. The use of shear wave elastography in the diagnosis and staging of liver fibrosis has been increasing. Being a non-invasive technique proves advantageous because repeat measurements can be obtained in patients with chronic progressive liver diseases. However, this non-invasive procedure does have some pitfalls. It is subject to intra- and inter-observer variability, validated cut-offs have mainly only been demonstrated in hepatitis C; Acute hepatitis can have false positives. In patients with a high body mass index, erroneous values may be obtained. A very practical pitfall is confounding factors such as edema, inflammation, cholestasis and congestion. All these



must be put in context and a multidisciplinary clinical approach used in the interpretation of the results<sup>[5,7,8,10]</sup>.

A limitation of prior studies is the lack of integration of the accuracy and limitations of elastography. No prior study has combined elastography with the use of APRI and histology to guide management of liver fibrosis. This study addresses the gaps and makes practical inferences that focus on accurate early diagnosis and staging. The focus in our study is "interpretation within a clinical context". This multi-institutional study performed in Kenya aims to capture and highlight factors based on the disease burden in this region. Ultrasound elastography has only recently been made available in East Africa. The findings, therefore, could be of wider benefit because of the high burden of other etiologies of liver disease such as hepatitis B in the region. Most studies thus far have been carried out in the West with the disease burden focused on hepatitis C. In addition, the literature largely reports data from middle-high economic areas whereas adherence to clinical guidelines may not be as feasible in poor/resource challenged facilities.

## MATERIALS AND METHODS

### Objectives

The primary objective was to analyze the accuracy of shear wave elastography in comparison to liver biopsy in differentiating the various stages of liver fibrosis. The secondary objective was to evaluate whether the addition of the APRI score to SWE would improve the accuracy of this differentiation. With these, illustrate the role of the shear wave elastography, APRI score, and biopsy solely and or in combination in the diagnostic algorithm of accurate quantification of liver fibrosis. We also sought to assess the role of other covariates, namely, age, gender and steatosis, and their influence on the relative importance of SWE and the APRI score in predicting the extent of liver fibrosis.

### Design

Three hospitals were included in this prospective study: Aga Khan University (AKU) Hospital, Kenyatta Teaching and Referral Hospital and St Mary's Mission Hospital. Approval was obtained from the relevant Scientific and Ethics committees. All consecutive patients referred for an ultrasound guided liver biopsy at all three institutions were subject to recruitment based on the inclusion and exclusion criteria as well as informed written consent. The study included patients above eighteen years of age with chronic progressive diffuse liver disease. Patients were excluded from the study if they did not have any of the three diagnostic tests, *i.e.*, liver biopsy, APRI score or SWE.

Consecutive patients were recruited by the principle investigator in three ways: (1) referral for an ultrasound guided liver biopsy at the AKU Radiology Department; (2) referral from St Mary's Hospital with biopsy performed by the AKU Radiology department and pathological

analysis performed at AKU department; and (3) referral for a liver biopsy request to be analyzed at the Pathology department of Kenyatta National Hospital.

At recruitment, a study file was opened for each patient by the principle investigator at the AKU. Routine liver function tests, platelet counts, and demographic information related to confounding factors of chronic liver disease, including information on alcohol use, Human Immunodeficiency Virus status, viral load and CD4 levels, hepatitis B and C status was collected. Men who had been drinking more than 30 g of alcohol per day and women who had been drinking more than 20 g of alcohol per day were considered current drinkers. Patients who had stopped drinking completely for more than six months before the biopsy were considered ex-drinkers<sup>[11]</sup>.

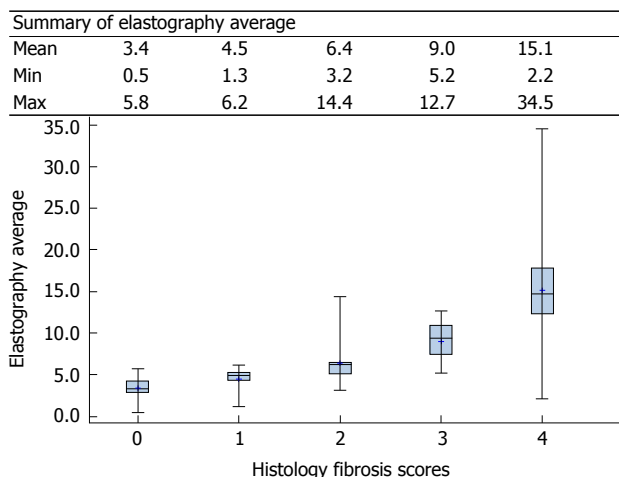
At the AKU routine ultrasound of the liver was performed to qualitatively record presence (grade 0-3) or absence of steatosis using established criteria published by Lupşor-Platon *et al*<sup>[12]</sup>. Any other diffuse or focal lesions were documented followed by SWE. At AKU elastography measurements were taken from the right lobe<sup>[13]</sup> of the liver with the patients holding their breath. Measurements were considered successful using validated criteria established by Castéra *et al*<sup>[14]</sup>: "(1) 10 valid shots; (2) a ratio of valid shots to the total number of shots of 60% or higher; and (3) variability of measurements less than 30% of the median value of liver stiffness measurements". Philips iU22 ultrasound machine with its C5-1 curvilinear transducer was used. The units for SWE readouts (liver stiffness) were kilopascals (kPa). Four sonologists each with more than 5 years' experience in routine liver scanning and validated ultrasound elastography experience from uniform training performed each exam independently. The median stiffness (used to grade the fibrosis), average stiffness and standard deviation of measurements generated by the software were recorded and interpreted by the four sonologists independently<sup>[7,10]</sup>. Each patient had one liver biopsy specimen taken from the right lobe<sup>[13]</sup> after the ultrasound elastography which was graded histologically for fibrosis based on the Metavir classification system<sup>[15,16]</sup>. This was done by two experienced histopathologists at the AKU and KNH Pathology departments. Each specimen was evaluated by the two histopathologists from each respective pathology department. Discrepancies were resolved by consensus between the two. The histopathologists from the two sites were full-time faculty, certified by the Kenya Medical Practitioners and Dentists Board, practicing in University Hospitals each with greater than ten years' experience in liver biopsy assessment for fibrosis. In each patient the time interval between ultrasound, elastography and histology was not more than one month. The pathologists and sonologists were blinded to clinical data and elastography or histology grade.

Interpretation of liver fibrosis by shear wave elastography in kPa divided the entity into no fibrosis (F0), mild fibrosis (F1), severe fibrosis (F2), significant fibrosis (F3)

**Table 1** Summary illustrating sample size calculation above

AUROC	Total (n)	% Positive	# Positive	# Negative	SE	Confidence interval	
						Lower	Upper
0.8	110	30	33	77	0.050	0.701	0.899
0.8	130	30	39	91	0.046	0.709	0.891

AUROC: Area under the receiver operating characteristic.

**Figure 1** Elastography average vs histology fibrosis score (Box Plot).

and cirrhosis (F4). Automatic median value generated by the ultrasound software was used to establish the elastography grade as follows  $< 4.6 = F0$ ,  $4.6-5.6 = F1$ ,  $5.7-7.0 = F2$ ,  $7.1-12.0 = F3$  and  $> 12 = F4$ <sup>[10,17-19]</sup>. APRI score was calculated using a formula proposed by original study of Wai *et al.*<sup>[20]</sup>:  $APRI = [(AST \text{ level}/ULN)/platelet \text{ counts } (10^9/L)] \times 100$ . A score of  $< 0.5$  was graded as F0,  $0.5-1.5$  as F1-3 and  $> 1.5$  as F4. The corresponding histology grade was assessed<sup>[21,22]</sup>.

### Sample size estimates

The sample size was determined with the aim to keep the standard error of the AUROC at 0.05. This set the difference between the upper and lower 95%CI limits to 0.20 ( $\pm 2$  standard errors). From previous publications the range for the AUROC for significant fibrosis, as determined from non-invasive tests, is approximately 0.69 to 0.89; for cirrhosis the range is from 0.81 to 0.98. Assuming the AUROC to be approximately 0.8<sup>[20]</sup> the sample size of 110 patients would yield a standard error of 0.05 (Table 1).

Shear wave elastography has been shown to have a lower operator error technique than transient elastography (3%-16%)<sup>[23-25]</sup>. As a precautionary measure we raised the sample size from 110 to 130.

### Statistical analysis

The statistical review of the study was performed by a biomedical statistician. Logistic regression models with backward elimination, using SAS version 9.3, were utilized to assess the significance of SWE median,

the APRI score and the covariates age (categorized as below and above the median), gender and steatosis. Besides the *P*-values, the analysis provided the ORs and their respective 95%CI limits. The sensitivity and the specificity were computed based on the variables included in the model. This in turn enabled the ROCs and the AUROC to be determined. Summary statistics and correlation coefficients, where appropriate, were computed.

## RESULTS

### Demographics and baseline characteristics

One hundred and twenty-eight patients were recruited for the study. AKU, KNH and St. Mary's contributed 54 (42.2%), 53 (41.4%) and 21 (16.4%) patients, respectively. The most prevalent viral infection was hepatitis B that was noted in 30 (23.4%) patients. This was followed by human immunodeficiency virus (HIV) with 18 (14.1%) and hepatitis C with 13 (10.2%) patients (Appendix 1); fifteen patients had 2 or more infections. Sixty-three (49.2%) of the patients had a steatosis score of 0 and 61 (47.7%) of the patients had a histology fibrosis score of 0. Eighty-one (63.3%) patients fell in the histology fibrosis score subgroup F0-1; the remaining 47 (36.7%) fell in the F2-F4 subgroup. Fifty percent of the patients had an elastography score of F0 and 59 (46%) of the patients the APRI score of F0 (Appendix 2). The elastography median scores were the lowest among HIV subjects followed by those with hepatitis B and then a hepatitis C infection. The highest scores were from those with multiple viral infections. The APRI scores also follow the pattern described above for the elastography median scores (Appendix 3). There appears to be a good correlation between the elastography median scores and the histology fibrosis scores (Appendix 4). The elastography and APRI fibrosis score are statistically significantly correlated with the histology fibrosis scores (Appendix 5) (Figure 1 and Table 2).

### Logistics regression results

Table 3 summarizes some of the key results that stemmed from the analysis of the SWE median and APRI score data using logistic regression. Both variables show a high degree of statistical significance in their individual ability to distinguish between the lower stages of fibrosis compared to the higher stages. This is true across all possible partitions of the Metavir fibrosis scores. However, the AUROCs for SWE medians are much higher than

**Table 2** Summary of liver disease scores by histology fibrosis score (discrete variables) *n* (%)

	Histology fibrosis scores					Total ( <i>n</i> = 128)	$\chi^2$ <i>P</i> value
	0 ( <i>n</i> = 61)	1 ( <i>n</i> = 20)	2 ( <i>n</i> = 20)	3 ( <i>n</i> = 10)	4 ( <i>n</i> = 17)		
Elastography fibrosis score							
F0	51 (83.6)	7 (35.0)	5 (25.0)	0 (0.0)	1 (5.9)	64 (50.0)	< 0.0001
F1	10 (16.4)	10 (50.0)	2 (10.0)	2 (20.0)	1 (5.9)	25 (19.5)	
F2	0 (0.0)	3 (15.0)	9 (45.0)	1 (10.0)	0 (0.0)	13 (10.2)	
F3	0 (0.0)	0 (0.0)	3 (15.0)	7 (70.0)	1 (5.9)	11 (8.6)	
F4	0 (0.0)	0 (0.0)	1 (5.0)	0 (0.0)	14 (82.4)	15 (11.7)	
APRI fibrosis score							
0	45 (73.8)	6 (30.0)	6 (30.0)	1 (10.0)	1 (5.9)	59 (46.1)	< 0.0001
1 to 3	14 (23.0)	13 (65.0)	9 (45.0)	9 (90.0)	8 (47.1)	53 (41.4)	
4	2 (3.3)	1 (5.0)	5 (25.0)	0 (0.0)	8 (47.1)	16 (12.5)	
Steatosis							
Grade 0	48 (78.7)	8 (40.0)	2 (10.0)	1 (10.0)	4 (23.5)	63 (49.2)	< 0.0001
Grade 1	6 (9.8)	8 (40.0)	5 (25.0)	1 (10.0)	1 (5.9)	21 (16.4)	
Grade 2	6 (9.8)	2 (10.0)	7 (35.0)	4 (40.0)	0 (0.0)	19 (14.8)	
Grade 3	1 (1.6)	2 (10.0)	6 (30.0)	4 (40.0)	12 (70.6)	25 (19.5)	

APRI: Aminotransferase platelet ratio index.

**Table 3** Shear wave elastography median and aminotransferase platelet ratio index score to differentiate between metavir subgroups

		F0-3 vs F4	F0-2 vs F3-4	F0-1 vs F2-4	F0 vs F1-4
SWE median	<i>P</i> value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	OR	1.708	1.789	2.983	2.683
	95%CI	1.379, 2.115	1.432, 2.236	1.839, 4.838	1.789, 4.025
	AUROC	0.926	0.929	0.908	0.879
APRI score	<i>P</i> value	0.0202	0.039	0.0005	0.0008
	OR	1.511	1.404	3.482	4.651
	95%CI	1.067, 2.141	1.018, 1.938	1.727, 7.018	1.895, 11.416
	AUROC	0.812	0.784	0.780	0.803
SWE and APRI <sup>1</sup>	AUROC	0.927	0.931	0.920	0.890
	APRI influence	0.001	0.002	0.012	0.011

<sup>1</sup>SWE median and APRI score. OR: Odds ratio; SWE: Shear wave elastography; APRI: Aminotransferase platelet ratio index; AUROC: Area under the receiver operating characteristic.

those for APRI score.

The SWE median differentiates the Metavir fibrosis subgroups F0-1 and F2-4 with an AUROC of 0.908 compared to 0.780 for the APRI score. These results imply that the SWE on its own is a better predictor of the differentiating the subgroups than the APRI score. When we utilize both variables simultaneously, the increase in the AUROC attributed to the APRI score is less than 1.2% higher than that predicted by SWE median. This amounts to about 13% of the 9% not predicted correctly by the SWE median. The results for other partitions of the histology fibrosis scores mimic those described above with the AUROC for SWE median being around 0.9 and that of the APRI score being about 0.8.

Additional logistic regression models incorporated several other variables in the analysis to evaluate their impact on the AUROC. The variables considered, in addition to the SWE median and the APRI score were the covariates age (categorized as below and above the median), gender and steatosis score. The results of the logistic regression analysis (Table 4) with all of the aforementioned variables in the model showed that SWE and the steatosis score were the only two variables that

made significant contributions to the predictive power of the model.

Using the backward elimination method, all of the variables that were not making a significant contribution at the 0.1 level were dropped from the model. The results of these analysis (Table 5) show that for the primary objective of differentiating between F0-1 and F2-4 is accomplished quite well with an AUROC of 0.944; the two variables that made a significant contribution were SWE and steatosis. The steatosis score adds significantly to the prediction model that tries to identify the fibrosis group that a patient belongs to (Table 5); this is true in every case except for the F0-3 vs F4 partition. The APRI score on the other hand makes a significant contribution to only the partition F0-2 vs F3-4. Given that the APRI score appears in only one partition as an important predictor, an additional analysis was performed by dropping the APRI score from the model. This resulted in adding a few additional observations to the data set used for analysis since missing APRI scores had contributed to a slightly reduced sample size. In addition, the steatosis score was added to the F0-3 vs F4 model in order to have a unique set of predictors across

**Table 4** Prediction of histology fibrosis score (Grouping: F0-1 vs F2-4) using elastography median, aminotransferase platelet ratio index score, age category<sup>1</sup>, sex and steatosis maximum likelihood and odds ratio estimates

Variable	DF	Coefficient estimate	Standard error	Wald $\chi^2$	Pr > $\chi^2$	OR estimate	Lower 95%CI limit for OR	Upper 95%CI limit for OR
Intercept	1	-6.6482	1.3510	24.2158	< 0.0001			
Age category	1	0.0971	0.3219	0.0910	0.7629	1.214	0.344	4.288
APRI score	1	0.1946	0.3873	0.2526	0.6153	1.215	0.569	2.595
Elastography median	1	0.9041	0.2513	12.9464	0.0003	2.470	1.509	4.042
Sex	1	0.3330	0.3551	0.8797	0.3483	1.947	0.484	7.830
Steatosis	1	1.1317	0.3462	10.6843	0.0011	3.101	1.573	6.112

<sup>1</sup>Age category (years): *n* = 128. Mean (SD) 46 (16.99). Median 42; Minimum 18; Maximum 108. OR: Odds ratio; APRI: Aminotransferase platelet ratio index.

**Table 5** Significance of predictive values associated with key pre-identified variables

	F0-3 vs F4	F0-2 vs F3-4	F0-1 vs F2-4	F0 vs F1-4
SWE median	< 0.0001	< 0.0001	0.0003	0.0002
APRI score	NS <sup>1</sup>	0.0404	NS	NS
Age	NS	NS	NS	NS
Gender	NS	NS	NS	NS
Steatosis	NS	0.0263	0.0002	0.0007
AUROC	0.926	0.962	0.944	0.902

<sup>1</sup>NS: Not significant at the 0.1 level. SWE: Shear wave elastography; APRI: Aminotransferase platelet ratio index; AUROC: Area under the receiver operating characteristic.

all partitions. These results are presented in Table 6. The fact that the results from Tables 5 and 6 are very similar implies that the missing data points did not influence the outcome in any meaningful way.

The F0-2 vs F3-4 data shows that the AUROC obtained with the use of the SWE median and the steatosis score is 0.954 (Table 6). By adding the APRI score (Table 5) the AUROC increase of 0.008 or 0.8%. This represents a decrease of about 13% (0.008 of 0.046) in the error rate. On the other hand, adding steatosis to the model after including the SWE median and the APRI score, the AUROC increase from 0.931 (Table 3) to 0.962 (Table 5), an increase of 0.031 or 3.1%. This represents a decrease of about 44.9% (0.031 of 0.069) in the error rate.

## DISCUSSION

### Summary of findings

The infection with the highest prevalence was hepatitis B. There were also patients with HIV, hepatitis C and the co-infections in this cohort. While the prevalence of hepatitis B, C and HIV in Sub-Saharan Africa has not been conclusively established<sup>[26]</sup>, it has been postulated that hepatitis B is relatively more prevalent than hepatitis C as compared to the western world where hepatitis C is more prevalent<sup>[27]</sup>. Most studies on liver fibrosis quantification have been carried out in the western world therefore it is important to have the same studies carried out in Sub-Saharan Africa where the epidemiology of the viral infections is likely to be different. The elastography median scores were the lowest among HIV subjects

**Table 6** Elastography median and steatosis

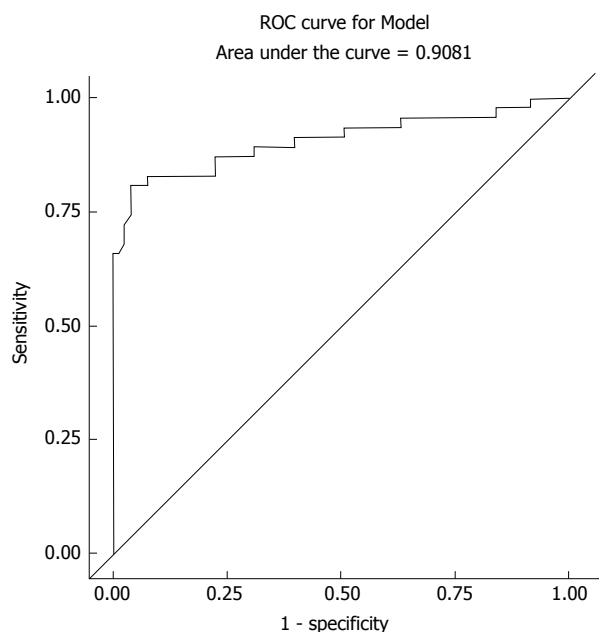
Variable	Pr > $\chi^2$	OR estimate	Lower 95%CI limit for OR	Upper 95%CI limit for OR
F0-3 vs F4				
Intercept	< 0.0001			
Elastography median	< 0.0001	1.681	1.347	2.099
Steatosis	0.6529	1.187	0.563	2.502
AUROC = 0.936				
F0-2 vs F3-4				
Intercept	< 0.0001			
Elastography median	< 0.0001	1.684	1.353	2.097
Steatosis	0.0568	1.846	0.982	3.467
AUROC = 0.954				
F0-1 vs F2-4				
Intercept	< 0.0001			
Elastography median	0.0003	2.397	1.500	3.828
Steatosis	0.0002	3.135	1.703	5.772
AUROC = 0.944				
F0 vs F1-4				
Intercept	< 0.0001			
Elastography median	0.0002	2.221	1.463	3.370
Steatosis	0.0007	2.496	1.473	4.230
AUROC = 0.902				

OR: Odds ratio; AUROC: Area under the receiver operating characteristic.

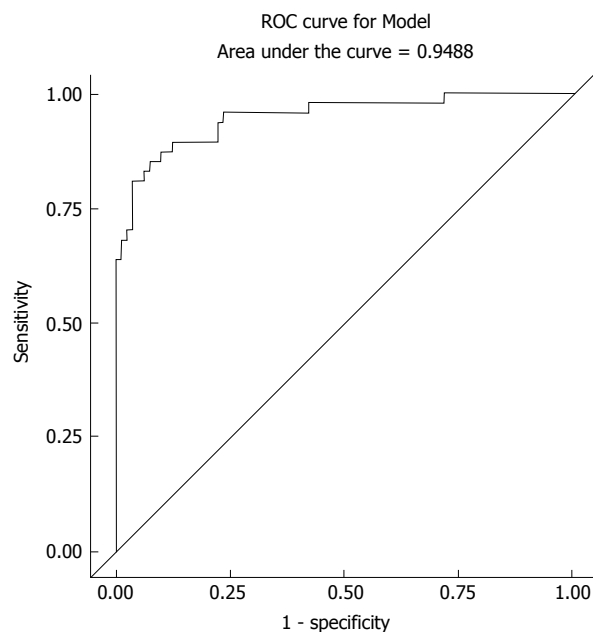
followed by those with hepatitis B infection; the next highest score was for patients with a hepatitis C infection. The lowest score was from those with multiple infections. The APRI scores also follow the pattern described above for the elastography scores. This pattern corresponds with what has previously been described. Hepatitis C most likely has higher levels of quantified liver fibrosis because of the three viruses it has the most indolent and chronic clinical course. The mortality from these infections is correlated to chronic liver disease and not due to progression of the virus due to the success of antiretroviral therapy<sup>[27,28]</sup>.

Analysis categorized fibrosis as, F0 vs F1-4, F0-1 vs F2-4, F0-2 vs F3-4, F0-3 vs F4. The results focus on F0-1 vs F2-4 which is of most clinical significance (no and non-significant fibrosis vs significant fibrosis that demands intervention). There is a good correlation between the elastography median scores and the histology fibrosis scores. The OR of 3.0 implies that the elastography median scores are 3 times more likely to correctly identify a fibrosis score of F2-4 compared to F0-1. The upper and lower limits for the OR with 95%CI do not cross 1 which





**Figure 2** Prediction of histology fibrosis score (Grouping: F0-F1 vs F2-F4) using elastography median. ROC: Receiver operating characteristic.



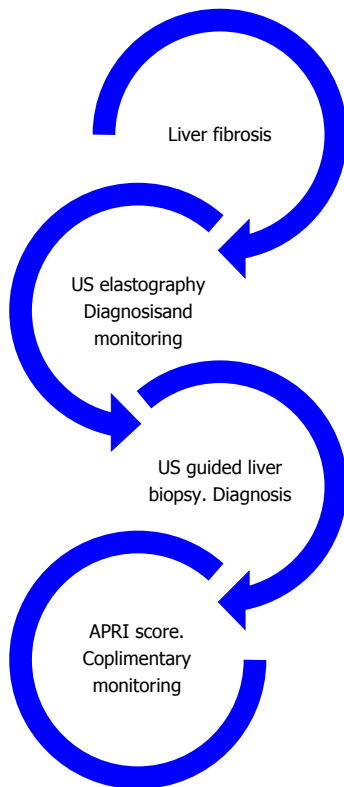
**Figure 3** Prediction of histology fibrosis score (Grouping: F0-1 vs F2-4) using elastography median, aminotransferase platelet ratio index score, age categor, sex and steatosis.

strengthens this result. However, the limits are wide 1.8-5. This may be due to the small sample size and because close to 50% of the sample at F0. If F0 were to be eliminated from the analysis this may reduce the limits with a questionable effect on clinical significance. The accuracy of the elastography median depends on the sensitivity and specificity. This is clear from the raw data used to generate the ROC curve. An elastography median of approximately 3.8 is the point at which you get the best sensitivity matched with specificity. The ROC curve for model, which depicts sensitivity and specificity, illustrates how for a very high sensitivity, specificity is low and as sensitivity reduces; one arrives at a point where specificity is acceptable clinically. That is, one can identify disease with a high sensitivity and be correct (specifically know that you are also picking the non-diseased). The AUROC for the elastography median was 0.91. The elastography and APRI fibrosis score are statistically significantly correlated with the histology fibrosis scores. However, APRI score in itself or when combined with elastography median score does not significantly increase the accuracy of elastography in the differentiation of non-significant vs significant fibrosis. APRI had an AUROC of 0.78. APRI and elastography median had an AUROC of 0.92. Therefore APRI does not have a statistically significant effect on the prediction of F0-1 from F2-4 when added to elastography. And when used alone it is significantly less accurate than elastography. However in patients with chronic progressive liver fibrosis who need repeated analysis to categorize and monitor the progress of liver fibrosis APRI does have a clinically significant role in the management algorithm of liver fibrosis.

Previous studies vary on the accuracy of elastography. The sensitivity, specificity and diagnostic accuracy of shear wave elastography in the determination of liver

stiffness compared with biopsy results is comparable to<sup>[8-10]</sup>. The accuracy of elastography mirrors those depicted by these studies albeit a slightly higher accuracy in this study. This may be due to the difference in grouping of the fibrosis scores for analysis. Also, if the F0 of this study are removed from the analyses this may lead to more similar figures since the F0 constitute approximately 50%. The diagnostic accuracy of shear wave elastography and APRI score in the determination of liver stiffness has not been reported before<sup>[8-10]</sup>.

Ordinal regression and backward elimination was used to analyze significance of HIV, hepatitis B, alcohol use, steatosis, age and gender. It showed that steatosis has a significant OR and *P*-value in the analysis for fibrosis. Ferraioli *et al*<sup>[10]</sup> showed that steatosis does not affect the performance of elastography. The challenge as stated in this paper is the confounding effect of various pathologies in the diagnosis and staging of liver fibrosis. This is particularly relevant in the generation and given the wide use of reference ranges for all modalities used in the diagnosis and staging of liver fibrosis. It is for this reason that to date several studies have used variable reference ranges for F0-F4<sup>[8,10,17-19]</sup>. Our results highlight the potential effect of the presence of steatosis (EF-S\_1\_10\_3\_Logistic\_All Variables\_F0-F1VsF2-F4 document) on the diagnosis and characterization of liver fibrosis. Of note from this result is the increase in the AUROC from 0.91 (Figure 2) to 0.95 (Figure 3) in the logistic regression backward elimination analysis that is attributable to the elastography median and steatosis each with a significant *P*-value and OR (Table 4). This is an area that needs further study, especially since steatosis was measured subjectively in this study. The data on HIV and alcohol use as variables were not adequately



**Figure 4** Link between diagnostic tests used in the evaluation of liver fibrosis. US: Ultrasound; APRI: Aminotransferase platelet ratio index.

powered to add to this analysis.

### Strengths

This study is timely because of the growing use of elastography in the developed world. In the developing world the use of this diagnostic tool needs to grow *via* an evidence based approach that is tailored to the local disease burden. Most research in the west has focused on hepatitis C and alcohol or nonalcoholic steatosis. The wider disease burden covered in this study is thus a more homogenous representation of chronic liver fibrosis pathology. The complimentary use of APRI score is especially relevant in resource limited setups.

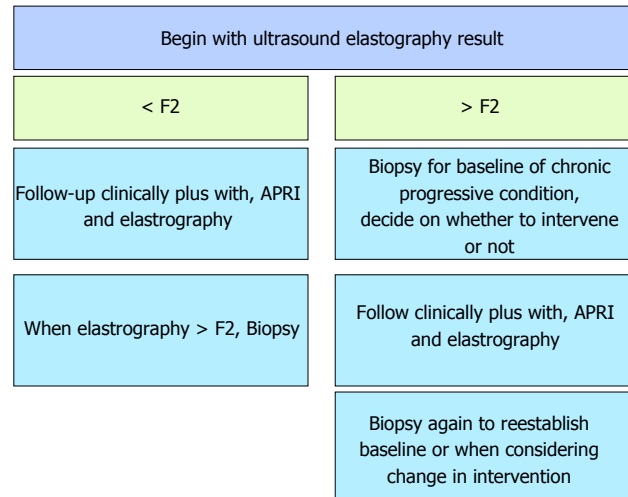
### Theoretical and practical implications of these findings

This study brought to light theoretical implication of the need to standardize the diagnosis and accurate staging and follow-up of liver fibrosis. Figure 4 depicts the link between all tests used in the evaluation of liver fibrosis (Figure 4).

The use of all tests should be complimentary and histology has its place but the noninvasive tests may be more logical in the beginning and for progressive monitoring. Inference from the results generates a flowchart below describing a management algorithm (Figure 5).

### Inference from the results: Consider the following flowchart at diagnosis

Practical unique and reported challenges exist. Care needs to be taken in interpretation and training for elasto-



**Figure 5** Flow chart depicting use of diagnostic tests in liver fibrosis. Begin with US SWE result. US: Ultrasound; SWE: Shear wave elastography; APRI: Aminotransferase platelet ratio index.

graphy. Technical and interpretation skill for elastography specifically when choosing the ten values to include in the report; this includes excluding/ignoring far outliers, though these outliers may represent focal areas of different fibrotic stages! Use of the median to give a final conclusion of the ten chosen readings is appropriate especially in the setting of variable readings. But, care must be exercised. There should be a low threshold to recommend a biopsy (still the gold standard) to confirm the findings especially if the mean elastography reading is in keeping with a diagnosis of no fibrosis yet the standard deviation is high in the presence of individual readings of cirrhosis<sup>[5]</sup>. The challenge of the heterogeneously fibrotic liver or presence of lesions, *e.g.*, metastasis causing heterogeneity of the liver rendering fibrosis assessment questionable in terms of using just the median to conclude on the level of fibrosis especially when there is a big difference in the individual stiffness values must be remembered. These issues red flag the danger of not allowing room to vary an impression and advise further evaluation with an United States guided liver biopsy to correlate<sup>[5]</sup>. Indeed, one must also bear in mind that the suggestion to further evaluate with a liver biopsy is inherently flawed because of the attend pitfalls of the tool. Chronic progressive liver fibrosis needs accurate early diagnosis and interval monitoring. Elastography is a validated tool. APRI can be used as a complimentary tool though its effect is not of statistical significance but clinical significance. Biopsy remains the gold standard. We propose a flow chart at diagnosis. Further, the probability of a correct diagnosis is significantly enhanced with the addition of steatosis as a prognostic factor (Figure 5).

### Limitations and problems encountered in the method

The number of ultrasound guided liver biopsies for the assessment of liver fibrosis has continued to reduce because of the increasing use of elastography which is noninvasive and without the side effects associated with

liver biopsy. This diagnostic trend is reinforced by the continued validation of elastography<sup>[5,7,10,12]</sup>. Therefore during this study the recruitment rate was low. As such participants were recruited from three different sites to meet the sample size requirement to adequately power the results. The ultrasound and elastography were all performed at the same site (AKU). Biopsy results were analyzed by pathologists at two of the three sites (AKU and KNH). This may have potentially led to variable histological inter-observer variability. To counter this each sample was read by each pathologist at each respective site and discrepant values were resolved by consensus. However, major and minor discrepancy analyses of histology for inter-observer discrepancies were not assessed.

There are limitations associated with elastography, including the confounding effects of inflammatory activity, and to a lesser extent, steatosis<sup>[13]</sup>, on liver stiffness evaluation. There is also reduced accuracy observed in lower fibrosis stages (F0-F2). Furthermore, the incidences of failed and unreliable scans have been reported to be approximately 3% to 16% in transient elastography but less in shear wave elastography (figures not reported yet)<sup>[22]</sup>. The sample size was inflated by 5% to cater for this. A typical liver biopsy covers 1/10000<sup>th</sup> of the liver while elastography covers a larger area. Matching the two sites covered by the two exams may not have been 100%.

The information sort in the data collection form to analyze the secondary objectives was sensitive in nature including queries about alcohol use and HIV status. This precluded complete disclosure from participants and led to inadequate data on related parameters. This led to a reduction in the power of inferences regarding the role of alcohol and HIV.

### Suggestions for improvement and further work

This has been an East African experience: Unique challenges and similar differences to those published. More comprehensive analysis needs to be done to further reveal the extent of confounding factors affecting the use of elastography in the diagnosis and staging of liver fibrosis. The role of steatosis needs further objective assessment. Further work needs to be done to describe the in cooperation of magnetic resonance elastography in the diagnostic algorithm of liver fibrosis.

Our study validates the use of ultrasound shear wave elastography in the diagnosis and staging of fibrosis within the context of liver disease in a LMIC.

### ACKNOWLEDGMENTS

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## COMMENTS

### Background

Chronic progressive liver diseases cause liver fibrosis whose end result is decompensated liver failure. Liver fibrosis that results from these diseases can be reversed if diagnosed early. The current gold standard in the diagnosis of liver fibrosis is a liver biopsy preferably ultrasound guided, which is an invasive procedure with limitations and risks. Recent research have validated the use of shear wave ultrasound based liver elastography which is a non-invasive imaging based tool that has a sensitivity and specificity that almost parallels histological diagnosis from a liver biopsy. The staging of liver fibrosis at diagnosis uses a Metavir scoring system that has been adapted by elastography. Aminotransferase to platelet ratio index is a liver function test that has some usefulness in the diagnosis of liver fibrosis. The combined use of histology, elastography and aminotransferase to platelet ratio index has not been elucidated.

### Research frontiers

Previous studies have shown that ultrasound based elastography can substitute liver biopsy in the accurate diagnosis of liver fibrosis.

### Innovations and breakthroughs

This is the first study to evaluate the combined role of ultrasound based elastography, histology and aminotransferase to platelet ratio index in a low to middle income country for the management of progressive liver fibrosis.

### Applications

The use of the three tests should be complimentary and histology has its place but the noninvasive tests may be more logical in the beginning and for progressive monitoring. More comprehensive analysis needs to be done to further reveal the extent of confounding factors affecting the use of elastography in the diagnosis and staging of liver fibrosis. Further work needs to be done to describe the in cooperation of magnetic resonance elastography in the diagnostic algorithm of liver fibrosis.

### Terminology

Elastography is radiological based software that can diagnose and quantify the degree of liver fibrosis. It is either ultrasound or magnetic resonance based. Ultrasound based elastography uses sound wave to assess for and quantify liver stiffness that is directly related to liver fibrosis. Aminotransferase to platelet ratio is a laboratory parameter derived from part of the routine liver function tests and platelet count.

### Peer-review

The authors have validated the use of ultrasound shear wave elastography in the diagnosis and staging of fibrosis within the context of liver disease in a low to middle income country. Practical management algorithms that in cooperate the use of ultrasound based elastography, histology and aminotransferase to platelet ratio index have been demonstrated. More comprehensive analysis needs to be done to further reveal the extent of confounding factors affecting the use of ultrasound or magnetic resonance based elastography.

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Retrospective Study

## Efficacy and safety of tenofovir in chronic hepatitis B: Australian real world experience

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**Author contributions:** Lovett GC data collection, data analysis and manuscript composition; Nguyen T, Iser DM, Chen R, Demediuk B, Shaw G and Bell SJ data collection; Holmes JA data collection and manuscript reviewing; Desmond PV planned study, supervised data collection and analysis and revised manuscript; Thompson AJ planned study, supervised data collection and analysis and revised manuscript.

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**Informed consent statement:** Informed consent was not required for this study as only a retrospective audit was undertaken. The confidentiality of participant records has been maintained at all times, with patient information remaining deidentified.

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### Abstract

#### AIM

To evaluate the long-term treatment outcomes of tenofovir therapy in patients in a real world Australian tertiary care setting.

#### METHODS

We performed a retrospective analysis of treatment outcomes among treatment-naïve and treatment-experienced patients receiving a minimum 3 mo tenofovir therapy through St Vincent's Hospital Melbourne, Australia. We included patients receiving tenofovir [tenofovir disoproxil fumarate (TDF)] monotherapy, as well as patients treated with TDF in combination with a second antiviral agent. Patients were excluded if they demonstrated human immune-deficiency virus/hepatitis C virus/hepatitis delta virus coinfection or were less than 18 years of age. We considered virological and biochemical

response, as well as safety outcomes. Virological response was determined by measurement of hepatitis B virus (HBV) DNA using sensitive assays; biochemical response was determined *via* serum liver function tests; histological response was determined from liver biopsy and fibroscan; safety analysis focused on glomerular renal function and bone mineral density. The primary efficacy endpoint was complete virological suppression over time, defined by HBV DNA < 20 IU/mL. Secondary efficacy endpoints included rates of biochemical response, and HB e antigen (HBeAg)/HB surface antigen loss and seroconversion over time.

## RESULTS

Ninety-two patients were identified who fulfilled the enrolment criteria. Median follow-up was 26 mo (range 3-114). Mean age was 46 (24-78) years, 64 (70%) were male and 77 (84%) were of Asian origin. 55 (60%) patients were treatment-naïve and 62 patients (67%) were HBeAg-negative. Complete virological suppression was achieved by 45/65 (71%) patients at 12 mo, 37/46 (80%) at 24 mo and 25/28 (89%) at 36 mo. Partial virological response (HBV DNA 20-2000 IU/mL) was achieved by 89/92 (96.7%) of patients. Multivariate analysis showed a significant relationship between virological suppression at end of follow-up and baseline HBV DNA level (OR = 0.897, 95%CI: 0.833-0.967,  $P = 0.0046$ ) and HBeAg positive status (OR = 0.373, 95%CI: 0.183-0.762,  $P = 0.0069$ ). There was no difference in response comparing treatment-naïve and treatment-experienced patients. Three episodes of virological breakthrough occurred in the setting of non-compliance. Tenofovir therapy was well tolerated.

## CONCLUSION

Tenofovir is an efficacious, safe and well-tolerated treatment in an Australian real-world tertiary care setting. Our data are similar to the reported experience from registration trials.

**Key words:** Tenofovir; Hepatitis B virus; Australia; Real-life; Virological suppression; Chronic hepatitis B

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**Core tip:** Clinical trials have demonstrated that tenofovir is a safe and efficacious treatment for patients with chronic hepatitis B, with high rates of sustained virological suppression. There are limited data evaluating the safety and efficacy of tenofovir in real-world settings. The aim of this study was to evaluate the long-term treatment outcomes of tenofovir therapy in patients in an Australian tertiary care setting. We performed a retrospective analysis of treatment outcomes among treatment-naïve and treatment-experienced patients.

Lovett GC, Nguyen T, Iser DM, Holmes JA, Chen R, Demediuk B, Shaw G, Bell SJ, Desmond PV, Thompson AJ. Efficacy and safety of tenofovir in chronic hepatitis B: Australian real world

experience. *World J Hepatol* 2017; 9(1): 48-56 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i1/48.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i1.48>

## INTRODUCTION

Chronic hepatitis B (CHB) affects 240-400 million people around the world<sup>[1]</sup>. It is estimated that 218000 people in Australia live with CHB, a population prevalence of approximately 1%<sup>[2]</sup>. CHB is associated with the long-term complications of cirrhosis, liver failure and hepatocellular carcinoma (HCC), in 15%-40% of patients. CHB is one of the most common causes of HCC, the most rapidly rising cause of cancer deaths in Australia<sup>[3-5]</sup>.

The goal of treatment for CHB is to improve survival by preventing disease progression to cirrhosis, liver failure and HCC<sup>[6]</sup>. This can be achieved by long-term suppression of hepatitis B virus (HBV) DNA levels<sup>[7-10]</sup>. In long-term follow-up, sustained virological suppression has been associated with histological improvement and regression of cirrhosis, as well as reduced risk of hepatic decompensation and HCC<sup>[6,11-14]</sup>. Surrogate endpoints used in clinical trials include rates of biochemical [serum alanine aminotransferase (ALT) < upper limit of normal (ULN)], virological (undetectable HBV DNA level), serological [HB e antigen (HBeAg)/HB surface antigen (HBsAg) loss ± seroconversion] and histological (improvements in necro-inflammatory grade and fibrosis stage) response<sup>[13]</sup>. Current therapies approved for CHB include peginterferon-alpha, lamivudine (LMV), adefovir (ADV), telbivudine, entecavir (ETV) and tenofovir (TDF).

Tenofovir is a nucleotide analogue (NA) recommended as first-line treatment for CHB. Tenofovir was first developed as an antiviral for the treatment of human immune-deficiency virus (HIV). The safety and efficacy of TDF for the treatment of chronic HBV infection was confirmed in two phase-III clinical trials, enrolling patients with HBeAg-positive and HBeAg-negative CHB respectively. Rates of virological suppression were 76% and 93% at week 48 in HBeAg-positive and HBeAg-negative patients respectively<sup>[13]</sup>, and > 98% overall at week 240<sup>[15]</sup>. Among HBeAg-positive patients, rates of HBeAg seroconversion were 21% and 40%, and rates of HBsAg seroconversion were 3% and 7%, at weeks 48 and 240, respectively<sup>[15]</sup>. Genotypic resistance to TDF has not been described. TDF is effective for the treatment of both treatment-naïve and treatment-experienced patients. TDF has a reported good safety profile. Reversible renal toxicity has been reported in < 2% of patients in registration/post-registration studies<sup>[16]</sup>. Decreased bone mineral density has been reported in HIV-infected patients treated with TDF, but the effect in HBV-mono-infected patients remains unclear<sup>[17,18]</sup>.

There are limited data that describe the safety and efficacy of TDF in the "real world". The few studies that have been published describe populations in Europe and North America<sup>[19-21]</sup>. There have been no reports

of the experience with TDF in Australia. Such data are important. Australia is a multi-cultural country, and the CHB population is unique for the diversity of HBV genotypes, reflecting immigration patterns from Southern Europe, South-East Asia and Sub-Saharan Africa<sup>[2]</sup>. The rates of TDF response and resistance in Australia are unknown.

The aim of this study was to evaluate the efficacy and safety of long term TDF therapy in an Australian single-centre real-world cohort of CHB patients.

## MATERIALS AND METHODS

### Data collection

Data were collected retrospectively from a comprehensive clinical database of CHB patients receiving TDF through liver clinics at St Vincent's Hospital Melbourne (Australia) between 7 March 2006 and 18 February 2014.

### Selection criteria

All patients receiving TDF 300 mg daily therapy for HBV mono-infection through St Vincent's Hospital Melbourne were considered for analysis. Inclusion criteria included age > 18 years and treatment duration > 3 mo. Patients could be treatment-naïve or treatment-experienced. We included patients receiving TDF monotherapy, as well as patients treated with TDF in combination with a second antiviral agent. Patients were excluded in the setting of HIV, hepatitis C or hepatitis D co-infection.

### Prescription of TDF therapy

TDF was prescribed in accordance with the Australian Pharmaceutical Benefit Scheme. Patients were required to satisfy the following criteria: Non-cirrhotic patients must demonstrate documented chronic liver injury confirmed *via* liver function tests or liver biopsy and must demonstrate appropriate HBV DNA levels according to HBeAg status (HBeAg positive patients HBV DNA > 20000 IU/mL; HBeAg negative patients HBV DNA > 2000 IU/mL). Patients with cirrhosis are required to demonstrate detectable HBV DNA. Patients may be NA naïve or experienced (having failed previous therapy).

### HBV DNA assay

Prior to 2010, HBV DNA levels were measured using the versant HBV DNA 3.0 assay (bDNA) (Siemens Healthcare Diagnostics, Tarrytown, NY) with a lower limit of detection (LLD) of 351 IU/mL. From 2010, HBV DNA levels were measured using the Cobas Taqman assay (LLD = 20 IU/mL, Roche Molecular Systems, Pleasanton, CA, United States).

### Definitions of response

Complete virological suppression was defined as plasma HBV DNA level < 20 IU/mL. Partial virological suppression was defined as plasma HBV DNA level of  $\geq$  20 IU/mL and < 2000 IU/mL. Virological breakthrough (VBT) was

defined as an increase in viral load > 1 log<sub>10</sub> from nadir, or by a detectable HBV DNA level on two serial measures in a patient who had previously achieved an undetectable HBV DNA level. Biochemical response was defined as the normalisation of serum ALT to < 45 IU/L. Serological response was defined as the loss of detectable HBeAg and/or HBsAg from serum (HBeAg/HBsAg loss)  $\pm$  the development of antibodies against these antigens (HBeAg/HBsAg seroconversion).

### Clinical endpoints

The primary efficacy endpoint was complete virological suppression over time, defined by HBV DNA < 20 IU/mL. Secondary efficacy endpoints included rates of biochemical response, and HBeAg/HBsAg loss and seroconversion over time. We also measured rates of VBT and the occurrence of clinical events including hepatic decompensation and HCC. The assessment of safety was specifically focussed on renal function and, where available, bone mineral density.

### Statistical analysis

All statistical analyses were performed using SAS 9.4. For descriptive statistics, continuous variables were summarised as median (25<sup>th</sup>-75<sup>th</sup> centile). Categorical variables were described as frequency and percentage. Comparisons between groups for demographic, clinical and virological data were performed using the Wilcoxon signed pair test for continuous data and Fisher's exact test for categorical data. Significance was defined at *P*-value < 0.05. Kaplan Meier analysis was used to determine influences on the time to virological suppression. The associations between baseline HBeAg status, baseline HBV DNA, treatment experience, age, gender, baseline ALT, fibrosis stage and end of follow-up virological suppression were tested using Cox proportional hazards regression analysis and direct multivariate analysis. Ninety-two patients were included in the analysis of demographics and on-treatment safety and efficacy. Patients who had undetectable HBV DNA levels at time of commencement of TDF were excluded from the multivariable analysis (*n* = 18).

This study was approved by the Human Research Ethics Committee at St Vincent's Hospital Melbourne (QA: 009/14).

## RESULTS

### Study population

A total of 92 patients were identified. Patient characteristics are summarised in Table 1. The majority of patients were male (70%), of Asian ethnicity (84%) and had HBeAg-negative disease (69%). Fifty-five (60%) were treatment-naïve at the time TDF was commenced. Thirty-seven (40%) patients had been previously treated with NA therapy. Compared to treatment-naïve patients, treatment-experienced patients were more likely to have a lower serum HBV DNA level, and a normal serum ALT

**Table 1** Baseline demographics

Baseline demographics	Total population ( <i>n</i> = 92)	Treatment naïve ( <i>n</i> = 55)	Treatment experienced, viraemic ( <i>n</i> = 20)	Treatment experienced, non-viraemic ( <i>n</i> = 17)
Age (yr)				
Mean (IQR)	46 (36-54)	42 (32-53)	48 (41-57)	55 (44-60)
Gender <i>n</i> (%)				
Male	64 (69.6)	39 (70.9)	11 (55)	14 (82.4)
Female	28 (30.4)	16 (29.1)	9 (45)	3 (17.6)
Ethnic origin <i>n</i> (%)				
African	4 (4.3)	3 (5.5)	0	1 (5.9)
Asian	77 (83.7)	48 (87.3)	16 (80)	13 (76.5)
Caucasian	5 (5.4)	3 (5.5)	0	2 (11.8)
Mediterranean	2 (2.2)	1 (1.8)	1 (5)	0
Middle Eastern	1 (1.1)	0	1 (5)	0
Duration of therapy (mo)				
Median (IQR)	24 (6-42)	24 (12-36)	24 (6-54)	24 (12-42)
HBe antigen status <i>n</i> (%)				
HBeAg positive	30 (32.6)	19 (34.5)	10 (50)	1 (5.9)
HBeAg negative	62 (67.4)	36 (65.5)	10 (50)	16 (94.1)
Treatment history <i>n</i> (%)				
Experienced	37 (40.2)	0	20 (100)	17 (100)
Adefovir	10 (27)		4 (20)	6 (35.3)
Adefovir/lamivudine	13 (35.1)		6 (30)	7 (41.2)
Lamivudine	6 (16.2)		4 (20)	2 (11.8)
Lamivudine/entecavir	1 (2.7)		1 (5)	0
Entecavir	5 (13.5)		4 (20)	1 (5.9)
Entecavir/adefovair	2 (5.4)		1 (5)	1 (5.9)
Naïve	55 (59.8)	55 (100)	0	0
HBV DNA load (IU/mL) <i>n</i> (%)				
< 20	17	-	-	17 (100)
20-2000	29 (31.5)	6 (10.9)	6 (30)	0
2000-100000	11 (12)	8 (14.5)	3 (15)	0
> 100000	52 (56.5)	41 (74.5)	11 (55)	0
Median (IQR)	1.8 × 10 <sup>5</sup> (302-1.6 × 10 <sup>7</sup> )	9.4 × 10 <sup>5</sup> (9.7 × 10 <sup>4</sup> -3.7 × 10 <sup>7</sup> )	1.8 × 10 <sup>5</sup> (790-4.1 × 10 <sup>6</sup> )	N/A
ALT (U/L) <i>n</i> (%)				
0-20	11 (12)	2 (3.6)	4 (20)	5 (29.4)
20-40	26 (28.3)	10 (18.2)	8 (40)	8 (47.1)
40-400	51 (55.4)	39 (70.9)	8 (40)	4 (23.5)
> 400	4 (4.3)	4 (7.3)	0	0
Median (IQR)	30 (22-41.8)	73 (41-140)	34 (22.3-62.3)	24 (19-44)
Serum creatinine (IU/mL)				
Median (IQR)	70 (60-81.5)	66.5 (50.8-71.5)	71 (63.5-84.5)	83 (69-93)
Pre-treatment biopsy <i>n</i> (%)	69 (75)	40 (72.7)	16 (80)	14 (82.4)
Fibrosis score <i>n</i> (%)				
0	9 (9.8)	5 (9.1)	3 (15)	3 (17.6)
1	31 (33.7)	21 (38.2)	6 (30)	4 (23.5)
2	13 (14.1)	8 (14.5)	3 (15)	2 (11.8)
3	7 (7.6)	1 (1.8)	2 (10)	4 (23.5)
4	9 (9.8)	5 (9.1)	2 (10)	2 (11.8)
Genotype <i>n</i> (%)	35 (38)	6 (10.9)	9 (45)	12 (70.6)
A	3 (3.3)	2 (3.6)	0	1 (5.9)
B	7 (7.6)	2 (3.6)	1 (5)	4 (23.5)
C	13 (14.1)	2 (3.6)	7 (35)	4 (23.5)
D	3 (3.3)	0	0	2 (11.8)

"Treatment experience" refers to previous NA therapy. "Viraemia" refers to HBV DNA > 20 IU/mL. Liver biopsy was scored using the METAVIR scoring system. HBe: Hepatitis B "e"; HBV DNA: Hepatitis B viral deoxyribonucleic acid; ALT: Alanine aminotransferase; N/A: Not applicable.

at the time TDF therapy was commenced. Seventeen treatment-experienced patients had a baseline HBV DNA level less than the lower limit of detection, and had directly switched to TDF for convenience. Nity-seven percent of patients received TDF monotherapy. Median duration of follow-up was 24 mo (6-42 mo).

### Virological outcomes

Virological response to TDF is detailed in Table 2 and

Figure 1. Overall, 77 (83.7%) patients achieved complete virological suppression by the end-of-follow-up, with a median time to suppression of 6 mo (IQR = 3-12 mo). The rates of complete virological suppression were 71% (45/65) at 12 mo, 80% (37/46) at 24 mo and 89% (25/28) at 36 mo. Eighty-nine/ninety-two (96.7%) achieved a partial virological response (HBV DNA 20-2000 IU/mL).

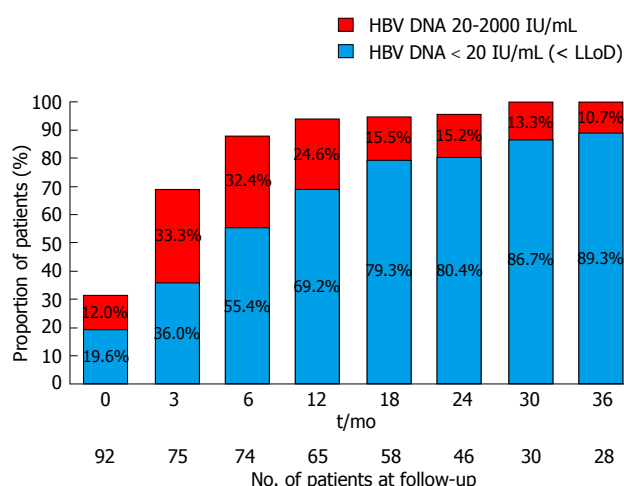
**Treatment-naïve individuals:** Complete virological



**Table 2** Virological suppression at on-treatment time-points (*n* = 92)

Follow-up (mo)	0	6	12	18	24	30	36
Patients with viral load <i>n</i> (%)	92 (100)	74 (80.4)	65 (70.7)	58 (63)	46 (50)	30 (32.6)	28 (30.4)
Virological suppression <i>n</i> (%)	18 (19.6)	41 (55.4)	45 (69.2)	46 (79.3)	37 (80.4)	26 (86.7)	25 (89.3)

"Patients with viral load" refers to the number of patients at each time point who had an available HBV DNA reading. "Virological suppression" refers to the number of patients with HBV DNA < 20 IU/mL. HBV: Hepatitis B virus.



**Figure 1** Complete virological suppression and partial virological suppression at on treatment time points. The proportion of patients who achieve complete virological suppression (HBV DNA < 20 IU/mL) or partial virological suppression (HBV DNA 20-2000 IU/mL) while on tenofovir therapy. The number of patients followed up at each time point is recorded below the Time axis. HBV: Hepatitis B virus.

suppression was achieved in 43/55 (78%) of patients with a median time to suppression of 6 mo (IQR = 3-12 mo). Rates of complete virological suppression were 70% (29/44) at 12 mo, 87% (26/30) at 24 mo and 100% at 36 mo (18/18). This was maintained by 50/55 (91%) of patients throughout follow-up. While a total of five patients failed to maintain complete virological suppression, only three patients experienced VBT. This was associated with reported non-compliance. In the first patient, HBV DNA levels rose from undetectable viral load at 12 mo to 55 IU/mL at 18 mo and 23 IU/mL at 24 mo. In the second patient, HBV DNA levels rose from undetectable at 18 mo to 1940 IU/mL at 24 mo and 578 IU/mL at 30 mo. In the final patient, HBV DNA levels increased from undetectable at 24 mo to 46300 IU/mL at 42 mo and 29 IU/mL at 48 mo (results for the intervening 12 mo were unavailable). A transient low level viraemia not meeting the definition for VBT (single HBV DNA level of 23 IU/mL and 21 IU/mL, respectively, following achievement of complete virological suppression) was observed in two additional patients before returning to undetectable levels.

**Treatment-experienced individuals:** Viraemia was seen in 54% (20/37) of treatment-experienced individuals at the time TDF therapy was commenced. Complete virological suppression was achieved among 85% (17/20) of viraemic patients with a median time to

suppression of 6 mo (IQR = 3-18 mo). Rates of complete virological suppression were 64% (9/14) at 12 mo, 58% (7/12) at 24 mo and 63% (5/8) at 36 mo. While 3 patients showed persistent viraemia at 36 mo, all had an HBV DNA level < 2000 IU/mL and subsequently achieved complete virological suppression by 60 mo. This was maintained in 17/20 (85%) patients throughout follow-up. No patient met the strict definition for virological breakthrough. Two patients demonstrated a single instance of HBV DNA > 20 IU/mL (28 IU/mL and 27 IU/mL) before returning to undetectable levels, but did not meet the criteria for VBT. Among patients with an undetectable plasma HBV DNA level at baseline, 16/17 patients (94%) maintained complete virological suppression throughout follow-up. One patient experienced a single HBV DNA level of 40 IU/mL.

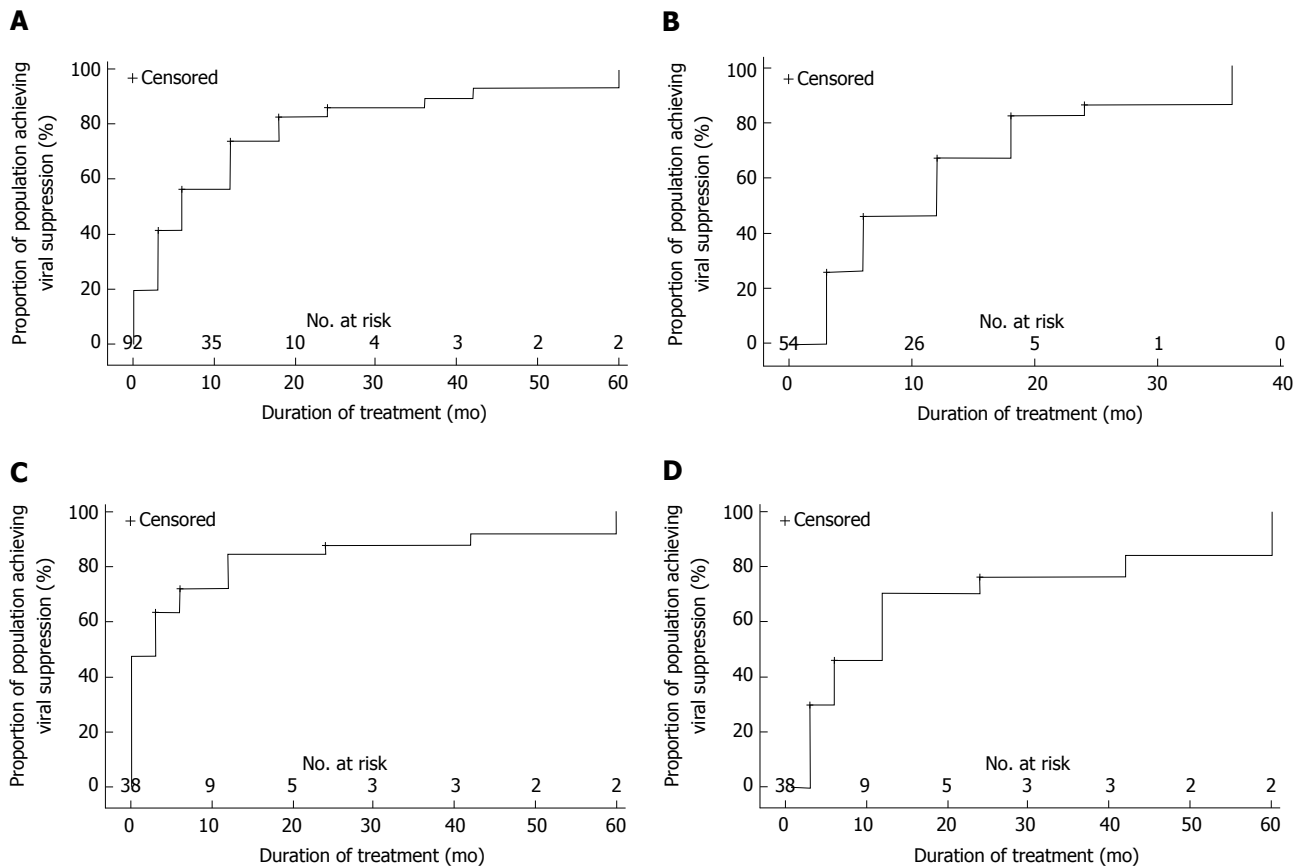
### Predictors of virological outcome

Survival analysis of the influence of treatment experience on complete virological suppression is presented in Figure 2. Cox proportional hazards analysis was carried out on viraemic patients at baseline with the final model including baseline HBV DNA, HBeAg status treatment experience, age and baseline ALT (Table 3). Multivariate analysis showed a significant relationship between virological suppression at end of follow-up and baseline HBV DNA (OR = 0.897, 95%CI: 0.833-0.967, *P* = 0.0046) and HBeAg status (HR = 0.373, 95%CI: 0.183-0.762, *P* = 0.0069).

### Serological outcomes

**HBeAg loss/seroconversion:** Among 30 HBeAg-positive patients at baseline, 5 (16.7%) underwent HBeAg loss and seroconversion. Median time to seroconversion was 30 mo (9-60 mo). Mean age was 38 years (24-48 mo) and median baseline HBV DNA was  $1.7 \times 10^7$  IU/mL. There was no significant difference in HBeAg seroconversion rates between treatment-naïve and treatment-experienced patients (*P* = 0.87). Two patients showed documented HBeAg seroreversion while on TDF treatment. One patient who was HBeAg-negative at baseline underwent HBeAg seroreversion at 24 mo of TDF therapy. HBeAg seroconversion then reoccurred at 36 mo and was sustained for the remainder of follow-up. The second case showed HBeAg loss without seroconversion at 12 mo, followed by seroreversion at 36 mo of treatment. This patient had only been on therapy for 36 mo at end of follow-up.

**HBsAg:** One treatment-naïve male underwent HBsAg loss and seroconversion following 12 mo of TDF therapy.



**Figure 2** Survival analysis of the influence of treatment experience on complete virological suppression. A: Time to virological suppression according to duration of treatment,  $n = 92$ ; B: Amongst treatment naïve patients  $n = 54$ ; C: Treatment experienced patients,  $n = 37$ ; D: Treatment experienced viraemic patients,  $n = 20$ . Number at risk describes the number of target group patients captured at each time period.

**Table 3** Cox regression model of predictors of end of follow-up virological suppression ( $n = 74^1$ )

Covariates	Multivariable	
	Hazard ratio (95%CI)	P value
Baseline HBV DNA ( $\log_{10}$ IU/mL)	0.897 (0.833-0.967)	0.0046
HBeAg status (HBeAg pos vs neg)	0.373 (0.183-0.762)	0.0069
Treatment experience (Naïve vs experienced)	1.189 (0.598-2.364)	0.6207
Age (yr)	1.018 (0.992-1.044)	0.1760
ALT ( $\log_{10}$ IU/mL)	1.093 (0.816-1.465)	0.5505

<sup>1</sup>Excludes patients who were not viraemic at baseline. HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

This individual was 28 years of age and HBeAg-negative at the time TDF was started.

### Biochemical outcomes

Mean ALT at baseline was  $134 \pm 340$  U/mL and  $33 \pm 13$  U/mL at end of follow-up, with a mean change of  $-101.3 \pm 340.4$  U/mL. Treatment experienced patients had a lower mean baseline ALT than treatment naïve patients ( $52 \pm 70.2$  U/mL vs  $190 \pm 431.1$  U/mL,  $P = 0.02$ ). They consequently had a lower mean change in ALT at the end of follow-up ( $-21 \pm 68$  U/mL vs  $-155 \pm 432$  U/mL,  $P = 0.28$ ). Baseline serum ALT levels were within the

normal range in 42/92 (45.7%) patients. By the end of treatment, 76/92 (83%) patients were within the normal range. Of the 50 patients who were above the ULN at baseline, 38 (76%) achieved ALT normalisation by the end of follow-up.

### Clinical outcomes

Hepatocellular cancer was diagnosed in two patients within 12 mo of starting TDF treatment. Both patients were diagnosed with cirrhosis prior to commencing TDF and one patient died as a result of their malignancy. A third patient was diagnosed with HCC 12 mo after ceasing TDF. No episodes of hepatic decompensation were recorded in the study population.

### Treatment discontinuation and safety

Treatment was discontinued at the discretion of individual clinicians in 11 patients (12%). Treatment was discontinued as a result of a rise in serum creatinine levels in 3 patients (3%). All 3 patients had a peak serum creatinine  $< 1.5 \times$  ULN. Two patients had only been taking TDF for 3 mo, and both had previously been treated with long-term LMV plus ADV therapy. Creatinine returned to the normal range on switch to ETV in one patient and LMV plus ADV in the other. The clinical decision to return the latter patient to LMV plus

ADV was determined by the treating physician and is not a standard treatment recommendation. One treatment-naïve patient was noted to have a rising serum creatinine at month 42 of treatment (peak creatinine = 118  $\mu\text{mol/L}$ , ULN = 104). Serum phosphate levels were normal. Treatment was switched to ETV and creatinine returned to the normal range. Bone mineral density measurements were not routine and were only performed in a minority. There were 4 patients who were noted to have osteopenia or osteoporosis after treatment durations of 18–42 mo. Two of these patients were treatment naïve at the time TDF was started, one patient had previously been treated with adefovir for 5 years, and one patient had previously received LMV for 4 years. None of the 4 patients had a baseline bone mineral density measure available for comparison. Tenofovir was discontinued in another 4 patients after reports of non-specific adverse events including nausea, dizziness, fatigue, weight loss and myalgia.

## DISCUSSION

Tenofovir is a potent antiviral therapy for CHB. It has been associated with high rates of virological suppression in clinical trials and virological resistance is yet to be described in clinical practice<sup>[13]</sup>. Post-registration real-world studies provide confirmation of therapy efficacy outside of the selected clinical trial situation, and monitor for rare adverse events. This is the first real-life study of TDF in an Australian setting. It validates the efficacy and safety of TDF in NA-naïve and experienced patients with CHB.

Similarly to registration trials and real-life studies, the study population was predominantly male and HBeAg negative, with 75% over the age of 40. However, while registration trials studied predominantly Caucasian populations, this population was mostly of Asian origin. Other ethnic minorities were also represented, reflecting Australian migration patterns. HBV genotype data were available for a minority of patients (35/92). Genotypes C and B were the most common genotypes, with A and D also represented. Studies from Europe and Asia are dominated by genotype A/D (Europe) and genotype B/C (Asia), limiting cross genotype comparisons. The tenofovir registration studies included mainly Western genotype A/D individuals, as have most of the real world data<sup>[13,22,23]</sup>. While this study's patient size may be limited, the population studied here are unique for the breadth of ethnicity and HBV genotypes and comprise the first dataset described in an Australian population. Liver fibrosis ranged predominately between stages 1 and 2, with 10% of patients diagnosed with cirrhosis at baseline. Forty percent of the population were NA treatment-experienced.

The efficacy of TDF therapy in our cohort largely reflects the clinical trial experience. A daily dose of 300 mg of TDF was found to achieve at least partial virological suppression in 97% of patients and complete virological suppression in 84% of patients, demonstrating

robust efficacy. Complete virological suppression was sustained by 94% of patients over time. Patients with persistent viraemia had HBV DNA levels < 2080 IU/mL, except for two patients who had a viral load  $1.2\text{--}2.6 \times 10^5$  IU/mL after 3 mo on therapy. Virological breakthrough was only observed in one patient with documented non-compliance. The clinical variables that were independently associated with time to suppression were high HBV DNA level at baseline, and HBeAg seropositivity. Previous NA therapy was not associated with reduced response rate. HBeAg seroconversion was achieved in 17% of HBeAg positive patients, with median duration of follow-up of 24 mo. One patient underwent HBsAg loss and seroconversion after 12 mo of treatment. The efficacy data are therefore broadly consistent with the experience in the registration studies<sup>[13,22,24]</sup>.

Our findings are also in keeping with "real life" international studies. Pol *et al.*<sup>[23]</sup> reviewed safety and efficacy data from two real-life cohorts in the United Kingdom and Europe. The cohorts had a combined sample size of 362 NA-naïve patients with a median follow-up of 9–28 mo. Virological suppression was achieved in 80%–89% of patients with breakthrough identified in 2% of patients, without any corresponding resistance mutations. HBeAg seroconversion occurred in 7%–18% of patients and HBsAg loss occurred in 2% of the European cohort. Eighty-seven percent of patients achieved ALT normalisation by 30 wk<sup>[5]</sup>. Pan *et al.*<sup>[21]</sup> analysed the real-life safety and efficacy of TDF in 90 Asian-American patients over 48 wk. Ten percent of the population had a history of prior treatment with lamivudine or adefovir. Virological suppression was achieved in 82% of patients, 12% of patients underwent HBeAg seroconversion and 66% of patients showed ALT normalisation by the end of follow-up. No resistance to TDF was detected and the treatment was considered well-tolerated with few related adverse events. While our results reflect those of other "real life" data, few studies have included treatment-experienced patients and if so they compose only a small minority. This is an area for future focus considering clinical practice of switching patients over to TDF from older less effective NAs.

Therapy was ceased in 12% of patients at the discretion of individual clinicians due to concern about renal (3%) and bone impairment. Tenofovir was self-ceased by 4% of patients due to non-specific adverse events. It was not possible to establish causality for these events; all possible renal events were mild and reversible with discontinuation. No confirmed cases of proximal tubular dysfunction were observed. Isolated cases of osteomalacia and osteopenia concurrent with TDF therapy have also been reported in the HBV literature<sup>[25]</sup>; there are more numerous reports in the HIV literature. In our cohort, although cases of osteopaenia and osteoporosis were noted, the absence of baseline bone mineral density scans meant that causality could not be speculated. Chronic liver disease itself is a risk factor for osteoporosis. We now perform routine monitoring of renal function and fasting serum phosphate levels every

six months, as well as bone mineral densitometry at baseline and every 3 years to screen for osteoporosis. This approach needs prospective validation.

In conclusion, our Australian experience shows TDF to be an effective and safe therapy for patients with CHB. Rates of sustained virological suppression were very high. Elevated baseline HBV DNA level and HBeAg-positive disease were associated with slower time to suppression, but TDF resistance was not observed, and most patients achieved complete virological suppression with continued therapy. Tenofovir was generally well tolerated. This study supports the findings of other real-life experience into the efficacy and safety of TDF in the treatment of CHB.

## COMMENTS

### Background

Chronic hepatitis B (CHB) affects 240-400 million people around the world. It is estimated that 218000 people in Australia live with CHB, a population prevalence of approximately 1%. CHB is associated with the long-term complications of cirrhosis, liver failure and hepatocellular carcinoma (HCC), in 15%-40% of patients. CHB is one of the most common causes of HCC, the fastest increasing cause of cancer death in Australia.

### Research frontiers

Tenofovir is a nucleotide analogue recommended as first-line treatment for CHB. The safety and efficacy of tenofovir disoproxil fumarate (TDF) for the treatment of chronic HBV infection has been confirmed in two phase-III clinical trials. There are limited data that describe the safety and efficacy of TDF in the "real world". The few studies that have been published describe populations in Europe and North America. There have been no reports of the experience with TDF in Australia.

### Innovations and breakthroughs

Out of 92 patients, 89 (96.7%) achieved partial virological response and 77 (83.7%) achieved complete virological suppression by the end-of-follow-up. Predictors of virological suppression included lower baseline HBV DNA and HBeAg negative disease.

### Applications

The authors' Australian experience shows that TDF is an effective and safe therapy for patients with CHB. Rates of sustained virological suppression were very high and most patients achieved complete virological suppression with continued therapy. TDF resistance was not observed and treatment was generally well tolerated. This study supports the findings of other real-life experience into the efficacy and safety of TDF in the treatment of CHB.

### Terminology

The hepatitis B virus (HBV) is transmitted vertically, parenterally or *via* mucosal exposure to infected blood or bodily fluids. CHB is associated with long-term complications of cirrhosis, liver failure and hepatocellular carcinoma. They carry high rates of morbidity and mortality and affect 15%-40% of patients at some point in their life. Tenofovir disoproxil fumarate is a nucleotide analogue used in the treatment of CHB. Prior to its role in CHB, TDF was used in the treatment of HIV type 1 infection.

### Peer-review

This is a well-designed and well-written real life data study of tenofovir treatment for hepatitis B.

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Retrospective Study

## Clinical usefulness of ursodeoxycholic acid for Japanese patients with autoimmune hepatitis

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## Abstract

### AIM

To evaluate the therapeutic effects of ursodeoxycholic acid (UDCA) on autoimmune hepatitis (AIH).

### METHODS

A total 136 patients who were diagnosed with AIH were included in our study. All of the patients underwent a liver biopsy, and had at least a probable diagnosis on the basis of either the revised scoring system or the simplified scores. Initial treatment included UDCA monotherapy (Group U,  $n = 48$ ) and prednisolone (PSL) monotherapy (Group P,  $n = 88$ ). Group U was further classified into two subgroups according to the effect of UDCA: Patients who had achieved remission induction with UDCA monotherapy and showed no sign of relapse (Subgroup U1,  $n = 34$ ) and patients who additionally received PSL during follow-up (Subgroup U2,  $n = 14$ ). We compared the clinical and histological findings between each groups, and investigated factors

contributing to the response to UDCA monotherapy.

## RESULTS

In Group U, 34 patients (71%) achieved and maintained remission over 49 (range: 8-90) mo (Subgroup U1) and 14 patients (29%) additionally received PSL (Subgroup U2) during follow-up. Two patients in Subgroup U2 achieved remission induction once but additionally required PSL administration because of relapse (15 and 35 mo after the start of treatment). The remaining 12 patients in Subgroup U2 failed to achieve remission induction during follow-up, and PSL was added during 7 (range: 2-18) mo. Compared with Subgroup U2, Subgroup U1 had significantly lower alanine aminotransferase (ALT) levels at onset (124 IU/L *vs* 262 IU/L,  $P = 0.023$ ) and a significantly higher proportion of patients with mild inflammation (A1) on histological examination (70.6% *vs* 35.7%,  $P = 0.025$ ). When multivariate analysis was performed to identify factors contributing to the response to UDCA monotherapy, only a serum ALT level of 200 IU/L or lower was found to be associated with a significant difference ( $P = 0.013$ ).

## CONCLUSION

To prevent adverse events related to corticosteroids, UDCA monotherapy for AIH needs to be considered in patients with a serum ALT level of 200 IU/L or lower.

**Key words:** Autoimmune hepatitis; Japanese patients; Adverse events; Corticosteroids; Ursodeoxycholic acid

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**Core tip:** Autoimmune hepatitis (AIH) is generally responsive to immunosuppressive treatment, and corticosteroids are commonly used for the initial and maintenance treatments. However, corticosteroid treatment must be discontinued in some patients because of several side effects. This study aimed to evaluate the therapeutic effects of ursodeoxycholic acid (UDCA), which has high tolerability and no severe side effects, on AIH. Our results suggest that to prevent adverse events related to corticosteroids, treatment with UDCA alone for AIH needs to be considered in selected patients, especially those with an alanine aminotransferase level of 200 IU/L or lower. This utility of UDCA must be confirmed in a prospective study.

Torisu Y, Nakano M, Takano K, Nakagawa R, Saeki C, Hokari A, Ishikawa T, Saruta M, Zeniya M. Clinical usefulness of ursodeoxycholic acid for Japanese patients with autoimmune hepatitis. *World J Hepatol* 2017; 9(1): 57-63 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i1/57.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i1.57>

## INTRODUCTION

Autoimmune hepatitis (AIH) is an unresolving pro-

gressive liver disease that affects females preferentially and is characterized by interface hepatitis, hypergammaglobulinemia, circulating autoantibodies, and a favorable response to immunosuppression. The aim of treatment in AIH is to obtain complete remission of the disease and to prevent further progression of liver disease, which generally requires permanent maintenance therapy. Corticosteroids have been widely used as the first choice drug treatment of AIH<sup>[1,2]</sup>. However, long-term treatment with a generous corticosteroid dosage may induce predictable side effects, such as cosmetic changes (facial rounding, dorsal hump formation, striae, weight gain, acne, alopecia, and facial hirsutism) or even more dreadful complications, such as osteopenia, brittle diabetes, psychosis, pancreatitis, opportunistic infections, labile hypertension, and malignancy<sup>[3-7]</sup>. Consequently, corticosteroid treatment must be discontinued in 13% of patients. Of those withdrawn from therapy, most have intolerable cosmetic changes or obesity (47%), osteoporosis with vertebral compression (27%), and/or difficult-to-control diabetes (20%)<sup>[4,8]</sup>. Because AIH predominantly affects middle-aged women, the presence of cosmetic issues is one of the key factors for maintaining drug compliance. Cosmetic issues may lead to emotional problems that result in treatment failure and a poor prognosis. Thus, a strategy to reduce the adverse effects of corticosteroid treatment is needed.

Ursodeoxycholic acid (UDCA) has been widely used as the first choice for treating primary biliary cirrhosis (PBC) and has been established as clinically useful<sup>[9-11]</sup>. No severe side effects have been reported during UDCA therapy for PBC<sup>[12]</sup>. Although there are reports that UDCA is also useful for treating similar autoimmune liver diseases, its clinical value has not as yet been established<sup>[13-15]</sup>. In this study, patients with a confirmed diagnosis of AIH who started treatment with UDCA alone were analyzed, and the results are reported.

## MATERIALS AND METHODS

### Patients

The present study included 136 patients who were diagnosed with AIH between 1975 and 2011 at the Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University Hospital (Tokyo, Japan). All of the patients had at least a probable diagnosis on the basis of either the revised scoring system, as proposed by the International Autoimmune Hepatitis Group in 1999<sup>[16]</sup>, or the simplified scores<sup>[17]</sup>. All of the patients underwent a liver biopsy. In this study, patients with no histological fibrosis (F0) were excluded. Chronic viral hepatitis B and C were excluded by serological testing in all of the patients. Patients with an overlapping syndrome or a coexistent liver disease (e.g., primary biliary cirrhosis, primary sclerosing cholangitis, nonalcoholic fatty liver disease, or alcohol-induced liver injury) were also excluded by medical history, serological data and histological finding. So, patients with positive antimitochondrial antibody were excluded. Of the 136

**Table 1 Clinical features of the two groups classified according to the therapeutic agent**

	Group U (n = 48)	Group P (n = 88)	P
Age (yr)	45 (17-74)	51 (15-78)	ns
Sex (female)	45 (93.8%)	65 (73.9%)	< 0.01
Acute presentation	5 (10.4%)	31 (36.5%)	< 0.01
Laboratory data			
AST (IU/L)	104 (46-1234)	303 (31-2215)	< 0.001
ALT (IU/L)	149 (52-1000)	431 (38-2801)	< 0.001
T.Bil (mg/dL)	0.8 (0.3-18)	1.3 (0.4-19.3)	< 0.05
ALP (U/L)	300 (144-1184)	369 (145-4420)	ns
$\gamma$ -GTP (U/L)	82 (13-875)	183 (12-1256)	< 0.05
IgG (mg/dL)	1954 (1096-3793)	2336 (1051-5776)	< 0.01
ANA ( $\geq 1:40$ )	46 (95.8%)	83 (94.3%)	ns
SMA ( $\geq 1:40$ )	11/23 (47.8%)	35/45 (77.8%)	< 0.05
HLA DR4	6/16 (37.5%)	33/57 (57.9%)	ns
Histological finding			
Grading			
A1	29 (60.4%)	25 (28.4%)	< 0.01
A2	18 (37.5%)	44 (50%)	
A3	1 (2.1%)	19 (21.6%)	
Staging			
F1	35 (72.9%)	43 (48.9%)	< 0.05
F2	6 (12.5%)	28 (31.8%)	
F3	6 (12.5%)	10 (11.4%)	
F4	1 (2.1%)	7 (8.0%)	
AIH score			
Revised score	15 (10-20)	16 (7-23)	ns
Simplified score	6 (4-8)	6 (3-8)	ns

Continuous variables are expressed as median (range) values. The Mann-Whitney *U* test was used to evaluate differences in continuous variables between two groups. Dichotomous variables were compared by Pearson's  $\chi^2$  test. Values of  $P < 0.05$  were considered significant. ALT: Alanine aminotransferase; AIH: Autoimmune hepatitis; AST: Aspartate transaminase; HLA: Human leukocyte antigen; ANA: Antinuclear antibody; ALP: Alkaline phosphatase;  $\gamma$ -GTP:  $\gamma$ -glutamyltransferase; SMA: Smooth muscle antibody; ns: No significant difference; T.Bil: Total bilirubin; IgG: Immunoglobulin G.

patients, 48 received UDCA (Group U) after diagnosis, and the remaining 88 received prednisolone (PSL) (Group P). Furthermore, Group U was divided into the following subgroups: Subgroup A, consisting of 33 patients with a serum alanine aminotransferase (ALT) level of 200 IU/L or lower at the start of treatment; Subgroup B, consisting of 29 patients in whom histological activity on liver biopsy before the start of treatment was determined to be A1 on the basis of the classification of Desmet *et al.*<sup>[18]</sup>; Subgroup C, consisting of 24 patients who were included in both Subgroups A and B; Subgroup D consisting of 15 patients with a serum ALT level of 200 IU/L or higher at the start of treatment; Subgroup E consisting of 19 patients in whom histological activity was A2 or A3 before the start of treatment; and Subgroup F consisting of 10 patients who were included in both Subgroups D and E. The clinical characteristics of the study subjects are presented in Table 1.

In each group and subgroup described above, subsequent clinical courses, changes in treatment, and histological findings at the time of diagnosis were evaluated. Moreover, Group U was divided into Subgroup U1, consisting of patients who had achieved remission

**Table 2 Clinical features of the two subgroups classified according to the effect of ursodeoxycholic acid**

	Subgroup U1 <sup>1</sup> (n = 34)	Subgroup U2 <sup>2</sup> (n = 14)	P
Age (yr)	42 (17-74)	48 (21-66)	ns
Sex (female)	33 (97.1%)	12 (85.7%)	ns
Acute presentation	4 (11.8%)	1 (7.1%)	ns
Laboratory data			
AST (IU/L)	93 (46-505)	144 (50-1234)	0.024
ALT (IU/L)	124 (52-742)	262 (65-1000)	0.023
T.Bil (mg/dL)	0.7 (0.3-18)	1.0 (0.3-2)	ns
ALP (U/L)	300 (144-1184)	300 (168-924)	ns
$\gamma$ -GTP (U/L)	86 (16-875)	67 (13-405)	ns
IgG (mg/dL)	1959 (1096-3800)	1960 (1476-3793)	ns
ANA ( $\geq 1:40$ )	32 (94.1%)	14 (100%)	ns
SMA ( $\geq 1:40$ )	8/17 (47.1%)	3/6 (50%)	ns
HLA DR4	4/11 (36.4%)	2/5 (40%)	ns
Histological finding			
Grading			
A1	24 (70.6%)	5 (35.7%)	0.025
A2	10 (29.4%)	8 (57.1%)	
A3	0 (0%)	1 (7.1%)	
Staging			
F1	25 (73.5%)	10 (71.4%)	ns
F2	4 (11.8%)	2 (14.3%)	
F3	5 (14.7%)	1 (7.1%)	
F4	0 (0%)	1 (7.1%)	
AIH score			
Revised score	15 (10-19)	17 (12-20)	ns
Simplified score	6 (4-8)	6 (6-7)	ns

<sup>1</sup>Subgroup U1, normalized ALT and sustained remission; <sup>2</sup>Subgroup U2, non-normalized ALT or relapse. Continuous variables are expressed as median (range) values. The Mann-Whitney *U* test was used to evaluate differences in continuous variables between two groups. Dichotomous variables were compared by Pearson's  $\chi^2$  test. Values of  $P < 0.05$  were considered significant. ALT: Alanine aminotransferase; AIH: Autoimmune hepatitis; AST: Aspartate transaminase; HLA: Human leukocyte antigen; ANA: Antinuclear antibody; ALP: Alkaline phosphatase;  $\gamma$ -GTP:  $\gamma$ -glutamyltransferase; SMA: Smooth muscle antibody; T.Bil: Total bilirubin; IgG: Immunoglobulin G; ns: No significant difference.

induction with UDCA monotherapy and showed no sign of relapse, and Subgroup U2, consisting of patients who additionally received PSL during follow-up. Laboratory test results and histopathological findings at the time of diagnosis were compared between Subgroups U1 and U2 (Table 2).

This study complied with the standards of the Declaration of Helsinki and the current ethical guidelines, and was approved by the institutional ethics board. Written, informed consent for participation in this study was not obtained from the patients, because this study did not report on a clinical trial and the data were retrospective in nature and analyzed anonymously.

### Treatment

PSL was used as the standard initial treatment. Taking into account body weight, the initial dose was set between 30 and 40 mg/d, with subsequent reduction after improvement in liver function had been confirmed.

In mild clinical cases with both histological low-grade inflammatory activity and adequate residual capacity of



liver function, the initial treatment was UDCA alone. The initial dose of UDCA was set at 600 mg/d (10–13 mg/kg per day) in accordance with Japanese guideline for the treatment of PBC. The dosage was neither increased nor decreased during the treatment period. PSL was also administered, as described above, when an incomplete response to UDCA monotherapy or relapse was observed.

### Follow-up

Each patient underwent a comprehensive clinical review and physical examination at each follow-up visit. Conventional laboratory blood tests were performed every 1–3 mo.

### Criteria for the remission and relapse of AIH

Remission was defined as a normalization of serum ALT levels after the start of treatment. The judgement of remission for UDCA monotherapy was carried out within at least 18 mo after initiation of therapy. Relapse was defined as an increase in serum ALT levels to more than twice the upper normal limit following the normalization of serum ALT levels with medical treatment.

### Statistical analysis

Statistical analysis was performed using the SPSS statistical program (release 16.0.1 J, SPSS, Inc., Chicago, IL). Continuous variables are expressed as medians and ranges. The Mann-Whitney *U* test was used to evaluate differences in continuous variables between two groups. Dichotomous variables were compared by Pearson's  $\chi^2$  test. Multivariate analyses by logistic regression were used to identify independent factors contributing to the response to UDCA monotherapy. Values of  $P < 0.05$  were considered significant.

## RESULTS

### Comparison of clinical features among two groups classified according to initial treatment

As the initial treatment, of the 136 patients, 48 received UDCA monotherapy (Group U) and 88 received PSL monotherapy (Group P). There were no differences between Groups U and P in age, serum levels of alkaline phosphatase, the frequencies of positivity for antinuclear antibody or human leukocyte antigen DR4, and scores derived from either the old or the new scoring system. However, compared with Group P, Group U had significantly lower serum levels of aspartate transaminase (AST) (104 IU/L vs 303 IU/L,  $P < 0.001$ ), ALT (149 IU/L vs 431 IU/L,  $P < 0.001$ ), total bilirubin (0.8 mg/dL vs 1.3 mg/dL,  $P < 0.05$ ),  $\gamma$ -glutamyltransferase (82 U/L vs 182 U/L,  $P < 0.05$ ), and immunoglobulin G (1954 mg/dL vs 2336 mg/dL,  $P < 0.01$ ), and lower frequencies of male sex, acute presentation, and positivity for smooth muscle antibody at the onset. Additionally, Group U had a significantly higher proportion of patients with mild inflammation and fibrosis (A1 and F1) on histological examination (28.4% vs 60.4%,  $P < 0.01$ , and 48.9% vs

72.9%,  $P < 0.05$ ) (Table 1). Cumulative incidence of the normalization of serum ALT levels was 80% in Group P.

### UDCA monotherapy as initial treatment

The follow-up durations were 49 (range: 8–156) mo in Group U. In Group U, 34 patients (71%) achieved and maintained remission over 49 (range = 8–90) mo (Subgroup U1), and 14 patients (29%) additionally received PSL during follow-up (Subgroup U2). Two patients in Subgroup U2 achieved remission induction once but additionally required PSL administration because of relapse (15 and 35 mo after the start of treatment). The remaining 12 patients in Subgroup U2 failed to achieve remission induction during follow-up, and PSL was added during 7 (range: 2–18) mo.

### Comparison of clinical features among two subgroups classified according to the effect of UDCA

The rate of numbers was 73% in Subgroup U1 and 27% in Subgroup U2. Compared with Subgroup U2, Subgroup U1 had significantly lower ALT levels at onset (124 IU/L vs 262 IU/L,  $P = 0.023$ ) and a significantly higher proportion of patients with mild inflammation (A1) on histological examination (70.6% vs 35.7%,  $P = 0.025$ ) (Table 2). However, there were no differences between Subgroups U1 and U2 in other clinical features, as shown in Table 2.

### Predictive factors associated with normalized ALT and sustained remission with UDCA monotherapy in AIH patients

When multivariate analysis was performed to identify factors contributing to the response to UDCA monotherapy, a serum ALT level of 200 IU/L or lower was found to be associated with a significant difference (Table 3).

On subgroup analysis, remission was induced and maintained by UDCA in 85%, 83% and 92% of patients in Subgroups A, B, and C, respectively. In these subgroups, high rates of remission induction and successful maintenance were achieved by UDCA. On the other hand, the rates of remission induction and successful maintenance in Subgroups D, E and F were low, at 40%, 53%, and 50%, respectively (Figure 1).

## DISCUSSION

UDCA has been widely used as the first choice drug for the treatment of PBC<sup>[9–11]</sup>. This is because of its efficacy for cholestasis, exerted through its choleretic action which is well understood<sup>[19]</sup>. In addition to its choleretic action, UDCA reportedly has a protective action on hepatocytes and an immunomodulatory action<sup>[20]</sup>. In fact, it has also been reported that the administration of UDCA reduces elevated serum immunoglobulin levels in patients with PBC, which is one of the clinical characteristics of PBC<sup>[9,21]</sup>. *In vitro* studies have also shown that UDCA inhibits immunoglobulin production by peripheral lymphocytes in a concentration-dependent manner<sup>[22]</sup>. Although the

**Table 3** Multivariate logistic regression analysis of factors associated with normalized alanine aminotransferase and sustained remission of ursodeoxycholic acid monotherapy in autoimmune hepatitis patients

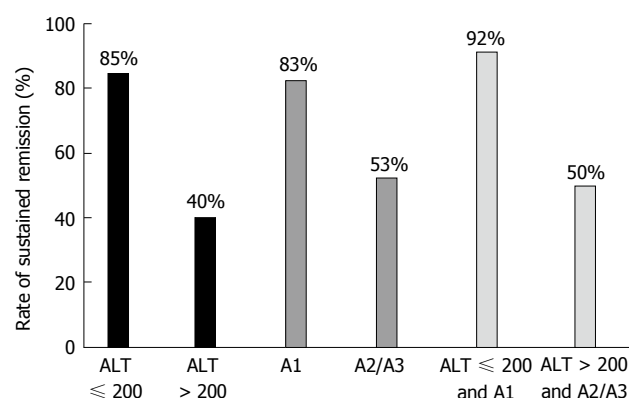
Factor	Category	Odds ratio (95%CI)	P
ALT (IU/L)	> 200	1	0.013
	≤ 200	10.8 (1.64-71.0)	
Age	> 50	1	0.86
	≤ 50	1.16 (0.21-6.38)	
IgG	> 2000	1	0.66
	≤ 2000	0.65 (0.10-4.32)	
Acute presentation	No	1	0.4
	Yes	4.13 (0.15-110.5)	
Histological Grading	A2 or A3	1	0.8
	A1	0.76 (0.10-5.88)	
Histological Staging	F2 or F3 or F4	1	0.46
	F1	0.41 (0.04-4.44)	
AIH score (International diagnostic criteria)	> 15	1	0.29
	≤ 15	2.66 (0.43-16.48)	
AIH score (Simplified criteria)	> 6	1	0.22
	≤ 6	5.46 (0.37-81.3)	

ALT: Alanine aminotransferase; AIH: Autoimmune hepatitis.

UDCA level required to inhibit immunoglobulin production is approximately 10 times the blood concentration after administration of UDCA at routine doses<sup>[22]</sup>, similarly high levels apparently exist in hepatocytes secreting bile, in other words, in the liver. Thus, UDCA may exert a liver-specific immunosuppressive action. This indicates that UDCA can be administered to achieve immunosuppression in patients with AIH. Miyake *et al.*<sup>[13]</sup> demonstrated in a small-scale study that UDCA is effective for AIH. Moreover, the administration of UDCA has also been shown to allow corticosteroid doses to be tapered<sup>[14]</sup>.

In this study, 71% of the UDCA group achieved and maintained the normalization of serum ALT levels with UDCA monotherapy. Especially, the present study also identified that in 85% of the patients with ALT levels of 200 IU/L or lower at the start of treatment, AIH remission could be induced and maintained by UDCA monotherapy. So, UDCA monotherapy will be effective in some Japanese AIH patients. However, in this study, patients treated with UDCA monotherapy had lower serum ALT levels and milder histological activity and fibrosis at presentation than those treated with PSL as shown in Table 1. Hence, it is necessary to consider that usefulness of UDCA was presented in mild AIH group. In the future, utility of UDCA must be confirmed in a prospective study.

On the other hand, among these mild AIH patients, the proportion indicated for UDCA monotherapy was low. On the bases of this finding, the patients in Group U can be considered to have no indications for treatment. In fact, 10-year survival in untreated patients with mild disease was reported to be 67%-90%<sup>[23,24]</sup>, and in an uncontrolled study, untreated asymptomatic patients had similar survival to those receiving immunosuppression<sup>[25]</sup>.



**Figure 1** Remission rate of each subgroup with ursodeoxycholic acid therapy. Remission was induced and maintained by ursodeoxycholic acid in 85%, 83% and 92% of patients in Subgroups A, B and C, respectively. On the other hand, the rates of remission induction and successful maintenance in Subgroups D, E, and F are low, at 40%, 53% and 50%, respectively. ALT: Alanine aminotransferase.

However, it also has to be acknowledged that untreated AIH has a fluctuating, unpredictable disease behavior, and a substantial proportion of asymptomatic patients become symptomatic during the course of their disease follow-up<sup>[25,26]</sup>, and progression towards end-stage liver disease with liver failure and development of HCC is possible<sup>[24]</sup>. Muratori *et al.*<sup>[27]</sup> also reported that patients with asymptomatic vs symptomatic AIH have similar courses of disease progression and responses to immuno-suppressive agents, and should therefore receive the same treatment. Additionally, to exclude patients with transient liver damage that may not have required treatment, patients with no histological fibrosis (F0) were not enrolled in the present study.

According to the AIH Guidelines issued by the American Association for the Study of Liver Diseases in 2010, no treatment is needed for patients with AST and ALT levels close to or below the standard levels<sup>[1]</sup>. The patients included in the present study did not meet these criteria, but largely met the indications for treatment. While the efficacy of corticosteroids for the treatment of AIH has been established, treatment with corticosteroids is currently the first choice only in patients with appropriate indications<sup>[1,2]</sup>. However, corticosteroids are associated with adverse events, such that there is often reluctance to administer these drugs. In patients with AIH in Japan, the age at onset and diagnosis has been increasing annually<sup>[28]</sup>. Particularly in elderly women, many of whom are postmenopausal, there is actually considerable concern regarding osteoporosis. Moreover, in the treatment of AIH, prevention of relapse is the most important issue, and maintenance therapy is thus important. However, because many patients are women, drug compliance can actually be poor due to cosmetic issues. In addition, it has also been pointed out that the incidence of other adverse events is high in elderly patients. The present study subjects had an age distribution between 17 and 74 years, demonstrating that elderly patients with AIH associated

with mild liver disorders could be treated with UDCA. Moreover, when the therapeutic effects of UDCA become inadequate, treatment can be continued by switching to corticosteroids, as shown in the present study. Furthermore, treatment with UDCA also has the benefit of eventually allowing the corticosteroid dose to be tapered<sup>[14]</sup>. A recent nationwide survey on AIH in Japan showed that UDCA monotherapy is administered as the initial treatment in 20% of patients<sup>[28]</sup>, so it is reasonable to assume that the treatment of AIH with UDCA is becoming clinically established. While Czaja *et al.*<sup>[15]</sup> found UDCA to be effective in a double-blind study, it is important to define criteria for UDCA treatment indications, as in the present study. Although the present study had a retrospective design, the results allow the conclusion to be drawn that UDCA use may be considered in patients with a serum ALT level of 200 IU/L at the time of diagnosis, especially in those who are elderly. Prospective studies on the long-term outcomes of patients receiving UDCA monotherapy are needed.

## COMMENTS

### Background

Autoimmune hepatitis (AIH) is an unresolving progressive liver disease that affects females preferentially and is characterized by interface hepatitis, hypergammaglobulinemia, circulating autoantibodies, and a favorable response to immunosuppression. The aim of treatment in AIH is to obtain complete remission of the disease and to prevent further progression of liver disease, which generally requires permanent maintenance therapy. Corticosteroids have been widely used as the first choice drug treatment of AIH. However, long-term treatment with a generous corticosteroid dosage may induce side effects. Ursodeoxycholic acid (UDCA) has been widely used as the first choice for treating primary biliary cirrhosis (PBC) and has been established as clinically useful. No severe side effects have been reported during UDCA therapy for PBC. Although there are reports that UDCA is also useful for treating similar autoimmune liver diseases, its clinical value has not as yet been established. In this study, patients with a confirmed diagnosis of AIH who started treatment with UDCA alone were analyzed.

### Research frontiers

There are few reports that UDCA monotherapy is effective for treating AIH. Moreover, the administration of UDCA has also been shown to allow corticosteroid doses to be tapered. However, its clinical value has not as yet been established.

### Innovations and breakthroughs

Few prior reports showed that UDCA is effective in some AIH patients. However, there is no report which showed independent predictive factors associated with normalized ALT and sustained remission of UDCA monotherapy in AIH patients. The present study showed that ALT levels of 200 IU/L or lower associated with to response to UDCA monotherapy. The results of the authors' study contribute to predict the therapeutic effect of UDCA for patients with AIH.

### Applications

This study suggests that that to prevent adverse events related to corticosteroids, treatment with UDCA alone for AIH needs to be considered in selected patients, especially those with an ALT level of 200 IU/L or lower.

### Terminology

UDCA: One of the secondary bile acids, which are metabolic byproducts of intestinal bacteria. It has been widely used as the first choice drug for the treatment of PBC. This is because of its efficacy for cholestasis, exerted through its choleretic action which is well understood. In addition to its choleretic

action, UDCA reportedly has a protective action on hepatocytes and an immunomodulatory action.

### Peer-review

This is a very interesting cut off point for future prospective studies to confirm these retrospective results.

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Prospective Study

## Shear wave elastography in hepatitis C patients before and after antiviral therapy

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**Author contributions:** Tamano M designed the research; Suda T, Okawa O, Masaoka R, Gyotoku Y and Tokutomi N performed the research; Suda T and Katayama Y analyzed the data; Suda T and Tamano M wrote the paper.

**Institutional review board statement:** This prospective study was reviewed and approved by the Ethics Committee of Dokkyo Medical University Koshigaya Hospital in Japan.

**Informed consent statement:** Written, informed consent was obtained from all participants and healthy volunteers in this study.

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**Data sharing statement:** No additional data are available.

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### Abstract

#### AIM

To investigate shear wave (SW) propagation velocity in patients with untreated hepatitis C and patients with sustained virological response (SVR).

#### METHODS

A total of 136 hepatitis C patients [85 patients who had not received antiviral therapy (naïve group) and 51 patients who had received antiviral therapy and subsequently achieved SVR of at least 24 wk (SVR group)] and 58 healthy volunteers and outpatients without liver disease (control group) underwent evaluation of liver stiffness by SW elastography (SWE). Various parameters were evaluated in the chronic hepatitis C patients at the time of SWE.

#### RESULTS

SW propagation velocity ( $V_s$ ) was  $1.23 \pm 0.14$  m/s in the control group,  $1.56 \pm 0.32$  m/s in the SVR group, and  $1.69 \pm 0.31$  m/s in the naïve group. Significant differences were seen between the control group and the SVR group ( $P = 0.0000$ ) and between the SVR group and the naïve group ( $P = 0.01417$ ). All four fibrosis markers were higher in the naïve group than in the SVR group. In the naïve group,  $V_s$  was positively correlated with alanine aminotransferase (ALT) ( $r = 0.5372$ ),  $\alpha$  fetoprotein (AFP) ( $r = 0.4389$ ), type IV collagen ( $r = 0.5883$ ), procollagen III peptide (P-III-P) ( $r = 0.4140$ ), hyaluronic acid ( $r = 0.4551$ ), and Mac-2 binding protein glycosylation isomer (M2BPGi) ( $r = 0.6092$ ) and negatively correlated with albumin ( $r = -0.4289$ ), platelets ( $r = -0.5372$ ), and prothrombin

activity ( $r = -0.5235$ ). On multiple regression analysis, Vs was the most strongly correlated with ALT (standard partial regression  $\beta = 0.4039$ ,  $P = 0.00000$ ). In the SVR group, Vs was positively correlated with AFP ( $r = 0.6977$ ), type IV collagen ( $r = 0.5228$ ), P-III-P ( $r = 0.5812$ ), hyaluronic acid ( $r = 0.5189$ ), and M2BPGi ( $r = 0.6251$ ) and negatively correlated with albumin ( $r = -0.4283$ ), platelets ( $r = -0.4842$ ), and prothrombin activity ( $r = -0.4771$ ). On multiple regression analysis, Vs was strongly correlated with AFP (standard partial regression  $\beta = 0.5953$ ,  $P = 0.00000$ ) and M2BPGi (standard partial regression  $\beta = 0.2969$ ,  $P = 0.03363$ ).

### CONCLUSION

In hepatitis C patients, liver stiffness is higher in treatment-naïve patients than in those showing SVR. SWE may be a predictor of hepatocarcinogenesis in SVR patients.

**Key words:** Hepatocarcinogenesis; Sustained virological response; Antiviral therapy; Shear wave elastography; Hepatitis C

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**Core tip:** This study is the first to compare liver stiffness in a group of hepatitis C patients in whom the virus was eliminated with antiviral therapy and a group of untreated hepatitis C patients using shear wave elastography. The liver stiffness value was higher in the untreated group than in the group in which the virus had been eliminated, which is thought to be due hepatitis activity. This study also suggests the possibility that liver stiffness measurements with shear wave elastography can be used as predictors of hepatocarcinogenesis in patients in whom the virus has been eliminated.

Suda T, Okawa O, Masaoka R, Gyotoku Y, Tokutomi N, Katayama Y, Tamano M. Shear wave elastography in hepatitis C patients before and after antiviral therapy. *World J Hepatol* 2017; 9(1): 64-68 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i1/64.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i1.64>

### INTRODUCTION

Shear wave elastography (SWE) is a new technology that gauges liver stiffness by measuring the propagation velocity of shear waves generated in liver tissue. At the same time, images are observed in real time using a normal B-mode ultrasound probe. The velocity of laterally propagated shear waves (lateral waves) is measured. SWE is useful for a diagnosis of breast tumor<sup>[1]</sup>, thyroid tumor<sup>[2]</sup>, muscle stiffness<sup>[3]</sup> as well as liver stiffness. SWE resembles acoustic radiation force impulse<sup>[4]</sup>, but it is new another technology.

Liver stiffness measurements with SWE are reported to be useful in diagnosing fibrosis in hepatitis C<sup>[5]</sup>. In

studies using transient elastography, liver stiffness was affected not only by liver fibrosis but also by necroinflammatory activity<sup>[6,7]</sup>. Therefore, the meaning of liver stiffness is predicted to differ in untreated patients with hepatitis activity and patients whose hepatitis has subsided with antiviral therapy.

The purpose of this study was to investigate the significance of SW propagation velocity in patients with untreated hepatitis C and patients with sustained virological response.

### MATERIALS AND METHODS

#### Patients

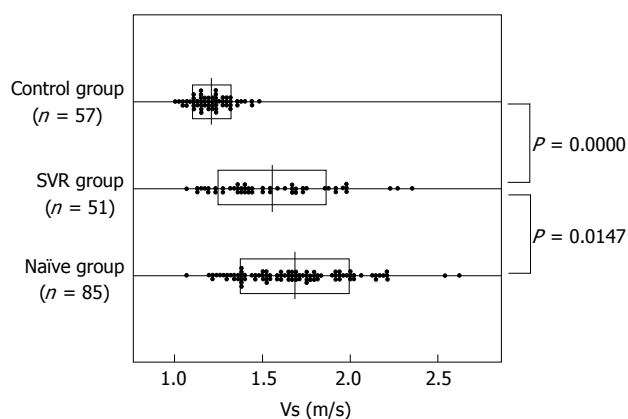
This prospective study was reviewed and approved by the Ethics Committee of Dokkyo Medical University Koshigaya Hospital, and written, informed consent was obtained from all participants and healthy volunteers. This study conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

The subjects were 136 chronic hepatitis C patients who were diagnosed in the Department of Gastroenterology of Dokkyo Medical University Koshigaya Hospital from April to October, 2015. The 136 patients included 85 patients in a naïve group who had not received antiviral therapy and 51 patients who had received antiviral therapy, either interferon-based therapy or direct-acting antiviral agent therapy (daclatasvir/asunaprevir), and subsequently achieved sustained virological response (SVR) of at least 24 wk (SVR group). Patients with decompensated liver cirrhosis, hepatocellular carcinoma, autoimmune disease, collagen disease, or chronic heart disease were excluded. Patients with a history of drinking  $\geq 20$  g alcohol per day and those diagnosed with obvious fatty liver on abdominal ultrasound were also excluded.

To obtain a standard liver stiffness value, SWE was performed in a total of 58 people including healthy volunteers and outpatients without liver disease (control group).

#### Measurement of SWE

Measurement of liver stiffness by shear wave elastography was performed using a LOGIQ E9 (GE Healthcare, Milwaukee, WI). The right lobe of the liver was visualized through an intercostal space while the patient was lying in a supine position with the right arm in maximum abduction. Measurements were taken while subjects held their breath during spontaneous breathing. The visual depth of the system was fixed at 8 cm, and the region of interest was 1-2 cm below the surface of the liver. The system was adjusted so that sample volume depth was 4 cm or less. Liver stiffness was automatically calculated by the apparatus, and the results are expressed as the velocity of shear wave velocity (Vs) (m/s). Measurements were performed by two investigators (Suda T and Tamano M) who have measurement experience of SWE more than 100 patients. They shot 10 to 12 times on liver segment 5, and the result was considered reliable only when 10



**Figure 1** Velocity based on shear wave elastography is shown for each group. Vs is  $1.23 \pm 0.14$  m/s in the control group,  $1.56 \pm 0.32$  m/s in the sustained virological response (SVR) group, and  $1.69 \pm 0.31$  m/s in the naïve group. Significant differences are seen between the control group and the SVR group ( $P = 0.0000$ ) and between the SVR group and the naïve group ( $P = 0.01417$ ). Vs: Velocity.

successful shots and a measurement success rate > 80% were obtained.

### Clinical parameters

The following clinical parameters were determined in chronic hepatitis C patients at the time SWE was performed: Age; aspartate aminotransferase (AST); alanine aminotransferase (ALT); total bilirubin, serum albumin; white blood cell (WBC) count; platelet count; prothrombin activity;  $\alpha$  fetoprotein (AFP); hyaluronic acid; type IV collagen; procollagen III peptide (P-III-P); Mac-2 binding protein glycosylation isomer (M2BPGi) measurements; and the Fib-4 index.

### Statistical analysis

Continuous data for Vs and other clinical parameters are expressed as means  $\pm$  SD. The Mann-Whitney *U* test was used for between-group comparisons. Correlations between Vs and other parameters were assessed using Spearman's rank correlation coefficient and multiple regression analysis. Values of  $P < 0.05$  were regarded as statistically significant.

## RESULTS

Figure 1 shows Vs (m/s) measured by SWE in each group. Vs was  $1.23 \pm 0.14$  m/s in the control group,  $1.56 \pm 0.32$  m/s in the SVR group, and  $1.69 \pm 0.31$  m/s in the naïve group. Significant differences were seen between the control group and the SVR group ( $P = 0.0000$ ) and between the SVR group and the naïve group ( $P = 0.01417$ ).

Table 1 shows the characteristics of the naïve group and the SVR group. Compared with the SVR group, the naïve group had significantly higher AST and ALT values ( $P = 0.00001$ ) and a significantly lower serum albumin value ( $P = 0.01049$ ). No significant differences were seen between the two groups in total bilirubin, WBC, platelet count, or prothrombin activity. AFP was significantly higher in the naïve group than in the SVR

**Table 1** Clinical characteristics in the Naïve and sustained virological response group

Characteristics	Naïve group ( <i>n</i> = 85)	SVR group ( <i>n</i> = 51)	<i>P</i> value
Age (yr)	$63.5 \pm 13.7$	$64.9 \pm 10.2$	0.9726
Sex (male/female)	43/42	27/24	0.79039
AST (IU/L)	$55.4 \pm 38.7$	$29.2 \pm 16.8$	0.00001
ALT (IU/L)	$61.9 \pm 50.1$	$23.9 \pm 18.0$	0.00001
Total bilirubin (mg/dL)	$0.89 \pm 0.36$	$0.95 \pm 0.33$	0.14736
Serum albumin (g/dL)	$4.10 \pm 0.46$	$4.32 \pm 0.44$	0.01049
WBC ( $\times 10^3/\text{mm}^3$ )	$5.21 \pm 1.83$	$5.24 \pm 1.56$	0.79015
Hb (g/dL)	$13.9 \pm 1.6$	$14.1 \pm 1.5$	0.47879
Platelet ( $\times 10^4/\text{mm}^3$ )	$14.5 \pm 6.4$	$14.5 \pm 5.7$	0.89078
Prothrombin activity (%)	$97.5 \pm 12.1$	$96.9 \pm 13.1$	0.98341
AFP (g/dL)	$9.6 \pm 10.6$	$5.1 \pm 3.3$	0.00773
Type IV collagen (ng/mL)	$180.5 \pm 71.8$	$179.2 \pm 71.6$	0.62913
P-III-P (U/mL)	$0.92 \pm 0.27$	$0.81 \pm 0.31$	0.00215
Hyaluronic acid (ng/mL)	$191.5 \pm 290.3$	$102.1 \pm 93.5$	0.24908
M2BPGi (COI)	$3.67 \pm 4.41$	$2.03 \pm 2.49$	0.00546
FIB-4 index	$4.44 \pm 4.21$	$3.39 \pm 2.63$	0.37443

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; WBC: White blood cell; P-III-P: Procollagen III peptide; M2BP: Mac-2 binding protein; COI: Cut-off index.

group ( $P = 0.00773$ ). All four fibrosis markers were higher in the naïve group than in the SVR group. No significant differences were seen in type IV collagen or hyaluronic acid, but significant differences were seen in P-III-P ( $P = 0.00215$ ) and M2BPGi ( $P = 0.00546$ ). The FIB-4 index tended to be higher in the naïve group than in the SVR group, but the difference was not significant ( $P = 0.37443$ ).

### Correlation between Vs and each parameter in the naïve group (*n* = 85)

Vs was positively correlated with ALT ( $r = 0.5372$ ), AFP ( $r = 0.4389$ ), type IV collagen ( $r = 0.5883$ ), P-III-P ( $r = 0.4140$ ), hyaluronic acid ( $r = 0.4551$ ), and M2BPGi ( $r = 0.6092$ ). Vs was negatively correlated with albumin ( $r = -0.4289$ ), platelets ( $r = -0.5372$ ), and prothrombin activity ( $r = -0.5235$ ). A multiple regression analysis was performed with the five parameters of ALT, platelets, prothrombin activity, type IV collagen, and M2BPGi that had correlation coefficients ( $r$ )  $\geq 0.5$ , and the results showed that Vs was the most strongly correlated with ALT in the naïve group (standard partial regression std  $\beta = 0.4039$ ,  $P = 0.00000$ ) (Table 2).

### Correlation between Vs and each parameter in the SVR group (*n* = 51)

Vs was positively correlated with AFP ( $r = 0.6977$ ), type IV collagen ( $r = 0.5228$ ), P-III-P ( $r = 0.5812$ ), hyaluronic acid ( $r = 0.5189$ ), and M2BPGi ( $r = 0.6251$ ). Vs was negatively correlated with albumin ( $r = -0.4283$ ), platelets ( $r = -0.4842$ ), and prothrombin activity ( $r = -0.4771$ ). A multiple regression analysis was performed with the five parameters of AFP, type IV collagen, P-III-P, hyaluronic acid, and M2BPGi that had correlation coefficients ( $r$ )  $\geq 0.5$ , and the results showed that Vs was strongly correlated with two parameters, AFP

**Table 2 Multiple regression analysis in the Naïve group**

	Coefficient (β)	SE(β)	Std β	t-value	df	P value
ALT	0.00261	0.00052	0.4069	5.02998	62	0.00000
Plt	-0.0121	0.00436	-0.27300	0.27300	62	0.00740
PT %	-0.0044	0.00245	-0.1712	1.79923	62	0.07685
Type IV collagen	0.00061	0.00040	0.1578	1.51030	62	0.13605
M2BPGi	0.00844	0.00709	0.1361	1.19098	62	0.23820

ALT: Alanine aminotransferase; Plt: Blood platelet; PT: Prothrombin time; M2BP: Mac-2 binding protein.

(standard partial regression  $\text{std } \beta = 0.5953$ ,  $P = 0.00000$ ) and M2BPGi (standard partial regression  $\text{std } \beta = 0.2969$ ,  $P = 0.03363$ ) (Table 3).

## DISCUSSION

The extent of hepatic fibrosis has classically been evaluated by histological procedures. However, the accuracy of this evaluation of hepatic fibrosis is limited by both sampling variability and inter-observer variability between pathologists<sup>[8,9]</sup>. In addition, liver biopsy is associated with patient discomfort and a risk of serious complications<sup>[10]</sup>.

Transient elastography (TE) has attracted attention as a noninvasive, objective diagnostic tool, and liver stiffness measured by TE is reported to be useful in diagnosing fibrosis in hepatitis C<sup>[11]</sup>. TE is useful in diagnosing non-alcoholic fatty liver disease<sup>[12]</sup> and in predicting carcinoma development in viral hepatitis patients<sup>[13]</sup>. However, TE is a test that is done blindly using a special probe in the right hepatic lobe confirmed with B mode, as a result of which measurement results are imprecise if vessels or other structures are present in the measured region. SWE is built into ultrasonic diagnostic equipment, and reliable measurements are possible in a short time under observation with normal B mode<sup>[14]</sup>.

The results of SWE measurements are expressed as the SW propagation velocity Vs (m/s). In this investigation, the Vs was  $1.23 \pm 0.14$  m/s in healthy livers,  $1.69 \pm 0.31$  m/s in the naïve group, and  $1.56 \pm 0.32$  m/s in the SVR group. The naïve group had a significantly higher Vs than the SVR group, suggesting that Vs decreases with virus elimination in hepatitis C patients. In this study, however, the naïve group and SVR group were different populations, and Vs measurements over time in the same population will be needed to accurately compare Vs before and after treatment.

Vs is determined not only by tissue elasticity (fibrosis), but it is also affected by viscosity. Thus, in cases of active hepatitis, propagation is expected to become faster due to increased tissue viscosity from increased exudate into the interstitium and cell infiltration, and in acute hepatitis that trend is marked<sup>[15,16]</sup>. In the naïve group, Vs was most strongly correlated with ALT. This is thought to be because the naïve group included many patients with active hepatitis with high ALT levels. Good positive correlations were seen between Vs and the liver fibrosis markers of hyaluronic acid, type IV collagen, P-III-P, and M2BPGi in the naïve group. Thus, in the naïve group, Vs is thought to reflect both hepatic activity (viscosity) and

**Table 3 Multiple regression analysis in the sustained virological response group**

	Coefficient (β)	SE (β)	Std β	t-value	df	P value
AFP	0.05564	0.0098	0.5953	5.6797	28	0.00000
Type IV Collagen	-0.0003	0.00066	-0.0626	0.42483	28	0.67420
P-III-P	0.13859	0.17013	0.146	0.81462	28	0.42217
Hyaluronic acid	0.00053	0.00053	0.1658	0.99713	28	0.32724
M2BPGi	0.03554	0.01591	0.2969	2.23421	28	0.03363

AFP: α feto protein; P-III-P: Procollagen III peptide; M2BP: Mac-2 binding protein.

fibrosis (elasticity).

In the SVR group, hepatitis had subsided for six months or more, and, in fact, the correlation between Vs and ALT in the SVR group in this study was very low. Therefore, Vs in the SVR group is presumed to almost purely reflect liver fibrosis (elasticity), and thus it is thought to have better correlations with fibrosis markers. Among the four different fibrosis markers, M2BPGi had the strongest positive correlation with Vs. M2BPGi is a new liver fibrosis marker that quantitatively measures changes in the carbohydrate structure of Mac-2 binding protein<sup>[17]</sup>, and it is also considered useful in predicting carcinogenesis in hepatitis C patients<sup>[18-20]</sup>.

In hepatitis C patients, AFP is a useful indicator of hepatocarcinogenesis following interferon therapy<sup>[21]</sup>. Although AFP had positive correlations in both the naïve group and the SVR group, a stronger correlation was seen in the SVR group. In the naïve group, AFP reflects inflammation and necrosis of hepatocytes and the accompanying hepatocyte regeneration. In the SVR group, on the other hand, AFP has a strong element as a surrogate marker of hepatocellular carcinoma, as mentioned previously. A very interesting finding in the SVR group in the present study was the strong correlations between Vs and AFP and between Vs and M2BPGi, which suggest the possibility that liver stiffness measurements with SWE may be used as predictors of hepatocarcinogenesis in hepatitis C patients following SVR.

In hepatitis C patients, liver stiffness with SWE was higher in the naïve group than in the SVR group, presumably due to hepatitis activity. In the SVR group, liver stiffness measurements with SWE may be a predictor of hepatocarcinogenesis.

## COMMENTS

### Background

Shear wave elastography (SWE) is a new technology that gauges liver stiffness by measuring the propagation velocity of shear waves generated in liver tissue. At the same time, images are observed in real time using a normal B-mode ultrasound probe. The velocity of laterally propagated shear waves (lateral waves) is measured.

### Research frontiers

The results of SWE measurements are expressed as the SW propagation velocity Vs (m/s). In this investigation, the Vs was  $1.23 \pm 0.14$  m/s in healthy livers,  $1.69 \pm 0.31$  m/s in the naïve group, and  $1.56 \pm 0.32$  m/s in the SVR group. The naïve group had a significantly higher Vs than the SVR group, suggesting that Vs decreases with virus elimination in hepatitis C patients. In this study, however,



the naïve group and sustained virological response (SVR) group were different populations, and Vs measurements over time in the same population will be needed to accurately compare Vs before and after treatment.

### Innovations and breakthroughs

A very interesting finding in the SVR group in the present study was the strong correlations between Vs and  $\alpha$  fetoprotein and between Vs and Mac-2 binding protein glycosylation isomer, which suggest the possibility that liver stiffness measurements with SWE may be used as predictors of hepatocarcinogenesis in hepatitis C patients following SVR.

### Applications

In the SVR group, liver stiffness measurements with SWE may be a predictor of hepatocarcinogenesis.

### Peer-review

This manuscript by Suda *et al* points to assess the changes of shear wave velocity in patients with untreated chronic hepatitis C and patients who received antiviral therapy and obtained SVR. The authors found significant differences between shear wave velocity between patients with SVR and those untreated.

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*WJH* covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## From the liver to the heart: Cardiac dysfunction in obese children with non-alcoholic fatty liver disease

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### Abstract

In the last decades the prevalence of non-alcoholic fatty liver disease (NAFLD) has increased as a consequence of the childhood obesity world epidemic. The liver damage occurring in NAFLD ranges from simple steatosis to steatohepatitis, fibrosis and cirrhosis. Recent findings reported that fatty liver disease is related to early atherosclerosis and cardiac dysfunction even in the pediatric population. Moreover, some authors have shown an association between liver steatosis and cardiac abnormalities, including rise in left ventricular mass, systolic and diastolic dysfunction and epicardial adipose tissue thickness. In this editorial, we provide a brief overview of the current knowledge concerning the association between NAFLD and cardiac dysfunction.

**Key words:** Cardiac dysfunction; Non-alcoholic fatty liver disease atherosclerosis; Children; Cardiovascular risk

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**Core tip:** Recently, growing scientific evidences suggest that obese children with non-alcoholic fatty liver disease are more predisposed to cardiovascular disease. Interestingly, this association seems to be independent from adiposity. In fact, based on recent findings, it has been proposed that liver steatosis plays an independent role in determining early atherosclerosis and cardiac dysfunction.

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## INTRODUCTION

Currently, non-alcoholic fatty liver disease (NAFLD) represents the major cause of chronic liver disease in childhood and is considered a multisystem disease that affects many extra-hepatic organs<sup>[1]</sup>. Experimental evidence suggests that children with NAFLD have a higher risk of developing end stage liver disease than the general population of United States of same age and gender<sup>[2]</sup>. Moreover, NAFLD has emerged as an independent risk factor for cardiovascular diseases (CVD)<sup>[3,4]</sup>, including coronary artery disease and cardiac dysfunction<sup>[5-8]</sup>.

The molecular mechanisms linking NAFLD to cardiovascular complications are still poorly understood<sup>[6,8]</sup>. Children with NAFLD display increased free fatty acids that may lead to myocardial lipid accumulation with consequent impairments in myocardial substrate metabolism and efficiency and, finally, cardiac dysfunction<sup>[7,8]</sup>. Moreover, the presence of a low-grade inflammatory state in these patients contributes to the release of several mediators that amplify this condition<sup>[6,8]</sup>. Therefore, it has been hypothesized that intra-hepatic fat might exert a key pathogenetic role in developing cardio-metabolic complications (Figure 1).

In the last 20 years, NAFLD has become the most common liver disease in pediatrics<sup>[1]</sup>, as result of the increased prevalence of early onset obesity<sup>[2-4]</sup>. Although NAFLD develops in the context of insulin resistance related to obesity, it is possible that a parental effect exists. As suggested by experimental studies in rats, in fact, paternal and maternal obesity during preconception could lead to obesity, glucose metabolism abnormalities and liver steatosis in the offspring<sup>[5]</sup>.

## ATHEROSCLEROSIS

NAFLD represents an independent risk factor for CVD as it is associated with dyslipidemia, insulin resistance and alterations of cardiac function independent of the degree of obesity<sup>[3,4]</sup>.

Recent studies<sup>[2,3,6]</sup> have shown that atherosclerosis and alterations of cardiac function may occur already during childhood, as demonstrated by the presence of early onset subclinical atherosclerosis as measured by impaired flow-mediated vasodilation and increased carotid artery intimal medial thickness as well as by the presence of abnormalities in myocardial structure and function in obese children and adolescents<sup>[3,4]</sup> (Table 1).

Despite this evidence, the relationship between NAFLD and cardiovascular alterations is still poorly understood. Moreover, it is unclear whether there is a causal relationship between intra-hepatic fat accumulation and alterations of cardiac dynamics or whether the

two phenomena are just independent complications of obesity.

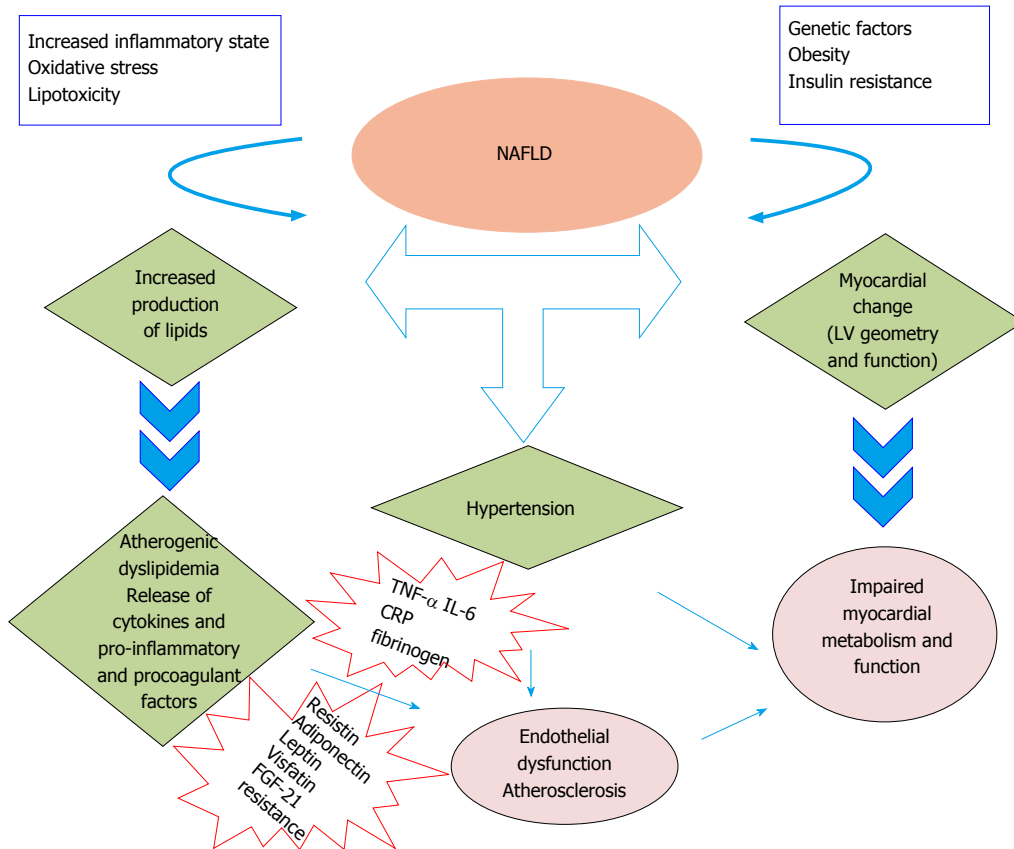
It is possible that intra-hepatic fat accumulation may be a pathogenic determinant of CVD. In fact, NAFLD can lead to atherosclerosis by causing an abundant secretion from the liver of lipoproteins [large very low density lipoproteins (VLDL)]<sup>[7]</sup>, which, in obese individuals with NAFLD, are abundant in oxidized fatty acids<sup>[8,9]</sup>. The higher concentration of large VLDL results in a high concentration in plasma of very small LDL, which in turn are a major contributor to the atherosclerotic plaque<sup>[7]</sup>, and their accumulation within the plaque would ultimately lead to ischemic events. NAFLD *per se*, in fact, is a hyperlipidemic state in which adipose tissue insulin resistance<sup>[10]</sup> and enhanced hepatic *de novo* lipogenesis<sup>[11]</sup> lead to an abnormal accumulation of fat in the liver, turning the hepatocytes in a fat producing factory.

It is important to remember that myocardial atherogenesis occurs very early in life. Seminal studies in the Bogalusa cohort showed that atherosclerosis often begins in pediatric age<sup>[12]</sup>, as demonstrated by the fact that fatty streaks are detected already in the aorta and the coronary arteries of children<sup>[6,13]</sup>. Since NAFLD can begin during childhood in obese children, it is reasonable to think that it can be an important determinant of these early events observed already in the pediatric population.

## CARDIAC ABNORMALITIES

While, the association between NAFLD and atherosclerosis could be easily explained by the abundance of circulating lipids present in obese children with NAFLD, it is more difficult to explain the relationship between NAFLD and cardiac function and geometry<sup>[14,15]</sup>. It has been reported that adolescents with NAFLD show an impaired systolic and diastolic function and an increased left ventricular mass compared to both healthy controls and age and gender matched obese adolescents without NAFLD<sup>[14,15]</sup> (Table 1).

To explain this observation several pathogenic mechanisms have been hypothesized, including the role of the liver as a generator of circulating mediators that could be involved in the cardiac remodeling<sup>[3,11,16,17]</sup>. In fact, the presence of a low-grade inflammatory state in patients obese patients with NAFLD and insulin resistance contributes to release of several cytokines and adipokines (e.g., IL-6, TNF-alpha, visfatin, FGF-21, adiponectin, resistin, leptin) that amplify this condition and worsen the metabolic phenotype<sup>[3,11,17]</sup>. Whether the trigger for this condition, usually referred to as "sterile inflammation", is NAFLD or insulin resistance *per se* is unclear, but a large body of evidence suggests that inflammatory cytokines may be the link among fatty liver, insulin resistance and myocardial changes<sup>[3,17]</sup>. In fact, adiponectin, a strong insulin sensitizer, seems to be



**Figure 1** The multifactorial mechanisms leading nonalcoholic fatty liver disease patients to unfavorable cardiac outcomes. CRP: C-reactive protein; IL-6: Interleukin-6; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; FGF-21: Fibroblast growth factor-21; NAFLD: Nonalcoholic fatty liver disease; LV: Left ventricular.

decreased in patients with NAFLD and some studies have suggested that low adiponectin levels may affect cardiac function and atherogenic risk. In contrast, patients with NAFLD experience also an increased production of pro-inflammatory and pro-atherogenic cytokines (IL-6, IL-12, TNF- $\alpha$ ), which in turn worsens insulin resistance, mostly through the down-regulation of insulin-receptor-substrate and by affecting gluconeogenesis<sup>[3,17]</sup>, these compounds could also affect the cardiac morphology and dynamics. More importantly, some data suggest that fibroblast growth factor 21 (FGF-21), a protein synthesized and secreted by the liver in high amounts in obese subjects with fatty liver, might play a role in cardiac hypertrophy<sup>[16]</sup>. Some investigators have speculated that FGF-21 resistance occurring in NAFLD could lead to cardiac damage in NAFLD patients<sup>[16]</sup>.

On the other hand, given that NAFLD is associated with high systolic blood pressure<sup>[18]</sup> it could be also argued that changes in myocardial structure might be the consequence of high blood pressure<sup>[18]</sup>. Against the latter hypothesis, there is the evidence that changes in cardiac morphology observed in children with NAFLD do not resemble the cardiac adaptation consequence of high blood pressure<sup>[19]</sup>. In fact, hypertension increases left ventricular (LV) mass and changes heart morphology as a result of overload. Recent findings reported that

obese children both with and without NAFLD showed no differences in LV mass and posterior wall thickness, while children with NAFLD were more likely to present low LV strain rate - a load - independent parameter that expresses LV elastance and deformation - than those without NAFLD. Therefore, the authors suggested that NAFLD affects myocardial fiber organization leading to systolic and diastolic dysfunction<sup>[19]</sup>.

## CONCLUSION

Besides the large amount of literature suggesting an association between NAFLD and CVD, the pathophysiologic mechanisms underlying these associations are far from being clear, therefore, in the future more studies should be focused on this area of research to unravel those mechanisms and to develop novel therapeutic strategies.

Moreover, we need to establish also whether NAFLD could be considered a marker of subclinical atherosclerosis as well as a cardiovascular risk factor even at a very early age. In fact, because of its strong relationship with CVD, NAFLD diagnosis could represent a red flag for the presence of high cardiovascular risk. In this scenario the prevention and treatment of NAFLD may play a crucial role in avoiding not only end-stage liver disease but also CVD.



**Table 1** Principal features and findings of the studies regarding the association between non-alcoholic fatty liver diseases and cardiac dysfunction

Ref.	Study design and methods	Population (n)	Main findings
Bonci <i>et al</i> <sup>[4]</sup>	Systematic review and meta-analysis Systematic literature search for papers from January 2000 to September 2014	12 observational studies: 9 studies based on adult population and 3 studies performed in pediatric population were selected	Children with NAFLD were not different from those without for LV mass Both children with and without NAFLD presented an increased LV mass compared to controls However children with NAFLD presented higher E/e' ratio rather those without NAFLD
D'Adamo <i>et al</i> <sup>[7]</sup>	Cross-sectional study NAFLD diagnosis performed by MRI Evaluation of VAT Lipoprotein particle characterized by MRS	Mean age 14.6 yr Obese African American (33) Obese Hispanic (33)	In multiple regression analyses liver fat accumulation resulted independently and significantly related to large VLDL concentrations
Sert <i>et al</i> <sup>[14]</sup>	Cross-sectional study NAFLD diagnosis performed by ultrasound and elevated serum alanine aminotransferase Pulsed and tissue doppler Echocardiography	Mean age 13.3 yr Healthy (68) Obese with NAFLD and elevated ALT (97)	NAFLD children showed increased CIMT and abnormalities of both LV structure and function LV CIMT and LV mass were positively related to HOMA-IR in obese children with NAFLD
Alp <i>et al</i> <sup>[15]</sup>	Cross-sectional study NAFLD diagnosis performed by liver biopsy Echocardiography	Obese without NAFLD and low ALT (83) Mean age 12 yr Healthy (150) Obese with NAFLD at United States (93)	NAFLD group had increased epicardial fat thickness, end-systolic thickness of the interventricular septum, and larger LV mass, as well as LV systolic and diastolic dysfunction
Pacifico <i>et al</i> <sup>[18]</sup>	Tissue doppler Echocardiography Cross-sectional study NAFLD diagnosis performed by MRI	Obese without NAFLD at United States (307) Mean age 12.5 yr Healthy (18)	Children with NAFLD presented signs of left ventricular dysfunction compared to children without NAFLD Subjects with a more severe NASH had a worse cardiac dysfunction
Singh <i>et al</i> <sup>[19]</sup>	Liver biopsy for NASH in 41 subjects Echocardiography Cross-sectional study NAFLD diagnosis performed by MRS Echocardiography	Obese with NAFLD at MRI (54) Obese without NAFLD at MRI (54) Mean age 15 yr Lean (14) Obese with Intrahepatic triglyceride content (15) Obese with increased Intrahepatic triglyceride content (15)	Obese adolescents with NAFLD show a worse systolic and diastolic functions rather than lean and obese adolescents without NAFLD independent from anthropometric parameters and blood pressure

MRI: Magnetic resonance imaging; NAFLD: Nonalcoholic fatty liver disease; MRS: Magnetic resonance spectrometry; VAT: Visceral adipose tissue; NASH: Non-alcoholic steatohepatitis; LV: Left ventricular; ALT: Alanine transaminase; CIMT: Carotis intima media thickness; VLDL: Very low density lipoproteins.

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## Hepatic structural enhancement and insulin resistance amelioration due to AT1 receptor blockade

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### Abstract

Over the last decade, the role of renin-angiotensin system (RAS) on the development of obesity and its comorbidities has been extensively addressed. Both

circulating and local RAS components are up-regulated in obesity and involved in non-alcoholic fatty liver disease onset. Pharmacological manipulations of RAS are viable strategies to tackle metabolic impairments caused by the excessive body fat mass. Renin inhibitors rescue insulin resistance, but do not have marked effects on hepatic steatosis. However, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers (ARB) yield beneficial hepatic remodeling. ARBs elicit body mass loss and normalize insulin levels, tackling insulin resistance. Also, this drug class increases adiponectin levels, besides countering interleukin-6, tumoral necrosis factor-alpha, and transforming growth factor-beta 1. The latter is essential to prevent from liver fibrosis. When conjugated with peroxisome proliferator-activated receptor (PPAR)-alpha activation, ARB fully rescues fatty liver. These effects might be orchestrated by an indirect up-regulation of MAS receptor due to angiotensin II receptor type 1 (AT1R) blockade. These associations of ARB with PPAR activation and ACE2-angiotensin (ANG) (1-7)-MAS receptor axis deserve a better understanding. This editorial provides a brief overview of the current knowledge regarding AT1R blockade effects on sensitivity to insulin and hepatic structural alterations as well as the intersections of AT1R blockade with peroxisome proliferator-activated receptor activation and ACE2-ANG (1-7) - MAS receptor axis.

**Key words:** Non-alcoholic fatty liver disease; Insulin resistance; Angiotensin receptor blockers; MAS receptor; Renin-angiotensin system

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**Core tip:** Intrahepatic renin-angiotensin system activation contributes to insulin resistance and non-alcoholic fatty liver disease onset. ANG II interaction with angiotensin II receptor type 1 (AT1R) mediates pro-inflammatory and pro-fibrogenic responses, besides enhancing the oxidative stress, which makes the liver more prone to

noxious liver diseases. AT1R blockers mitigate insulin resistance and fatty liver by enhancing beta-oxidation, reducing lipogenesis and controlling inflammation. The impact of the AT1R blockade on liver ACE2-angiotensin (1-7)-MAS receptor axis remains to be fully unraveled.

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## INTRODUCTION

Liver injuries can result from virus infection, alcohol and/or drugs abuse, and autoimmune diseases<sup>[1]</sup>. However, the increase in high-energy dense food availability combined with a sedentary lifestyle brought up unprecedented obesity rates, with the consequent increase in its comorbidities (hypertension, type 2 diabetes, and dyslipidemias) prevalences<sup>[2]</sup>. The metabolic disturbances caused by obesity also impair liver structure and physiology, with increasing prevalence of non-alcoholic fatty liver disease (NAFLD) and greater susceptibility to more harmful types of liver diseases such as non-alcoholic steatohepatitis (NASH) and liver fibrosis<sup>[3,4]</sup>.

Insulin resistance (IR) plays a central role in NAFLD pathogenesis<sup>[5]</sup>. Also, the low-grade inflammation observed in obese subjects and the increased adipocyte lipolysis are key factors for a more pronounced lipid droplet deposition within the hepatocytes<sup>[6]</sup>. Briefly, IR has opposite effects on adipose tissue and liver. On one hand, resistance to insulin action elicits enhanced lipolysis rate in the white adipose tissue as an attempt to compensate for the lack of glucose to be used as fuel by the adipocytes. Hence, increased free fatty acids (FFAs) are delivered to the liver<sup>[3,7]</sup>. On the other hand, insulin resistance impairs beta-oxidation within hepatocytes by reducing the expression of carnitine palmitoyltransferase 1 in the hepatic mitochondrion, besides reducing very low-density lipoprotein (VLDL) secretion. These conditions lead to unbalanced hepatic lipid metabolism as FFAs inflow surpasses fatty acid oxidation and lipoprotein exportation<sup>[8,9]</sup>. Therefore, excessive fatty acids are converted into triglycerides through the up-regulated lipogenic pathways, which accumulate as lipid droplets within hepatic parenchyma, characterizing the NAFLD<sup>[10]</sup>.

Considering that NAFLD is currently considered as the hepatic manifestation of the metabolic syndrome and that this condition, despite benign at first, can initiate a harmful spectrum of liver diseases, treatments should target hepatic alterations, but also alleviate others comorbidities such as hypertension, inflammation, and insulin resistance<sup>[11,12]</sup>. Recently, the activation of a local renin-angiotensin system (RAS) in the liver has been linked to NAFLD onset and progression towards liver fibrosis<sup>[13]</sup>. In

this way, the RAS emerges as a potential target to tackle hepatic alterations stemmed from obesity and other metabolic constraints imposed by increased body fat mass<sup>[14]</sup>.

## CIRCULATING RAS

From a classical view, the circulating RAS is implicated in the systemic hemodynamic regulation. Briefly, under a reduced renal perfusion, renin is secreted by the juxtaglomerular apparatus. This enzyme converts angiotensinogen (produced by the liver) in angiotensin 1 (ANG I), which is converted into angiotensin 2 (ANG II) by the angiotensin-converting enzyme (ACE). ANG II has countless physiological effects such as the stimulation of aldosterone release, which promptly reestablishes the hemodynamic control by enhancing water and sodium retention in the kidneys<sup>[15,16]</sup>.

ANG II exerts its main effects by interacting with two main receptors: Angiotensin II receptor type 1 (AT1R) or angiotensin II receptor type 2 (AT2R). AT1R has an important role in tissue repair and cell proliferation. However, when overexpressed, mediates pro-inflammatory and pro-atherogenic effects. Conversely, AT2R has anti-inflammatory effects, mainly by down-regulating tumoral necrosis factor-alpha (TNF-alpha) and nuclear factor-kappa B (NF-KB) pathways and by exerting anti-fibrogenic properties, besides reducing oxidative stress and cell proliferation<sup>[17,18]</sup>. Bearing this in mind, the angiotensin receptor blockers (ARBs) represents an evolution of the ACE inhibitors as they block exclusively the actions mediate by the interaction of ANG II with the AT1R<sup>[19,20]</sup>. Thus, important physiological effects stemmed from ANG II interaction with AT2R are maintained, leading to reduced atherogenesis, greater cardiac and endocrine pancreas functions, reduced glomerulosclerosis and fatty liver<sup>[21,22]</sup>.

Recently, with the discovery of ACE2, another branch of RAS has been described. ACE2 converts ANG II to ANG (1-7) and cleaves ANG I into ANG (1-9), which is also converted to ANG (1-7) by ACE. ANG (1-7) exerts its physiological effects through the MAS receptor. It can be argued that [ACE2-ANG (1-7)-MAS axis] counters the (ACE - ANG II - AT1R axis) effects. So, ACE2/ACE balance is an important target to tackle metabolic diseases<sup>[23-25]</sup>.

## LOCAL RAS: HEPATIC EFFECTS

Lately, apart from this circulating RAS, many local RAS have been described in organs such as heart, pancreas, adipose tissue, skeletal muscle, and liver<sup>[17,26,27]</sup>. Animal models of obesity show raised circulating renin, angiotensinogen, and ANG II<sup>[28]</sup>, besides higher expression of ACE and AT1R in the pancreas, which inhibit important steps of the insulin signaling cascade and contribute to IR and type 2 diabetes onset<sup>[29,30]</sup>. Intrahepatic activation of RAS favors NAFLD onset as it elicits greater triglycerides accumulation due to impaired beta-oxidation in conjunction with a significant fall in VLDL secretion.



These conditions comply with the increase of *de novo* lipogenesis (the formation of fatty acids from excessive dietary carbohydrate)<sup>[27,31]</sup>. Concomitantly, the increased production of reactive oxygen species by mitochondria and the raised expression of pro-inflammatory cytokines contribute to the progression to NASH<sup>[22]</sup>. These effects are mainly mediated by higher expression of ACE, ANG II, and AT1R concomitant to reduced ACE2 tissue expression in the hepatocytes of obese mice<sup>[32]</sup>.

Moreover, ANG II activates hepatic stellate cells (HSCs). Enhanced transforming growth factor-beta 1 (TGF-beta1) underlies this event, which implies a higher susceptibility to hepatic fibrosis, once HSCs acquire a myofibroblast phenotype<sup>[33,34]</sup>. These harmful effects of ANG II on liver structure and function are mediated predominantly by its interaction with the AT1R and results in collagen synthesis, pro-inflammatory cytokines release, stimulation of cell migration and proliferation<sup>[27,35]</sup>. These events altogether contribute to the second hit proposed by the two-hit theory, where inflammation and fibrogenesis play a decisive role in NAFLD progression to NASH<sup>[36]</sup>.

Obese mice show higher hepatic steatosis rate coupled with insulin resistance, a pro-inflammatory adipokine profile, reduced hepatic beta-oxidation of fatty acids and enhanced lipogenesis<sup>[37]</sup>. Recently, it has been shown that a mouse model of NAFLD, even without obesity, presents with enhanced ACE/AT1R expression locally in the liver<sup>[38]</sup>. Rats with liver fibrosis present with favored ACE-ANG II-AT1R axis over ACE2-ANG (1-7)-MAS receptor axis, confirming that AT1R is involved with NAFLD progression to NASH and fibrosis<sup>[24,25]</sup>. These observations suggest that the local expression of AT1R is related to NASH onset and AT1R blockade, with the consequent ACE2 induction, emerging as a potential approach to prevent liver fibrosis and chronic inflammation.

## BLOCKADE OF AT1 RECEPTOR EFFECTS ON INSULIN RESISTANCE AND FATTY LIVER

The impact of pharmacological manipulations of the RAS system on insulin resistance and liver structure is a new field of study. Evidence from animal studies shows that aliskiren (a direct renin inhibitor) rescued insulin resistance and hepatic steatosis, though its effects are not more advantageous than ARBs<sup>[39,40]</sup>.

Angiotensin-converting enzyme inhibitors (ACEi) inhibit ANG I to ANG II conversion and, therefore, enhances the availability of bradykinin<sup>[41]</sup>. This peptide yields cardiovascular protection by stimulating the release of important vasodilators such as nitric oxide and prostacyclin<sup>[42]</sup>. Bradykinin reduces the hepatic expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, inhibiting hepatic gluconeogenesis. Furthermore, isolated myocytes and adipocytes treated with bradykinin exhibited improved glucose uptake due to greater glucose transporter 4 translocation to the cell membrane<sup>[43]</sup>. These events show that by enhancing bradykinin availability,

ACEi are able to mitigate insulin resistance and counter NAFLD. Even though ACEi represent a potent approach as it combines benefits from bradykinin and ANG II inhibition, ARBs preserve AT2R-mediated benefits and favor ACE2-ANG (1-7)-MAS receptor axis. These properties make ARBs an attractive option to treat metabolic impairments.

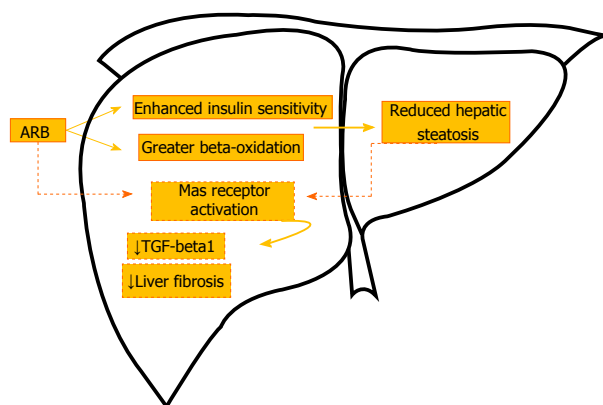
Olmesartan, a pure ARB, reduced body mass and hepatic triglyceride content, besides recovering the expression of hepatic antioxidant enzymes and sensitivity to insulin in rats<sup>[44,45]</sup>. The recovery of uncoupling protein 2 expression is put forward as the main mechanism that enhances hepatic lipid metabolism and antioxidant capacity after the blockade of AT1R<sup>[44]</sup>. Amelioration of IR after olmesartan treatment is also perceived in humans<sup>[46]</sup>.

Irbesartan, another ARB, and an ACEi (perindopril) prevented obese Zucker rats from developing fatty liver in a recent study. Both treatments elicited a marked reduction in hepatic steatosis percentage, with no difference with the lean control group<sup>[47]</sup>. A remarkable reduction in hepatic expression of TNF-alpha, interleukine-6, and TGF-beta1 is produced by enhanced ACE2-ANG (1-7)-MAS receptor, leading to the alleviation of hepatic IR and, consequently, reducing fatty liver<sup>[25]</sup>. Furthermore, low TGF-beta1 expression complies with the marked reduction in liver fibrosis in obese animals treated with irbesartan<sup>[44]</sup>. In agreement to this, losartan, an ARB, led to anti-proliferative and anti-fibrogenic effects in ANG II stimulated HSCs *in vitro*. Once again, a marked reduction in TGF-beta1 expression and AT1R down-regulation explain these findings<sup>[48]</sup>.

It was recently proposed a synergistic action between hepatic cholesterol metabolism and intrahepatic RAS activation in the physiopathology of NAFLD. In this context, chronic local RAS activation in the liver augments the extracellular matrix synthesis and disrupts LDL metabolism by impairing LDL receptor functioning. These alterations seem to rely on AT1R activation by ANG II. In agreement to this, telmisartan, an ARB that is also a partial peroxisome proliferator-activated receptor (PPAR)-gamma agonist, prevented from lipid deposition and overrode the translocation of SCAP/SREBP-2 complex from the endoplasmic reticulum to Golgi, blocking LDL receptor gene transcription in HepG2 cells<sup>[31]</sup>.

Animal studies show that telmisartan rescues the sensitivity to insulin, markedly reduces hepatic steatosis and augments the numerical density of mitochondria per area of hepatic tissue in diet-induced obese mice<sup>[49]</sup>. These events rely on PPAR-alpha activation in the liver coupled with dual AT1R blockade/partial PPAR-gamma agonist properties, which determine enhanced adiponectin levels, favored beta-oxidation over lipogenesis and reduced HSCs activity<sup>[49,50]</sup>.

Also, telmisartan limits hepatic fibrosis by enhancing mRNA levels of ACE2 and MAS receptor concomitant to reducing ACE, AT1R, collagen type II and TGF-beta1, besides blocking HSCs activation in bile duct-ligated rats<sup>[51]</sup>. However, some effects are stemmed from the partial PPAR-gamma agonist property, such as IR alleviation,



**Figure 1 Overview of angiotensin receptor blockers actions on the liver.** ARBs reduce hepatic steatosis through favored beta-oxidation and reduced insulin resistance. Concomitantly, it indirectly enhances Mas receptor activity, eliciting hepatoprotective effects by low TGF-beta1 expression, which limits hepatic stellate cells activation and prevents liver fibrosis. ARBs: Angiotensin receptor blockers; TGF-beta1: Transforming growth factor-beta 1.

reduced oxidative stress, and hepatic lipid deposition<sup>[52]</sup>.

It is likely that the favored activity of the ACE2-ANG (1-7)-MAS receptor action under the AT1R blockade mediates the beneficial findings<sup>[25]</sup>. With regard to this, the infusion of ANG (1-7) in bile duct-ligated rats elicited fibrosis attenuation by the suppression of HSCs activity, while the use of MAS receptor antagonist confirmed these findings as the animals presented with a maximization of liver fibrosis, supported by higher expression of collagen and TGF-beta1<sup>[53]</sup>. Figure 1 illustrates the main pathways related to ARBs effects on the liver.

## CONCLUSION

Increasing rates of obesity and NAFLD have drawn the attention of the scientific community to strategies to treat these metabolic diseases. Local RAS is up-regulated in the liver from obese individuals and in lean individuals with fatty liver. Among the pharmacological manipulations of RAS, AT1R blockade is considered the best approach as it favors AT2R effects and seems to activate indirectly the ACE2-ANG (1-7)-MAS receptor axis, with additional beneficial effects. The combination of AT1R blockers with oral ANG (1,7) treatment seems to be a promising approach to treating NAFLD and NASH and prevent liver fibrosis.

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## Systemic treatment for hepatocellular carcinoma: Still unmet expectations

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### Abstract

Many patients with hepatocellular carcinoma (HCC) are diagnosed in an advanced stage, so they cannot be offered the option of curative treatments. The results of systemic chemotherapy are unsatisfactory and this has led to molecular targeted approaches.

HCC develops in chronically damaged tissue due to cirrhosis in most patients. Several different cell types and molecules constitute a unique microenvironment in the liver, which has significant implications in tumor development and invasion. This, together with genome instability, contributes to a significant heterogeneity which is further enhanced by the molecular differences of the underlying causes. New classifications based on genetic characteristics of the tissue microenvironment have been proposed and key carcinogenic signaling pathways have been described. Tumor and adjacent tissue profiling seem biologically promising, but have not yet been translated into clinical settings. The encouraging first results with molecular - genetic signatures should be validated and clinically applicable. A more personalized approach to modern management of HCC is urgently needed.

**Key words:** Systemic; Chemotherapy; Hepatocellular carcinoma; Prognosis

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**Core tip:** The complete failure of chemotherapy in previous years gradually shifted hepatocellular carcinoma (HCC) treatment to the molecular targeted therapies. The initial-albeit limited - effectiveness of the currently approved systemic therapy, sorafenib, is due to the successful combination of targeting cancer cells and their microenvironment. Trials on drugs other than sorafenib, alone or in combination with drugs or transcatheter arterial chemoembolization were disappointing. Recently, genomic based analyses in HCC patients have proposed subclasses, based on molecular characteristics and a proliferative or non-proliferative genotypes. Combined targeted therapies, driven by specific molecular signatures for treatment selection and monitoring, potentially with immunotherapy, could be a future personalized approach.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) represents globally the fifth most common cancer and is considered the third most frequent cause of cancer related death<sup>[1]</sup>. In recent years there has been a significant progress in clarifying pathogenesis, etiology, and risk factors for hepatocarcinogenesis. Understanding the importance of underlying cirrhosis in the majority of HCCs led to more integrated approach, as in the majority of cases we have to deal with two diseases, cirrhosis and cancer.

The adoption of the barcelona-clinic liver cancer (BCLC) classification<sup>[2,3]</sup> offered the opportunity to better categorize HCC patients and select the best treatment option according to tumor stage, degree of liver function impairment and patient characteristics. The outcomes for surgical resection have improved and specific factors, as tumor and liver function characteristics, are being taken into account before the patient is referred for an operation<sup>[4]</sup>. Moreover, the widespread application of the Milan criteria in the field of transplantation, has changed the transplant procedure from an experimental approach to a standard of care therapy for HCC, which can treat at the same time the tumor and the underlying pre-neoplastic process (namely cirrhosis)<sup>[5]</sup>.

Despite screening patients at risk<sup>[6]</sup>, adopting regular surveillance rules and the impressive improvements in imaging, still many patients with HCCs are diagnosed in an advanced stage, thus being ineligible for radical treatments [transplantation, resection or Radiofrequency ablation (RFA)] or even for ablative techniques [transcatheter arterial chemoembolization (TACE)] that can also provide survival benefit<sup>[7]</sup>.

Patients with advanced HCC, especially if complicated with advanced cirrhosis, have a dismal prognosis. Several therapeutic efforts on this group of patients gave disappointing results in the past. The complete failure of systemic chemotherapy in previous years gradually shifted HCC treatment to the molecular targeted therapies. The first successful trials of sorafenib<sup>[8,9]</sup> provided a meaningful survival benefit in patients with advanced HCC, leaving at the same time many unresolved issues. This review attempts to present the effort of the scientific research to address the problem of HCC in multiple levels and to critically evaluate the inadequacies of the current trials of systemic treatments.

## THE STORY OF NEAR-FAILED SYSTEMIC TREATMENTS

Initial approaches with systemic therapy were ineffective,

as HCC is refractory to conventional chemotherapy and poorly tolerable in the context of liver cirrhosis due to altered drug metabolism and toxicity. Initial evidence for some efficacy of the anti-estrogen agent Tamoxifen in small trials were not confirmed in larger clinical trials and the drug has been abandoned<sup>[10]</sup>.

More interesting data came in to light with clinical studies of Somatostatin and its long acting analogues for advanced HCC with very promising initial results<sup>[11,12]</sup>, given the antiproliferative activity of the hormone and the positivity of HCC in somatostatin receptors in roughly 40% of the tumors<sup>[13]</sup>. Further publications have documented that somatostatin leads to apoptosis and has antineoplastic properties. Nevertheless, randomized trials - mainly from western countries - did not identify a clear survival benefit and this treatment is no longer recommended. There has been criticism for the methodology of these trials and the heterogeneity of selected patient population<sup>[14]</sup>.

Sorafenib, the only currently approved systemic treatment, that demonstrated statistically significant improvement in overall survival and prolonged time to progression in two large randomized controlled trials (Sharp and Asian Pacific)<sup>[8,15]</sup>. The efficacy of Sorafenib has been attributed to blockade of multiple kinases, most of them involved in the VEGF, PDGF, c-Kit and B-Raf and p38 signaling pathways<sup>[16]</sup>. Despite the low response rates and the associated toxicity, the drug showed survival benefit in Child's A patients with a good performance status.

The safety and efficacy of this treatment was further investigated in the Gideon trial (global phase IV, ongoing), focusing on patients with Child's B that were under represented in the registration trials. The interim analysis showed better outcomes for patients on the full dose (800 mg) as compared to the reduced (400 mg) dose, without significant differences in safety profile<sup>[17,18]</sup>. However, the median life expectancy of patients under Sorafenib treatment is generally less than one year, and this clearly needs to be improved. For the time being there are no validated factors to predict effectiveness or the possibility of adverse effects<sup>[19]</sup>.

More issues are still open, as what to do when the patient fails to respond or is intolerant to Sorafenib, or if Sorafenib could have a role as adjuvant treatment to other modalities like TACE. More data are expected and towards this direction is a recent study showed that tumor associated neutrophils (TAN) mediate the intratumoral infiltration of macrophages and Tregs by secreting the chemotactic C-C motif ligands CCL2 and CCL17. Thus neovascularization is being stimulated, and HCC growth and metastasis are promoted, all contributing to resistance to Sorafenib<sup>[20]</sup>. Thus, TAN infiltration is proposed as a potential biomarker.

Sunitinib, a potent multi-targeted receptor tyrosine kinase inhibitor of VEGFR, PDGFR, and c-KIT, reached to phase III study as compared to Sorafenib. The trial was terminated prematurely due to higher incidence of side effects in the sunitinib arm, besides demonstrating no superiority over sorafenib<sup>[21]</sup>.

Brivanib is a potent and selective inhibitor of VEGFR and FGFR and pre-clinical studies have shown *in vivo* antitumor activity<sup>[22]</sup>. Three phase III studies have been conducted, yielding negative results. The BRISK-FL study tested the efficacy of Brivanib vs Sorafenib, in patients with advanced HCC without prior systemic treatment<sup>[23]</sup>. The BRISK-PS study tested Brivanib vs placebo in patients that failed or were intolerant to Sorafenib<sup>[24]</sup>. In both studies Brivanib failed to improve OS but it did improve time to tumor progression (TTP), indicating some anti-tumor activity. Due to these results, a phase III trial in which Brivanib was used as an adjuvant to TACE was terminated prematurely<sup>[25]</sup>.

Linifanib is a multi-targeted receptor tyrosine kinase inhibitor effective on VEGFR and PDGFR. A phase III trial with 1035 patients comparing Sorafenib with Linifanib, showed similar overall survival in advanced HCC with a more favorable safety profile for Sorafenib; predefined superiority and non-inferiority overall survival boundaries were not met by Linifanib, which was more toxic than Sorafenib<sup>[26]</sup>.

Erlotinib is an orally active inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase. A phase III randomized trial (SEARCH) with 720 HCC patients (Child A cirrhosis) were assigned to Sorafenib/Erlotinib or Sorafenib/placebo<sup>[27]</sup>. The median OS and TTP were similar in both groups, thus adding Erlotinib to Sorafenib did not improve survival, but increased toxicity instead.

Dovotinib, a VEGFR, PDGFR, FGFR inhibitor was compared head to head with Sorafenib, in a randomized study in the Asian-Pacific in patients with advanced HCC. Although Dovotinib was well tolerated, it failed to show greater efficacy than sorafenib, and thus there will be no phase III trial<sup>[28]</sup>.

In patients who stopped Sorafenib due to disease progression or intolerance, a randomized phase III trial assessed Ramucirumab, a recombinant monoclonal IgG1 and VEGFR-2 blocking antibody (REACH). Despite acceptable safety profile, the study drug did not reach statistically significant survival benefit vs placebo<sup>[29]</sup>. However, a sub-population with  $\alpha$ FP > 400 ng/mL might have benefited from this 2<sup>nd</sup> line treatment and this is explored in an ongoing trial. Recently Codrituzumab, a humanized monoclonal antibody against Glypican-3 which is expressed in HCC, was studied vs placebo in a phase II randomized trial without showing any clinical benefit<sup>[30]</sup>.

Tivantinib is an oral selective small MET tyrosine kinase inhibitor with antitumor activity in MET-high patients. A phase II randomized placebo-controlled study in patients with advanced HCC, Child's A score and intolerant or progressing under the first line treatment, showed some promising results on time to progression, but with notable neutropenia in some patients<sup>[31]</sup>. A phase III study in patients with advanced HCC expressing high levels of c-MET after Sorafenib failure is underway.

Mammalian target of rapamycin (mTOR) regulates cell growth, metabolism and aging in response to nutrients, cellular energy state and growth factors<sup>[32]</sup>. It is

frequently up-regulated in cancer, including HCC, and is associated with poor differentiation and bad prognosis. Blocking this pathway appears an attractive option for HCC treatment. It is well known from the research on transplantation - given its immunosuppressive properties - that mTOR inhibitors (Sirolimus) are associated with better clinical outcomes in patients transplanted for HCC<sup>[33,34]</sup>.

Preliminary data in the non-transplant setting with Sirolimus and Everolimus treatment in HCC patients were encouraging. In the EVOLVE-1 phase III study, patients with advanced HCC and failure/intolerance to Sorafenib, randomized to Everolimus or placebo<sup>[35]</sup>. Everolimus did not improve OS with no difference to TTP vs placebo. Moreover, Everolimus led to hepatitis B virus (HBV) reactivation in 37% of the cases despite preventive antiviral therapies. A recent phase II randomized trial of the combination of Everolimus with Sorafenib vs Sorafenib alone, in patients with advanced HCC with Child's score  $\leq 7$ , showed that the combination was not more beneficial; in contrast it was more toxic<sup>[36]</sup>.

TACE is the treatment of choice for intermediate stage HCC. However, following TACE the hypoxic micro-environment promotes up-regulation of proangiogenic factors as VEGF and PDGF. This is the theoretical basis for the combination of TACE with drugs that inhibit angiogenesis, as Sorafenib and Brivanib. A recent review and meta-analysis reported that this combined approach may bring benefit to unresectable HCC in terms of TTP but not OS<sup>[37]</sup>. Recent studies (START, SOCRATES) that investigated the efficacy and safety of Sorafenib as an adjuvant to TACE displayed good tolerability and interesting response rate<sup>[38,39]</sup>. Clearly a better defined population of advanced HCC -that might have the maximal benefit from this approach- should be tested in clinical trial. Unfortunately, the recently published SPACE trial<sup>[40]</sup> showed that despite the combination of DC beads TACE with Sorafenib was feasible, this combination did not actually improve time to tumor progression in intermediate HCC.

Beyond TACE, efficacy and safety of Sorafenib was studied in a randomized phase III trial vs placebo, in patients with HCC after resection or local ablation (STORM trial)<sup>[41]</sup>. The recurrence free survival was identical in the two arms, whereas side effects were significantly more frequent in patients receiving Sorafenib in whom dose modification was necessary in 90% of the cases.

The combination of Sorafenib with other cytotoxic agents was tested to improve the disappointing results of conventional chemotherapy. In a phase II trial<sup>[42]</sup> the combination of Sorafenib/Doxorubicin was compared to Doxorubicin alone in Child-A cirrhotic patients with advanced HCC. The trial showed that the combination was better than doxorubicin alone as regards time to progression and overall survival. Whether there is benefit of the combination or this is an effect of sorafenib itself, will be clarified in an on-going phase III trial.

The efficacy and safety of GEMOX (Gemcitabin/Oxaliplatin) plus sorafenib, followed by sorafenib mono-

**Table 1 Randomized Phase III trials in advanced hepatocellular carcinoma**

Drug	<i>n</i> (patients)	OS (mo) SOR/Exp arm	HR
First line completed (Sorafenib standard)			
Brivanib	1155	9.9/9.5	1.06
Sunitinib	1074	10.2/7.9	1.3
Sorafenib/Erlotinib	720	8.5/9.5	0.92
Linifanib	1035	9.8/9.1	1.04
Second line completed (placebo standard)			
Brivanib	395	8.2/9.4	0.89
Everolimus	546	7.3/7.6	1.05
Ramucirumab	565	7.6/9.2	0.86

OS: Overall survival; SOR: Sorafenib arm.

therapy was examined in a small trial with 49 patients diagnosed with advanced HCC<sup>[43]</sup>. This approach was found effective (overall survival 15.7 mo) with manageable toxicity, and these results should be validated in a larger controlled trial. The data of a subsequent phase II randomized study on this combination, as well as the results of a single arm phase II study combining sorafenib with oxaliplatin/capecitabine, showed modest synergistic effect<sup>[44]</sup>. Further combinations that were tested, such as sorafenib with EGFR inhibitors or with mTOR inhibitors, both failed to show any meaningful antitumor activity.

Finally, the combination of Sorafenib with Octreotide was tested in a phase II study, recruiting 50 patients with advanced HCC and Child-Pugh score A or B<sup>[45]</sup>. The combination was well tolerated and displayed TTP 7 mo and median overall survival 12 mo. Nevertheless these results have not been confirmed in a larger phase III study as yet. We believe that this combination could provide an option for patients with inadequate response or intolerance to sorafenib (Figure 1).

The apparent failure of phase III trials beyond sorafenib, was disappointing but not discouraging for the scientific community (Table 1). Factors contributing to this failure and were related to drug toxicity (especially in cirrhotic patients), lack of significant antitumoural potency, lack of our understanding on diverse mechanisms of tumor progression and metastasis or biomarkers predictive of the efficacy of therapy<sup>[16]</sup>. Study design was another weak point for some trials. Trials in patients with advanced HCC should also pay attention to specific factors as portal vein invasion, the extrahepatic metastases, and the degree of liver impairment.

## EXPLORING THE ETIOPATHOGENESIS

### *Molecular and phenotypic diversity of HCC - Oncogenic pathways*

Beyond the success and wide adoption of the BCLC system on staging and prognosis of HCC<sup>[46]</sup>, recently new molecular classifications based on genetic characteristics of the tissue microenvironment have been proposed. However, HCC is a heterogeneous disease and each tumor is a result of unique combination of several genomic defects that lead to a significant diversity in the pathways

of carcinogenesis. It is documented that several differences exist not only amongst different patients, but also between different tumor nodules in the same liver, and even differences in the same nodule.

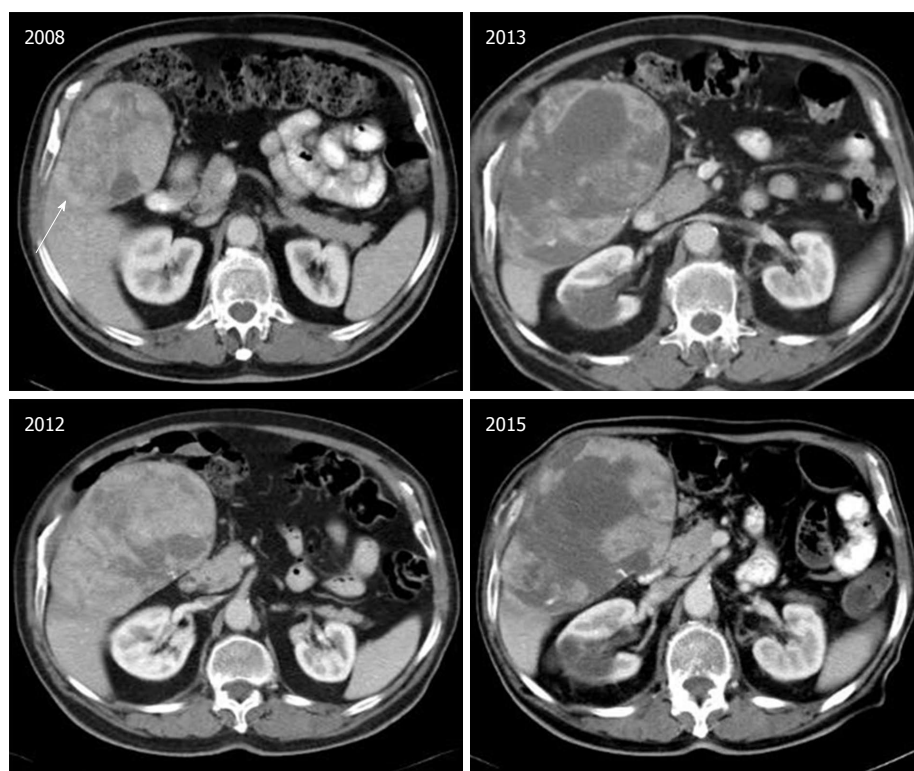
Cancer cells and stem cells have similar capacity as regards self-renewal, indefinite division, and generation of heterogeneous cell population. The concept of cancer stem cell, referring to a subset of cells bearing stem cell characteristics that is indispensable for tumour development and perpetuation, has been recently adopted<sup>[47]</sup>. Cancer stem cells are now considered an important target for the eradication of HCC. Furthermore, a 20%-40% of HCC subtypes show progenitor signature suggesting that these tumours derive from liver progenitor cell. These subtypes are highly aggressive and correlate with early recurrence after treatment and metastatic potential, thus correlated to worse prognosis. CD133 antigen (prominin-1) has been identified as a cancer stem cell marker in various cancers, including HCC. Patients with increased CD133 levels have shorter overall survival and higher recurrence rates compared to those with low expression. Recent data showed that IL-6/STAT3 signalling induced CD133 expression, through function co-operation with NF- $\kappa$ B and hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) during hepatocarcinogenesis<sup>[48]</sup> (Figure 2A).

Recently genomic based analyses in HCC patients have identified subclasses, based on molecular characteristics and proliferative and non-proliferative genotypes have been proposed<sup>[49]</sup>. The proliferative subclass - which is associated with a poor outcome - has been linked to the activation of RAS, mTOR, and/or IGF signaling. This has been further categorized into two phenotypic groups: The Wnt/TGF- $\beta$  group (activation of these pathways) and the progenitor cell group. In the former, the activation of the Wnt and the TGF- $\beta$  was the predominant feature, while the latter was enriched in progenitor cell, epithelial cell adhesion molecule and cytoskeletal markers and was associated with increased  $\alpha$ -fetoprotein at early stages<sup>[50]</sup>. On the other hand, the non-proliferative subclass was a heterogeneous one, with patients sharing only  $\beta$ -catenin in their molecular profiles<sup>[51]</sup>. The prognostic implications of these subclasses have been studied but there is no consensus on it and there is no translational clinical research has been done yet.

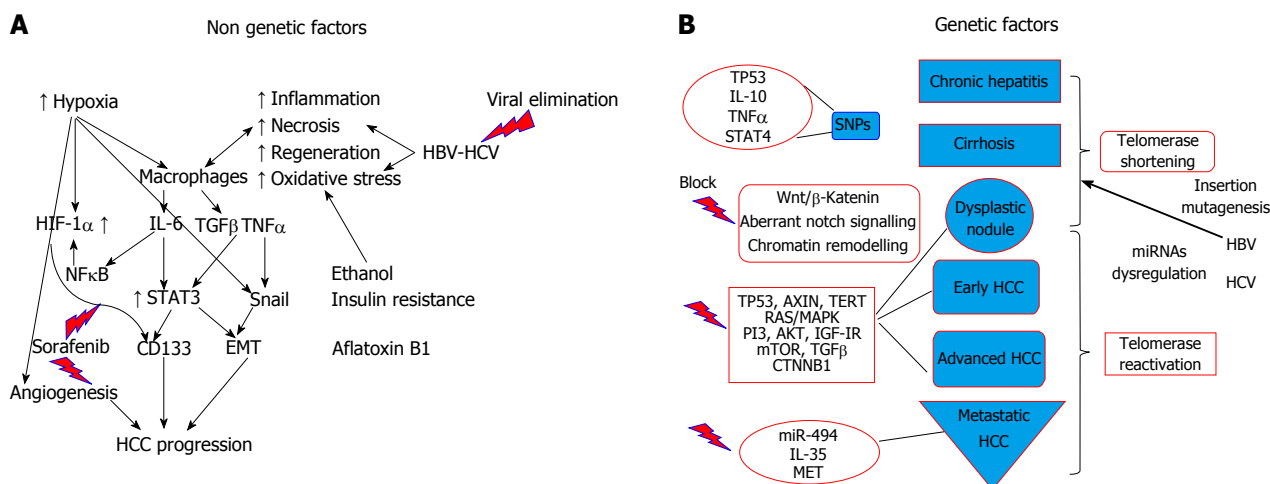
The paradigm from the management of other cancers such as colorectal cancer and non-small lung cell carcinoma, where mutations of K-Ras and EGFR drive the therapeutic choices, supports this new approach<sup>[52]</sup>. Unfortunately, HCC is still away from this path despite the success of sorafenib, a multi kinase inhibitor, which seems a proof towards the right direction. A key point may be that in HCC an average of 30-40 mutations were estimated per tumor, with 5-8 of them being the driver mutations<sup>[49]</sup> affecting cellular homeostasis and involved in the development of malignant phenotype.

A recent elegant study performing exome sequencing analysis of 243 surgically resected HCCs revealed mutational signatures associated with specific risk factors, as combined tobacco and alcohol use, or aflatoxin





**Figure 1** Serial computed tomography scans of a hepatocellular carcinoma patient with multiple co-morbidities precluding radical treatment, surviving 7 years with sequential approach in systemic treatment (Octreotide long acting release, followed by sorafenib). Despite an increase in tumor size, it is evident the central necrosis related to Sorafenib treatment (which was commenced when it became available).



**Figure 2** Interplay between genetic and non-genetic factors in the pathogenesis of hepatocellular carcinoma. Potential treatment targets. Hepatocellular carcinoma (HCC) is a complex entity with multifactorial pathogenesis. Control of non-genetic factors (A) (e.g., viral elimination, inhibition of CD133 positive cancer cell overexpression) may lead to alteration of the progress from cirrhosis to HCC. On the other hand, the various genetic irregularities (B) may lead to different HCC profiles with respect to invasiveness (miR-494) or response to treatment. New targeted treatments are also directed against Wnt/β-katenin. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIF-1α: Hypoxia-inducible factor 1 alpha; TNF: Tumor necrosis factor; IL-6: Interleukin-6; NFκB: Nuclear factor-kappa B.

B1. The researchers identified 161 putative driver genes associated with 11 recurrent pathways<sup>[53]</sup>. Moreover, a molecular 5 gene score (based on combined expression level of HN1, RAN, RAMP3, KRT19, and TAF9) was studied in surgical resected samples of 314 HCC, and was found significantly associated with outcomes<sup>[54]</sup>. Also recent data show that it is possible to modulate gene expression profiles (interfering with histone acetylation)

and thus increase the sensitivity to chemotherapeutic agents<sup>[55]</sup>.

Activation of telomerase is the earliest and most frequent alteration in the process of HCC development (mutations in TERT promoter in 60% - most frequently mutated gene - associated with increased telomerase expression)<sup>[56]</sup>. Genes as *TP53* and *CTNNB1* are also frequently mutated in HCC, whereas inactivating muta-

tions in TP53 are commonly found (especially with HBV etiology). Recently identified alterations in genes encoding metabolic enzymes, chromatin remodelers and a high rate of mTOR pathway activations could offer potential therapeutic targets<sup>[57]</sup>. Members of the Wnt pathway (crucial for hepatocarcinogenesis) are involved in the process of cell differentiation, which is frequently altered in cancer cells, whereas failure to control oxidative stress can favor additional DNA mutations and cellular damage.

Key carcinogenic signaling pathways have been described for HCC: Wnt/ $\beta$ -catenin [that can be triggered *via* both catenin  $\beta$ 1 (CTNNB1)-dependent and CTNNB1 independent pathways]<sup>[58]</sup>, a proliferation and hepatoblastoma-like pathway<sup>[59]</sup>. Nevertheless, their molecular signature is broad and for the time being this knowledge is unlikely to have clinical application. Notch signaling is important for normal liver development and aberrant Notch signaling is related to hepatocarcinogenesis (Figure 2B). Chromatin remodeling is important for the maintenance of DNA integrity, which is in turn crucial for cellular homeostasis. Aberrant chromatin remodeling has been implicated with HCC pathogenesis<sup>[57]</sup> as well as genes that are involved in oxidative stress (which induces mutations).

In respect to the receptor signaling pathways, RAS/MAPK pathway is activated in all patients with advanced HCC and in a large proportion of those with an early stage HCC<sup>[60]</sup>. PI3/AKT/mTOR and MAPK pathways related to proliferation, apoptosis and survival, as well as pro inflammatory cytokines (IL1, TNF $\alpha$ ) and growth factors, such as TGF $\beta$  (tumor stroma, progression, metastasis), are potential future clinical targets in HCC therapeutics (Figure 2B).

Very recently, IL-35 expression was found to correlate with HCC aggressiveness, conferring the rationale for another novel therapeutic target<sup>[61]</sup>. IFG-1R signalling is activated in a proportion of patients with HCC and its targeting had demonstrated antitumor activity in experimental models; however a phase II trial with an anti-IFG-1R monoclonal antibody did not show clinical benefit in unselected patients<sup>[62]</sup>. Finally, dysregulation of MET receptor and its ligand HGF, are crucial for hepatocyte regeneration after liver injury and are common events in HCC patients<sup>[49]</sup>. Activation of MET is found in half of advanced HCCs, and this pathway is currently tested in clinical trials. A MET inhibitor, cabozantinib, was found to suppress tumour growth and metastasis in a phase II study<sup>[63]</sup> and is further tested in a phase III second line clinical trial (in patients with high MET expression, treated with tivantinib).

### **Tissue microenvironment and the role of cirrhosis**

**Scientific basis:** Chronic liver injury triggers a sequence of cell death, inflammation, compensatory regeneration and genetic damage, which drives the development of HCC. In the majority of cases, HCC develops in chronically damaged tissue due to cirrhosis-irrespective of etiology - whereas the other malignancies develop on

an otherwise healthy tissue. This, together with genome instability, contributes to a significant heterogeneity which is further enhanced by the molecular differences of the underlying causes, *i.e.*, viral, alcohol, metabolic<sup>[52]</sup>. Moreover, epithelial plasticity is an important parameter in HCC, as strong inducers of epithelial to mesenchymal transition like TGF $\beta$  are able to co-ordinate both fibrogenesis and carcinogenesis, showing rising cytokine levels in cirrhosis as well as late stage HCC<sup>[64]</sup>.

Several different cell types and molecules constitute a microenvironment in the liver, which has significant implications in tumor development and invasion. Myeloid cells, including macrophages and neutrophils are the most abundant cells in the tumor microenvironment<sup>[65]</sup>. Tumor-associated macrophages acquire protumorigenic properties in primary and metastatic sites and support cancer development and progression, by stimulating cell proliferation and survival, angiogenesis, invasive behavior and suppression of cytotoxic T lymphocytes responses<sup>[66]</sup>. Tumor-associated neutrophils exhibit both antitumoral and protumoral functions. Dendritic cells, the main type of antigen presenting cells, play an important role in T cell priming. The generation and protective antitumour immunity depends on dendritic cell maturation and antigen presentation<sup>[67]</sup>.

It is generally accepted that dysregulated microenvironment affects tumorigenesis, based on the concept that chronic inflammation is associated with cancer<sup>[68]</sup>. Moreover, the stromal microenvironment has been recognized as a crucial element for cancer metastasis in general. A reasonable hypothesis is that an altered liver microenvironment, through reprogramming of the inflammatory milieu, may contribute to hepatocarcinogenesis, taking in account that HCC is an inflammation-associated cancer<sup>[69]</sup>. This microenvironment plays a major role in anti-tumor immunity.

**Therapeutic implications:** The effectiveness of the currently approved systemic therapy, sorafenib, is due to the successful combination of targeting cancer cells and their microenvironment, as a result of multiple kinases inhibition. Between sorafenib targets, an increasing amount of evidence has suggested that HSC are key regulators of hepatocarcinogenesis through a variety of mechanisms, including direct effects on malignant hepatocytes, and indirect *via* modulation of the peritumoral stroma and immune responses<sup>[70]</sup>. Moreover, activated stellate cells produce extracellular matrix.

Laminin-332 is produced and excreted by these cells in HCC but not in the surrounding non-neoplastic liver; this stimulates chemotaxis and migration of HCC cells in experimental models and promotes proliferation as well<sup>[52]</sup>. An association between Ln-332 and Keratin -19 has been documented, the latter being a marker of cholangiocytes<sup>[71]</sup>.

VEGF not only regulates tumor angiogenesis but also has important immunomodulatory functions. It inhibits dendritic cell maturation *in vitro* and *in vivo*, through activation of NF $\kappa$ B. Additionally VEGF may regulate T-cell

differentiation and its cytotoxic function and can enhance expression of immune checkpoint molecules<sup>[72]</sup>. This provides the rational of combining anti-VEGF therapy with checkpoint inhibitors.

Another area of active research is on the effect of mTOR inhibitors on advanced HCC in the non-transplant setting. Recent data showed that mTOR inhibition improves FGFR targeting<sup>[73]</sup> and reduces the activity level of Golgi protein 73, which is a serum marker for HCC<sup>[74]</sup>. However, the first results of trials with mTOR inhibitors were less than encouraging. Despite potential applications, the role of the whole tissue micro-environment is difficult to be reduced to the effect of just one molecule or protein.

### Immunity and implications

**Scientific basis:** Inflammation affects every single step of tumourigenesis from initiation, to tumor promotion and metastatic progress. Cancer development and its response to treatment are significantly influenced by innate and adaptive immunity, which either promote or attenuate tumourigenesis<sup>[66]</sup>. Various types of immune and inflammatory cells are present within tumours; these affect malignant cells through production of cytokines, chemokines, growth factors, prostaglandins and reactive oxygen and nitrogen species<sup>[68]</sup>.

The liver has been considered as an immunologically advantaged organ. A profound clinical paradigm is the development of tolerance in the context of transplantation. It is equipped with several myeloid and non-myeloid cell populations which affect both innate and adaptive responses in physiological conditions as well as in the context of defense against tumors<sup>[75]</sup>.

Kupffer cells represent the largest macrophage population in the human body and together with sinusoidal endothelial and hepatic stellate cells, play a critical role in physiology and disease. Local immunosuppression by these cells is induced by pro-inflammatory cytokines<sup>[69]</sup> whereas different immune cell subtypes have been related to antitumor immunity in HCC. Kupffer cells in analogy to the two subtypes of macrophages are now characterized as M1 and M2 types. In the case of HCC M2 cells are detrimental and M1 demonstrate anti-tumoral activity, contrary to the opposite effects of those cell subpopulations have in inflammation.

Among immunosuppressive cell populations, myeloid derived suppressor cells and T regulatory cells have the key role in cancer immunosurveillance<sup>[76]</sup>. A prominent humoral cytokine profile occurs in metastatic liver milieu and a shift towards anti-inflammatory/ immunosuppressive responses is significant for HCC metastases<sup>[77]</sup>.

**Therapeutic implications:** The liver is a privileged organ with respect to immune function and possesses a unique form of immune regulation: Tolerance is induced to avoid chronic inflammation caused by antigens coming from the portal vein blood. This may hampers an effective immune response against cancer cells<sup>[78]</sup>. Moreover this

is a challenge on the use of conventional immunotherapy is challenged. Immunotherapy trials have so far given suboptimal results. On the other hand, spontaneous immune responses as well as tumor regression have been reported in relation to systemic inflammatory responses<sup>[79]</sup>. This could as well be a result of M1 effect as previously mentioned.

Adaptive immune responses are well described in various conventional HCC treatments and are related to their effects. This has been extensively investigated in patients undergoing ablative therapies (TACE, RFA), and provide the theoretical basis for combined approaches. This applies to cytotoxic agents as well, and experience with sorafenib in experimental and clinical level is a paradigm.

While growing tumors acquire mutations, some of which create neoantigens that influence the response of patients to immune checkpoint inhibitors<sup>[80]</sup>. There are other studies supporting that cancers with high rate of somatic mutations respond best to immune check point blockade by triggering tumor rejection *via* activation of cytotoxic T-lymphocytes, a recent approach with acknowledged success in recent years in melanoma and non-small cell lung cancer<sup>[81]</sup>.

Preclinical and clinical studies have shown potential benefit of modulating immunogenicity of HCC and relevant approaches are currently being tested<sup>[82]</sup>. The rational to target immune-checkpoints is based on data that HCCs may evade the immune system by expressing molecules as PD-1, CTLA-4, TIM-3, LAG-3 and many more. Despite the fact that the blockade of PD-1 and CTLA-4 is already providing encouraging results in initial trials, overall the therapeutic relevance of blocking these agents is unclear<sup>[72]</sup>.

## CONCLUSION

HCC is one of the most lethal cancers and management still deems ineffective. Apart from the problems in prevention or early diagnosis, there are no persuasive answers for those (many) patients with advanced neoplasms. Systemic treatment was disappointing in the past, somehow improved with Sorafenib but with many weaknesses and grey zones, whereas the trials of new compounds beyond Sorafenib provided suboptimal results.

The complexity and heterogeneity of HCC pathogenesis is disregarded in treatment decisions. Is a personalized approach feasible with the limitations of current knowledge? Tumor and adjacent tissue profiling seems biologically significant, but not yet translated into the clinical setting. The role of liquid biopsy, *i.e.*, detection of circulating tumor cells, a hot topic in tumor biology is also inadequately explored in the case of HCC.

Nevertheless, encouraging first results with molecular - genetic signatures are promising towards -at least- prognosis. Additionally, miRNAs which are important regulators of gene expression, have been associated



with the occurrence of HCC. In addition, miRNAs are of potential value not only in diagnosis but also in the management of HCC.

Clinical scoring systems incorporating molecular profile characteristics, may better stratify patients at risk for HCC but further prospective validation is needed. The ideal future approach would be combined targeted therapies - driven by specific molecular signatures for the selection and the monitoring during treatment-potentially incorporating immunotherapeutic modalities, such as vaccination and/or check-point blockade.

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Case Control Study

## Predictors for advanced fibrosis in morbidly obese non-alcoholic fatty liver patients

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### Abstract

#### AIM

To investigate predictors for fibrosis specifically in a high risk population of morbidly obese patients, including detailed evaluation of lifestyle.

#### METHODS

We conducted a cross-sectional study among morbidly obese patients attending the bariatric clinic at the Tel-Aviv Medical Center between the years 2013-2014 with body mass index (BMI) above 40 or above 35 with co-morbidity. Patients with serum hepatitis B surface antigen or anti-hepatitis C virus antibodies, genetic liver diseases, autoimmune disease or high alcohol intake ( $\geq 30$  g/d in men or  $\geq 20$  g/d in women) were excluded from the study. Liver fibrosis was estimated by transient elastography (FibroScan®), using the "XL" probe. We collected data on age and gender, education, smoking status and amount, medical history, nutrition and lifestyle habits. All these data were collected using structured and validated questionnaires. Fasting blood test were available for a subsample.

#### RESULTS

Fibroscan was performed on a total of 91 patients, of which 77 had a valid examination according to the



accepted criteria. Of those, 21% had significant fibrosis (F2) and 39% had advanced or severe fibrosis (F3 or F4). In multivariate analysis, male gender and BMI had a positive association with advanced fibrosis; the OR for fibrosis  $F \geq 2$  was 7.93 (95%CI: 2.36-26.64,  $P = 0.001$ ) for male gender and 1.33 (1.11-1.60 kg/m<sup>2</sup>,  $P = 0.002$ ) for BMI. The OR for fibrosis  $F \geq 3$  was 2.92 (1.08-7.91,  $P = 0.035$ ) for male gender and 1.17 (1.03-1.33,  $P = 0.018$ ) for BMI. Subjects were categorized to subgroups based on the combination of male gender and BMI of 40 and above. A significant dose response association with stiffness level was noted across these categories, with the highest stiffness among men with a higher BMI ( $P = 0.001$ ). In addition, a significant positive correlation between pack-years cigarette smoking and liver stiffness was demonstrated among men ( $r = 0.54$ ,  $P = 0.012$ ).

## CONCLUSION

In the morbidly obese population, a higher BMI, male gender and degree of smoking in men bears a greater risk for advanced nonalcoholic fatty liver disease.

**Key words:** Non-alcoholic fatty liver disease; Morbid obesity; Fibrosis; Fibroscan; Diet

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**Core tip:** The presented results indicate that male gender and a higher body mass index (BMI) are risk factors for advanced fibrosis in morbidly obese patients. There is also a positive correlation between cigarette smoking and liver stiffness in men. Our study highlights the fact that even in the upper BMI ranges, higher BMI bears greater risk for advanced disease. Therefore, in the morbidly obese population it may seem useful to emphasize the importance of weight reduction, even within the range of obesity.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in the developed<sup>[1]</sup> and developing countries<sup>[2,3]</sup> with estimated prevalence of 20%-40%, and is predicted to become the leading indication for liver transplantation in the United States<sup>[4]</sup>. NAFLD is tightly associated with the metabolic syndrome and its complications. Obesity is the most important risk factor for NAFLD, which affects as much as 74% of obese individuals<sup>[5]</sup>. Two large electronic databases have demonstrated a clear association between a higher body mass index (BMI), diabetes and male gender and the risk for

NAFLD<sup>[6]</sup>. Furthermore, among morbidly obese patients, who are candidates for bariatric surgeries, the prevalence is even higher and reaches 96%<sup>[7-10]</sup>.

NAFLD encompasses a wide spectrum of histological and clinical manifestations, ranging from simple steatosis to steatohepatitis, fibrosis and cirrhosis<sup>[11]</sup>. It is estimated that approximately 6%-13% of patients with simple steatosis progress to steatohepatitis, of which approximately 10%-29% reach liver cirrhosis within 10 years<sup>[12]</sup>. Moreover, non-alcoholic steatohepatitis cirrhosis is a known risk factor for hepatocellular carcinoma<sup>[4]</sup>. Given the relative high prevalence of severe fibrosis (12%) in morbidly obese patients<sup>[7-10]</sup>, this population is especially prone to a detrimental course. Therefore, it is of utmost importance to identify those patients with high likelihood for advanced fibrosis who may later develop cirrhosis and hepatocellular carcinoma.

Several studies have aimed to find predictors and risk factors for advanced fibrosis, although most of them did not focus on morbidly obese population. In a study of 103 NAFLD patients who underwent serial liver biopsies to follow fibrosis progression rate<sup>[13]</sup>, only type-2 diabetes mellitus (T2DM), BMI and initial stage of fibrosis were associated with a higher rate of disease progression. Of note, in this study only 68% of patients were obese. Sub-analysis of 3041 subjects from the Rotterdam study<sup>[14]</sup> revealed that liver stiffness above 8 kilopascals (kPa), as measured by FibroScan, was strongly associated with steatosis and T2DM. In this cohort the average BMI was 27, thus not representing a morbidly obese population. With respect to lifestyle and other co-morbidities, smoking and obstructive sleep-apnea were demonstrated to be positively associated with liver fibrosis<sup>[15,16]</sup>. Once again, most of the patients were not morbidly obese with an average BMI of 34 and 28 respectively.

Given the scarce data regarding risk factors for advanced fibrosis in morbidly obese population, the aim of the present study was to investigate predictors for fibrosis, specifically in this high risk population, including detailed evaluation of lifestyle. To non-invasively assess liver fibrosis we used transient elastography (FibroScan®), which is a validated tool to determine liver stiffness<sup>[17]</sup>, and was demonstrated to be one of the most accurate tests for the non-invasive evaluation of liver fibrosis in NAFLD with a clinical prognostic value<sup>[18]</sup>.

## MATERIALS AND METHODS

We conducted a cross-sectional study among morbidly obese patients attending the bariatric clinic at the Tel-Aviv Medical Center between the years 2013-2014 with BMI above 40 or above 35 plus at least one co-morbidity (*i.e.*, hypertension, type 2 diabetes, cardiovascular disease, lung disease and respiratory disorders), according to the Israeli Health Ministry indications published on 2013 ([http://www.health.gov.il/hozer/mr33\\_2013.pdf](http://www.health.gov.il/hozer/mr33_2013.pdf)). Patients with serum HBsAg or anti-hepatitis C virus antibodies, genetic liver diseases, autoimmune disease or high alcohol intake ( $\geq 30$  g/d in men or  $\geq$

**Table 1 Clinical characteristics of the study population**

Variable	n <sup>1</sup>	Mean $\pm$ SD
Age (yr)	77	42.4 $\pm$ 12.98
Gender (male) %	77	46.8
BMI (kg/m <sup>2</sup> )	77	41.71 $\pm$ 4.68
Glucose (mg/dL) (< 100)	48	116.81 $\pm$ 35.08
Total cholesterol (mg/dL) (< 200)	42	191.97 $\pm$ 34.46
LDL (mg/dL)	42	112.80 $\pm$ 33.05
HDL (mg/dL)	40	44.19 $\pm$ 11.87
TG (mg/dL) (< 150)	42	182.38 $\pm$ 56.36
ALT (U/L) (5-39)	47	44.28 $\pm$ 44.49
AST (U/L) (7-40)	47	28.91 $\pm$ 20.11
Success rate %	77	84.31 $\pm$ 13.55
Stiffness	77	10.24 $\pm$ 6.27
Sedentary time <sup>2</sup> (min/d)	77	263.84 $\pm$ 176.56
Daily activity (score <sup>3</sup> )	77	20.56 $\pm$ 3.47
Alcohol servings/week	77	0.66 $\pm$ 1.67
Current or past smokers %	77	48.1

<sup>1</sup>Indicates on the number of people with available measure; <sup>2</sup>Time spent by using a computer, watching television or reading; <sup>3</sup>Climbing stairs, short walking and house-keeping chores, *etc.* The higher the score the lower is the activity (1-every day, 5-never). BMI: Body mass index; LDL: Low density lipoprotein; HDL: High density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate transaminase; TG: Triglycerides.

20 g/d in women)<sup>[19,20]</sup> were excluded from the study. All procedures performed in this study were approved by the institutional research committee of the Tel-Aviv Medical Center and in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all participants included in the study.

### Liver stiffness measurement

Liver stiffness measurement (LSM) was measured using the "XL" probe. LSM were considered representative only if they had at least 10 valid acquisitions with a success rate > 60%<sup>[21]</sup>. All measurements were taken by the same operator (experience, > 10000 measurements) which was blinded to other parameters of the patients. As previously described<sup>[22]</sup>, the examination was performed with the patient lying down in the dorsal decubitus position with the right arm in maximal abduction. The tip of the probe transducer was placed on the skin, between the ribs at the level of the right lobe of the liver. The results were expressed in kPa and each LSM corresponds to the median of 10 validated measurements. The cut-off values for fibrosis stage were according to the suggested best cutoffs to distinguish between fibrosis levels among NAFLD patients<sup>[23,24]</sup>: < 7.1; F0-F1: 7.1-9.5 kPa; F2: 9.6-11.5; F3: > 11.5; and F4: Significant fibrosis was defined as  $F \geq 2$ , and advanced fibrosis was defined as  $F \geq 3$ <sup>[25-27]</sup>.

### Demographic, health and lifestyle data

We collected data on age and gender, education, smoking status and amount, medical history including diabetes and medical treatment, nutrition, lifestyle habits and health status. All these data were collected using a structured and uniform questionnaire completed by all

participants, tailored for the current study based on validated questionnaires used in national Israeli surveys<sup>[28]</sup>. Fasting blood test were available for a subsample of 40-48 patients.

### Statistical analysis

Statistical analyses were performed using SPSS version 22 (SPSS Inc., Chicago, IL, United States) software. Continuous variables are presented as means  $\pm$  SD. To test differences in continuous variables between the two groups the independent samples *t*-test or the Mann-Whitney *U* test were performed. Associations between nominal variables were performed with the Pearson  $\chi^2$  test. The Pearson correlation was used for the evaluation of the correlation between liver stiffness and other measurements. A multivariate logistic regression analysis was performed to test the adjusted association between significant liver stiffness and potential predictors. Using a receiver operating characteristic curve, the best BMI cutoff point to predict significant fibrosis was 40, which represents grade-3 obesity. To test the combined effect of male gender and BMI of 40 and above (high BMI), we created a new variable with three categories: Lower BMI plus female gender, either male gender or high BMI, both male gender and high BMI. One way ANOVA of variance was used to test the difference in the distribution of liver stiffness between the categories with a *P* for trend test. Pearson  $\chi^2$  test was used to test the association between these categories and the categories of fibrosis severity with a *P* for trend test. *P* < 0.05 was considered statistically significant for all analyses.

## RESULTS

### Description of the study population

A total of 201 consecutive patients were recruited, of which 91 agreed to undergo Fibroscan exam, and 77 patients had a valid examination according to the accepted criteria<sup>[21]</sup>. As depicted in Table 1, the average BMI was 41.71  $\pm$  4.68 kg/m<sup>2</sup>, with a range between 32.25 to 56.36 kg/m<sup>2</sup>, 48 had a BMI of 40 and above. Alcohol consumption was very low and no patient had to be excluded due to excessive consumption. The available blood tests indicated impaired fasting glucose and mildly elevated triglycerides and ALT levels. Most patients (60%) had some level of fibrosis (F2 and above) according to the Fibroscan examination. Of note, 39% of the patients had advanced or severe fibrosis (F3 or F4), 21% had significant fibrosis (F2) and only 40% of the patients had minimal or no fibrosis (F0-F1) (Figure 1).

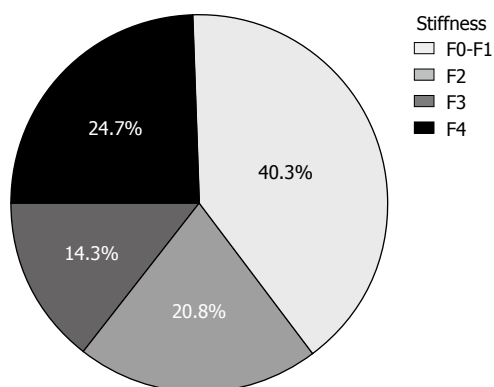
### Comparison between subjects with significant or advanced fibrosis and subjects with minimal or no fibrosis

Male gender was significantly more prevalent in subjects with fibrosis level of F2 and above or F3 and above, as compared to subjects with minimal or no fibrosis (63% vs 22.6%, *P* < 0.001 and 63.3% vs 36.2%, *P* = 0.020, respectively) (Tables 2 and 3). In addition, BMI was

**Table 2** Comparison between subjects with fibrosis degree  $F < 2$  and subjects with significant fibrosis degree  $F \geq 2$  (mean  $\pm$  SD, unless otherwise stated)

Variable	$F < 2$ ( $n = 31$ )	$F \geq 2$ ( $n = 46$ )	$P$
Gender (men) %	22.6	63	< 0.001
Age (yr)	42.32 $\pm$ 13.89	42.96 $\pm$ 12.29	0.838
Education (yr)	13.13 $\pm$ 2.05	13.78 $\pm$ 2.9	0.281
BMI ( $\text{kg}/\text{m}^2$ )	39.82 $\pm$ 3.16	42.99 $\pm$ 5.11	< 0.001
Type 2 diabetes drugs (%)	19.4	17.4	0.827
All sugared soft drinks (cups/d)	1.76 $\pm$ 2.74	1.38 $\pm$ 2.24	0.502
Carbonated sugared drinks intake (cups/d)	0.99 $\pm$ 1.52	0.89 $\pm$ 1.98	0.816
Diet carbonated drinks intake (cups/d)	0.46 $\pm$ 0.98	0.88 $\pm$ 1.6	0.196
Coffee intake (cups/d)	2.22 $\pm$ 1.87	1.77 $\pm$ 1.74	0.287
Alcohol servings/week	0.97 $\pm$ 1.80	0.46 $\pm$ 1.56	0.189
Current or past smokers (%)	48.4	47.8	0.961
Fruits intake (portions/d)	1.71 $\pm$ 1.35	1.74 $\pm$ 1.31	0.924
Vegetables intake (portions/d)	2.77 $\pm$ 2.38	2.64 $\pm$ 1.93	0.788
Fried food intake (portions/d)	1.18 $\pm$ 1.25	1.08 $\pm$ 0.77	0.690
Leisure time physical activity (min/wk)	189 $\pm$ 83.06	237.69 $\pm$ 437.89	0.733
Sedentary time <sup>1</sup> (min/d)	264.19 $\pm$ 171.7	263.61 $\pm$ 181.65	0.989
Daily activity (score <sup>2</sup> )	20.19 $\pm$ 2.83	20.8 $\pm$ 3.85	0.452
Glucose (mg/dL)	113.72 $\pm$ 42.35	119.42 $\pm$ 28.13	0.580
Total cholesterol (mg/dL)	202.68 $\pm$ 37.79	182.24 $\pm$ 28.6	0.054
LDL (mg/dL)	121.52 $\pm$ 38.77	104.04 $\pm$ 23.97	0.088
HDL (mg/dL)	46.55 $\pm$ 11.42	41.83 $\pm$ 12.13	0.213
Triglycerides (mg/dL)	170.73 $\pm$ 60.25	192 $\pm$ 52.3	0.228
ALT (U/L)	34.89 $\pm$ 37.77	50.66 $\pm$ 48.13	0.237
AST (U/L)	26.4 $\pm$ 24.47	30.78 $\pm$ 16.43	0.467
GGT (U/L)	22.52 $\pm$ 12.01	43.06 $\pm$ 23.53	0.090

<sup>1</sup>Time spent by using a computer, watching television or reading; <sup>2</sup>Climbing stairs, short walking and house-keeping chores, *etc.* The higher the score the lower is the activity (1-every day, 5-never). BMI: Body mass index; LDL: Low density lipoprotein; HDL: High density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate transaminase; GGT: Glutamyl transpeptidase.

**Figure 1** Distribution of liver stiffness levels according to Fibroscan.

significantly higher in subjects with fibrosis level of F2 and above or F3 and above as compared to subjects with minimal or no fibrosis (42.99  $\pm$  5.11  $\text{kg}/\text{m}^2$  vs 39.82  $\pm$  3.16  $\text{kg}/\text{m}^2$ ,  $P < 0.001$  and 42.32  $\pm$  5.3  $\text{kg}/\text{m}^2$  vs 40.68  $\pm$  3.96  $\text{kg}/\text{m}^2$ ,  $P = 0.023$ , respectively) (Tables 2 and 3). No other significant differences were noted between groups in nutritional, physical activity and biochemical parameters (Tables 2 and 3). Of note, there was no correlation between ALT and fibrosis (correlation  $r = 0.17$ ,  $P = 0.253$ ).

#### Multivariate analysis for the prediction of significant or advanced fibrosis

In multivariate analysis, including age, gender and BMI, the positive association between male gender (OR =

7.93, 95%CI: 2.36-26.64,  $P = 0.001$ ) and BMI (OR = 1.33, 95%CI: 1.11-1.60,  $P = 0.002$ ) and fibrosis  $F \geq 2$  was maintained. Similarly, the positive association between male gender (OR = 2.92, 95%CI: 1.08-7.91,  $P = 0.035$ ) and BMI (OR = 1.17, 95%CI: 1.03-1.33,  $P = 0.018$ ) and fibrosis  $F \geq 3$  was maintained (Table 4).

#### The dose response association of the combined categories of BMI of 40 and above (grade 3 obesity) and male gender with fibrosis level

Subjects were categorized to subgroups based on the combination of gender and BMI of 40 and above: Subgroup (1) women and BMI below 40; subgroup (2) either men or BMI of 40 and above; subgroup (3) both men and BMI of 40 and above. A significant dose response association was noted across these categories for stiffness as a continuous variable ( $P$  for trend = 0.001) (Figure 2A), for the rate of subjects with  $F \geq 2$  ( $P$  for trend < 0.001) (Figure 2B) and for the rate of subjects with  $F \geq 3$  ( $P$  for trend = 0.011) (Figure 2C). The highest stiffness or prevalence of significant/advanced fibrosis was among men with a higher BMI.

#### Cigarette smoking and fibrosis level

Twenty one of the men and 16 of the women were current or past smokers. Among them, there was a significant positive correlation between cigarette smoking measured by pack-years (number of packs multiplied with the number of years of smoking) and liver stiffness ( $r = 0.37$ ,  $P = 0.025$ ). However, stratification by gender

**Table 3** Comparison between subjects with fibrosis degree  $F < 3$  and subjects with advanced fibrosis degree  $F \geq 3$  (mean  $\pm$  SD, unless otherwise stated)

Variable	$F < 3$ ( $n = 47$ )	$F \geq 3$ ( $n = 30$ )	$P$
Gender (male) %	36.2	63.3	0.020
Age (yr)	42.47 $\pm$ 14	43.07 $\pm$ 11.09	0.836
Education (yr)	13.19 $\pm$ 2.59	14.03 $\pm$ 2.55	0.166
BMI (kg/m <sup>2</sup> )	40.68 $\pm$ 3.96	42.32 $\pm$ 5.3	0.023
Type 2 diabetes drugs (%)	17	20	0.741
All sugared soft drinks (cups/d)	1.64 $\pm$ 2.45	1.37 $\pm$ 2.46	0.635
Carbonated sugared drinks intake (cups/d)	0.9 $\pm$ 1.55	0.98 $\pm$ 2.16	0.850
Diet carbonated drinks intake (cups/d)	0.73 $\pm$ 1.44	0.68 $\pm$ 1.33	0.890
Coffee intake (cups/d)	1.96 $\pm$ 1.88	1.93 $\pm$ 1.69	0.951
Alcohol (servings/wk)	0.87 $\pm$ 1.76	0.33 $\pm$ 1.47	0.152
Current or past smokers (%)	48.9	46.7	0.846
Fruits intake (portions/d)	1.57 $\pm$ 1.25	1.97 $\pm$ 1.4	0.204
Vegetables intake (portions/d)	2.43 $\pm$ 2.11	3.12 $\pm$ 2.07	0.162
Fried food intake (portions/d)	1.12 $\pm$ 1.12	1.12 $\pm$ 0.75	0.999
Leisure time physical activity (min/wk)	155.62 $\pm$ 89.96	355.71 $\pm$ 585.97	0.403
Sedentary time <sup>1</sup> (min/d)	265.66 $\pm$ 178.79	261.00 $\pm$ 176.01	0.911
Daily activity (score <sup>2</sup> )	20.32 $\pm$ 3.49	20.93 $\pm$ 3.45	0.452
Glucose (mg/dL)	117 $\pm$ 39.93	116.53 $\pm$ 27.08	0.964
Total cholesterol (mg/dL)	197.75 $\pm$ 37.39	180.41 $\pm$ 25	0.083
LDL (mg/dL)	117.81 $\pm$ 35.94	101.61 $\pm$ 22.00	0.087
HDL (mg/dL)	44.85 $\pm$ 11.16	42.82 $\pm$ 13.6	0.619
Triglycerides (mg/dL)	176.28 $\pm$ 55.14	194.57 $\pm$ 58.84	0.328
ALT (U/L)	36.5 $\pm$ 33.26	55.76 $\pm$ 56.26	0.147
AST (U/L)	26.89 $\pm$ 21.99	31.89 $\pm$ 17.11	0.409
GGT (U/L)	29.89 $\pm$ 26.25	43.4 $\pm$ 16.96	0.241

<sup>1</sup>Time spent by using a computer, watching television or reading; <sup>2</sup>Climbing stairs, short walking and house-keeping chores, *etc.* The higher the score the lower is the activity (1-every day, 5-never). BMI: Body mass index; LDL: Low density lipoprotein; HDL: High density lipoprotein; ALT: Alanine aminotransferase; AST: Aspartate transaminase; GGT: Glutamyl transpeptidase.

**Table 4** Multivariate analysis for the prediction of significant or advanced fibrosis

Variable	Model 1: $F \geq 2$		Model 2: $F \geq 3$	
	OR (95%CI)	$P$	OR (95%CI)	$P$
Age (yr)	1.03 (0.99-1.08)	0.153	1.03 (0.98-1.07)	0.246
Gender (male)	7.93 (2.36-26.64)	0.001	2.92 (1.08-7.91)	0.035
BMI (kg/m <sup>2</sup> )	1.33 (1.11-1.60)	0.002	1.17 (1.03-1.33)	0.018

The models are adjusted for all variables listed in the model. BMI: Body mass index.

revealed that this association existed among men ( $r = 0.54$ ,  $P = 0.012$ ) (Figure 3), but not among women ( $r = -0.10$ ,  $P = 0.716$ ).

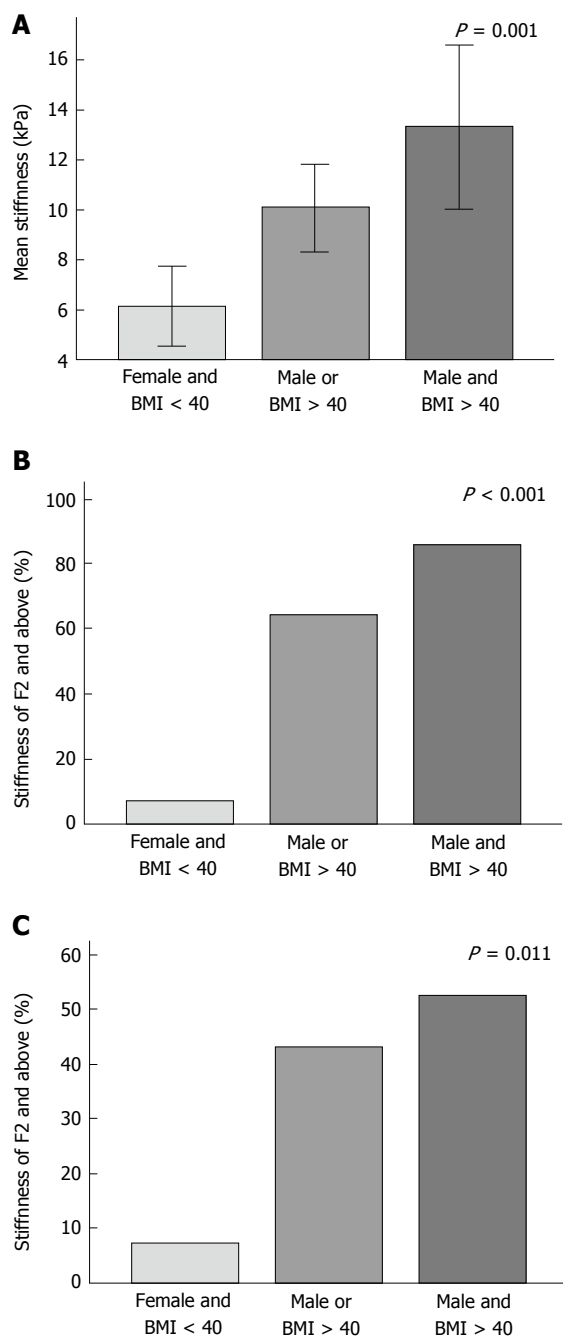
## DISCUSSION

The presented results indicate that male gender and a higher BMI are risk factors for advanced fibrosis in morbidly obese patients. Notably, there is also a positive correlation between cigarette smoking measured by pack-years and liver stiffness in men. Our data corroborate previous publications<sup>[5,7-10]</sup>, demonstrating high rate of NAFLD with significant percent of severe (F4) fibrosis in morbidly obese patients, which in our population reached approximately 25% of the patients. Given the magnitude of the disease in the morbidly obese patients, only few attempts have been performed to stratify the risk for advanced fibrosis in this unique

population, mostly among patients undergoing bariatric surgery. Ong *et al.*<sup>[29]</sup> have found in 212 consecutive morbidly obese patients in whom liver biopsy was taken during bariatric surgery, that waist to hip ratio and AST levels were independently associated with severe fibrosis. In our cohort, liver enzymes were not predictive of advanced fibrosis. In another study<sup>[7]</sup> which obtained liver biopsy from 181 patients undergoing bariatric surgery, only age was significantly associated with advanced disease (moderate and severe fibrosis), in contrast to our results which did not show such an association (Tables 2 and 3). In line with our results, Dixon *et al.*<sup>[8]</sup> have found in 105 consecutive biopsied bariatric patients that advanced fibrosis was associated with male gender and not with the presence of T2DM. In contrast, Beymer *et al.*<sup>[9]</sup> has found T2DM as the only predictor to advanced fibrosis. This discrepancy may be explained by systemic insulin resistance characterizing most of the patients in the aforementioned cohorts who have not developed overt diabetes yet. This assumption is supported by the higher C-peptide level found in patients with advanced fibrosis in Dixon's cohort<sup>[8]</sup>. Interestingly, a recent cohort analyzing 134 South Indian patients have found arterial hypertension as a sole independent risk factor for fibrosis<sup>[30]</sup>.

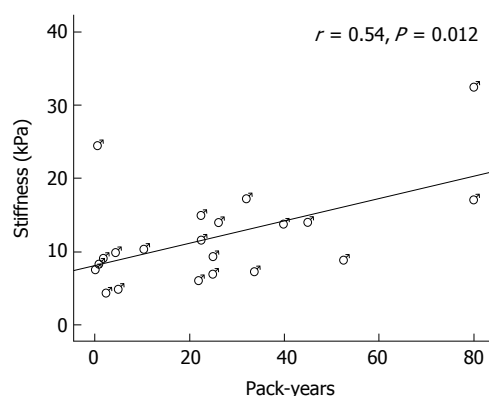
In our study we have extended the search for advanced liver fibrosis predictors toward diet elements, eating behavior and lifestyle variables, all of which have shown an association with obesity in general<sup>[31]</sup> and some of them with NAFLD in particular<sup>[32]</sup>. Whereas the





**Figure 2** Dose response relationship of the combined categories of body mass index of 40 and above (grade 3 obesity) and male gender with fibrosis level. Women + BMI < 40;  $n = 14$ , men or BMI  $\geq 40$ ;  $n = 42$ , men + BMI  $\geq 40$ ;  $n = 21$ . BMI: Body mass index.

association between active and passive smoking and NAFLD has been already demonstrated<sup>[33]</sup>, we succeeded to show a significant positive correlation between cigarette smoking measured by pack-years and liver stiffness in men (Figure 2). Our findings are corroborated by the data of the Multicenter Nonalcoholic Steatohepatitis Clinical Research Network generated from 1081 patients<sup>[16]</sup>, in which multivariate analysis has demonstrated 1.6 fold increased odds for advanced fibrosis among those with a smoking history of  $\geq 10$  pack-years. Among non-diabetics, a history of  $\geq 10$  pack-years was associated with even a higher chance of 2.5 fold for advanced fibrosis. Multiple mechanisms may be involved in smoking injury,



**Figure 3** Correlation between pack-years and liver stiffness among ever smoking men ( $n = 21$ ).

such as insulin resistance, oxidative stress and hypoxia<sup>[16]</sup>. Further studies are needed to confirm this finding among morbid obese patients and to elucidate this phenomenon.

We failed to find an association between liver stiffness and dietary parameters or eating patterns in this study. This lack of association may stem from potential sources of bias that need to be considered. First, recall and reporting bias may exist, especially on lifestyle habits and partially because of social desirability among obese population<sup>[34]</sup>. The bias was minimized by the use of structured and validated questionnaires. Also, no information about the purpose and the hypotheses of the study was provided to the participants in order to minimize the report bias. In addition, the interview was performed before receiving the results of the Fibroscan and thus the information bias is expected to be non-differential. Second, a measurement of liver stiffness is not the gold standard for the assessment of liver fibrosis. However, the Fibroscan test with the XL transducer is adjusted for patients with obesity and morbid obesity and compared with the standard transducer leads to lower rates of test failure (1.1% vs 16%) and an established validity<sup>[17,21]</sup>. However, among obese subjects, unreliable results may still be observed with the XL probe<sup>[35]</sup>. Nevertheless, despite this disadvantage, Fibroscan was demonstrated to be one of the most accurate tests for the non-invasive diagnosis of liver fibrosis in NAFLD<sup>[18]</sup>.

In conclusion, our study highlights the fact in even in the upper BMI ranges, higher BMI bears greater risk for advanced disease. In addition, male gender may a risk factor for advanced disease in the morbidly obese population. The suggested association with the degree of smoking in men will have to be confirmed in further studies with a larger sample size.

## ACKNOWLEDGMENTS

We would like to thank Ms. Stella Levit for performing the Fibroscan examinations.

## COMMENTS

### Background

Given the high prevalence of severe fibrosis (12%) in morbidly obese patients,

this population is especially prone to a detrimental course of nonalcoholic fatty liver disease (NAFLD). Therefore, it is of utmost importance to identify those patients with high likelihood for advanced fibrosis who may later develop cirrhosis and hepatocellular carcinoma.

### Research frontiers

The presented results indicate that male gender and a higher body mass index (BMI) are risk factors for advanced fibrosis in morbidly obese patients. There is also a positive correlation between cigarette smoking and liver stiffness in men.

### Innovations and breakthroughs

Their study highlights the fact in even in the upper BMI ranges, higher BMI bears greater risk for advanced disease. In addition, male gender may be a risk factor for advanced disease in the morbidly obese population.

### Applications

This study may indicate that weight reduction, even a modest one within the morbid obesity range, may be helpful in prevention of advanced fibrosis in morbid obese patients. Men may need more closer monitoring of fibrosis, and if supported by larger studies, may be advised to undergo smoking cessation.

### Terminology

NAFLD encompasses a wide spectrum of histological and clinical manifestations, ranging from simple steatosis to steatohepatitis, fibrosis and cirrhosis.

### Peer-review

This is an interesting and well-organized study. The results are clearly presented.

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Retrospective Cohort Study

# Impact of transjugular intrahepatic porto-systemic shunt on post liver transplantation outcomes: Study based on the United Network for Organ Sharing database

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## Abstract

### AIM

To determine the impact of transjugular intrahepatic porto-systemic shunt (TIPS) on post liver transplantation (LT) outcomes.

### METHODS

Utilizing the United Network for Organ Sharing (UNOS) database, we compared patients who underwent LT from 2002 to 2013 who had undergone TIPS to those without TIPS for the management of ascites while on the LT waitlist. The impact of TIPS on 30-d mortality, length of stay (LOS), and need for re-LT were studied. For evaluation of mean differences between baseline



characteristics for patients with and without TIPS, we used unpaired *t*-tests for continuous measures and  $\chi^2$  tests for categorical measures. We estimated the impact of TIPS on each of the outcome measures. Multivariate analyses were conducted on the study population to explore the effect of TIPS on 30-d mortality post-LT, need for re-LT and LOS. All covariates were included in logistic regression analysis.

## RESULTS

We included adult patients (age  $\geq 18$  years) who underwent LT from May 2002 to September 2013. Only those undergoing TIPS after listing and before liver transplant were included in the TIPS group. We excluded patients with variceal bleeding within two weeks of listing for LT and those listed for acute liver failure or hepatocellular carcinoma. Of 114770 LT in the UNOS database, 32783 (28.5%) met inclusion criteria. Of these 1366 (4.2%) had TIPS between the time of listing and LT. We found that TIPS increased the days on waitlist ( $408 \pm 553$  d) as compared to those without TIPS ( $183 \pm 330$  d),  $P < 0.001$ . Multivariate analysis showed that TIPS had no effect on 30-d post LT mortality (OR = 1.26; 95%CI: 0.91-1.76) and re-LT (OR = 0.61; 95%CI: 0.36-1.05). Pre-transplant hepatic encephalopathy added 3.46 d (95%CI: 2.37-4.55,  $P < 0.001$ ), followed by 2.16 d (95%CI: 0.92-3.38,  $P = 0.001$ ) by TIPS to LOS.

## CONCLUSION

TIPS did increase time on waitlist for LT. More importantly, TIPS was not associated with 30-d mortality and re-LT, but it did lengthen hospital LOS after transplantation.

**Key words:** Transjugular intrahepatic porto-systemic shunt; Shunt; Liver; Transplantation; Ascites; Model for end-stage liver disease; Mortality; Transjugular

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**Core tip:** The study was completed to determine the impact of transjugular intrahepatic porto-systemic shunt (TIPS) on post liver transplantation (LT) outcomes. Utilizing the United Network for Organ Sharing database, we compared patients who underwent LT from 2002 to 2013 who had undergone TIPS to those without TIPS for the management of ascites while on the LT waitlist. The impact of TIPS on 30-d mortality, length of stay (LOS), and need for re-LT were studied. TIPS was not commonly used in patients with ascites on the waitlist but did increase time on waitlist for LT. More importantly, TIPS was not associated with 30-d mortality and re-LT, but it did increase hospital LOS after transplantation.

Mumtaz K, Metwally S, Modi RM, Patel N, Tumin D, Michaels AJ, Hanje J, El-Hinnawi A, Hayes Jr D, Black SM. Impact of transjugular intrahepatic porto-systemic shunt on post liver transplantation outcomes: Study based on the United Network

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## INTRODUCTION

Transjugular intrahepatic portosystemic shunts (TIPS) play an important role in the treatment of recurrent esophageal varices, bleeding gastric varices and refractory ascites. Multiple randomized trials and meta-analyses have reported the superiority of TIPS over large volume paracentesis in controlling refractory ascites with no effect on long-term survival<sup>[1-8]</sup>. One study compared 149 patients with refractory ascites allocated to TIPS and 156 to paracentesis with significant improvement in the TIPS population regarding transplant-free survival of cirrhotic patients with refractory ascites<sup>[6]</sup>.

A few single-center studies have reported the impact of TIPS on liver transplant metrics<sup>[9-11]</sup>. When comparing TIPS vs non-TIPS patients, studies revealed comparable transfusion requirements and operative time between the two cohorts and also demonstrated operative mortality and early graft function not to be influenced by TIPS placement<sup>[9,10]</sup>. In fact, TIPS may offer an advantage in reducing ascites at the time of transplantation, which in turn may expedite the transplant time<sup>[11]</sup>.

Other single center studies explored the impact of TIPS on post-transplant survival and found no significant difference<sup>[12-14]</sup>. Guerrini *et al*<sup>[15]</sup>, however, found that patients who underwent TIPS pre-liver transplantation (pre-LT) had a lower risk of mortality at 1 year after LT. These potential advantages associated with the use of TIPS, however, are balanced by technical complications associated with it at time of LT<sup>[16]</sup>.

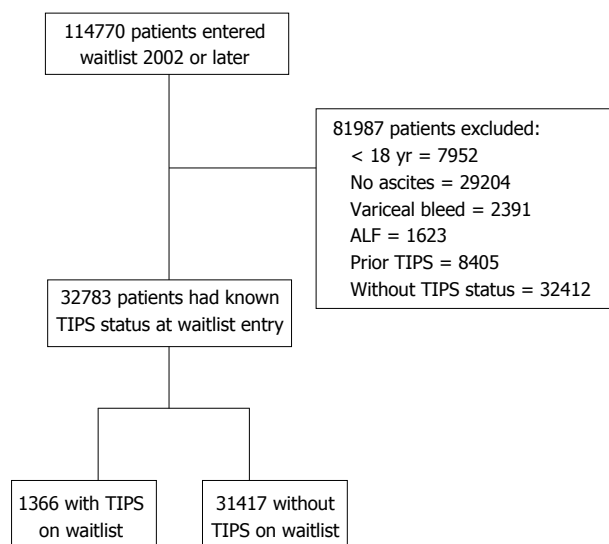
Previously, most single center studies and meta-analyses evaluating the utility of TIPS in the context of LT have explored the survival at 1 year or longer<sup>[12-14]</sup>. It appears that TIPS may improve portal hypertension related issues in immediate post-transplant setting by reducing the flow of blood in the collateral circulation, thus improving portal supply to the graft<sup>[15]</sup>. Keeping in mind the mechanism by which TIPS may be helpful or disadvantageous, it's prudent to study short-term outcomes such as 30-d mortality and re-LT.

We utilized the United Network for Organ Sharing (UNOS) database to determine if TIPS had an influence on short-term outcomes of LT. We hypothesized that TIPS is not associated with an increase in 30-d post LT mortality and rate of re-LT.

## MATERIALS AND METHODS

### Data collection

A retrospective cohort study was performed on adult LT candidates who were registered in the Organ Procurement and Transplant Network (OPTN) Standard Transplant Analysis and Research Database (Reference:



**Figure 1** Flow diagram of inclusion criteria. TIPS: Transjugular intrahepatic porto-systemic shunt; ALF: Acute liver failure.

UNOS/Organ Procurement and Transplantation Network Standard Transplant Analysis and Research Database. Available from: <https://optn.transplant.hrsa.gov/data/about-data/>, Accessed September 6, 2013). The study was approved by the Nationwide Children's Hospital Institutional Review Board with a waiver of individual consent (IRB14-00716). The UNOS/OPTN liver database was queried for all patients with cirrhosis listed from May 2002 to September 2013. Each first-time LT candidate listed was tracked until death. All patients with TIPS for ascites who ultimately underwent LT were included in this sample.

The data available from the UNOS Registry included status of TIPS in patients with ascites. Other variables included in analysis were gender, age, diabetes mellitus, body mass index (BMI) at listing, cold ischemia time (CIT), waitlist hepatic encephalopathy, etiology of liver disease (alcoholic vs other), model for end-stage liver disease (MELD) score at listing, MELD score at LT; biochemical tests including serum creatinine, bilirubin, albumin, and international normalized ratio (INR). We studied various outcomes including mortality at 30-d, need for re-LT and hospital length of stay (LOS) during admission for LT.

### Study sample

We included adult patients (age  $\geq 18$  years) who underwent LT from May 2002 to September 2013 [*i.e.*, after the inception of the MELD score and use of expanded-polytetrafluoroethylene (ePTFE) covered TIPS]. Only those undergoing TIPS after listing and before liver transplant were included in the TIPS group. We excluded patients with variceal bleeding within two weeks of listing (in order to exclude TIPS for variceal bleed) for LT and those listed for acute liver failure or hepatocellular carcinoma. After application of exclusion criteria (Figure 1) the analytic sample consisted of 32783/114770 (28.5%) patients with ascites who underwent LT and had a known

TIPS status. Among these 32783 patients with ascites, 1366 patients underwent TIPS while 31417 patients did not undergo TIPS.

### Statistical analysis

All values were expressed as means  $\pm$  SD for continuous measures, and counts and percentages for categorical variables. For all analyses, a  $P$ -value  $< 0.05$  was considered statistically significant. For evaluation of mean differences between baseline characteristics for patients with and without TIPS, we used unpaired  $t$ -tests for continuous measures and  $\chi^2$  tests for categorical measures. We estimated the impact of TIPS on each of the outcome measures. Multivariate analyses were conducted on the study population to explore the effect of TIPS on 30-d mortality post-LT, need for re-LT and LOS. All covariates were included in logistic regression analysis. All analyses were performed using Stata/MP, version 13.1 (College Station, TX: StataCorp LP). The statistical review of this study was performed by a biomedical statistician.

## RESULTS

### Study population

After applying the inclusion/exclusion criteria a total of 32783 patients with ascites from database were selected. A total of 1366 (4.2%) underwent TIPS for management of refractory ascites while awaiting LT (Figure 1). Those without TIPS ( $n = 31417$ ) were selected as a control group for comparison.

Demographics such as gender, age and BMI were comparable in the two groups; albumin and CIT were also equally distributed (Table 1). Patients with TIPS on waitlist had a lower mean MELD score at time of listing ( $16.6 \pm 6.7$ ) as compared to those without TIPS ( $19.7 \pm 8.9$ ), ( $P < 0.001$ ). Plausibly, TIPS group had a lower creatinine, bilirubin and INR. Interestingly, the MELD score at transplantation was higher in the TIPS group ( $23.2 \pm 9.2$ ) as compared to without TIPS group ( $22.6 \pm 9.8$ ) ( $P = 0.03$ ). Plausibly, there were less patients with severe hepatic encephalopathy (HE) in the TIPS group ( $n = 68$ ; 4.9%) as compared to without TIPS ( $n = 2218$ ; 7%) ( $P = 0.01$ ).

On univariate analysis (Table 2), we found that TIPS increases the days on LT waitlist ( $408 \pm 553$  d) as compared to those without TIPS ( $183 \pm 330$  d), ( $P < 0.001$ ). TIPS group had comparable 30-d post LT mortality as compared to non-TIPS group (46; 3.51% vs 915; 3.05%;  $P = 0.34$ ). There was also a comparable re-LT rate at 30 d (15; 1.1% vs 560; 1.78%;  $P = 0.06$ ) and hospital LOS (17.58 vs 16.62;  $P = 0.12$ ) between the two groups.

### Thirty-days post LT mortality predictors

On logistic regression, TIPS had no effect on 30-d post LT mortality (OR = 1.26; 95%CI: 0.91-1.75). However, the significant predictors of mortality at 30-d were advanced age (OR = 1.02; 95%CI: 1.01-1.03,  $P < 0.001$ ),

**Table 1** Demographics and clinical variables categorized by transjugular intrahepatic porto-systemic shunt status

Variables	TIPS on waitlist ( <i>n</i> = 1366; % or mean $\pm$ SD)	Non TIPS on waitlist ( <i>n</i> = 31417; % or mean $\pm$ SD)	<i>P</i> -values
Male candidate	943 (69)	21374 (68)	0.43
Candidate race			< 0.001
White	1072 (78.4)	23063 (73.4)	
Black	75 (5.4)	2865 (9.1)	
Other	219 (16)	5489 (17.4)	
Diabetes mellitus	380 (28)	7769 (24.8)	0.009
ALD	311 (22.7)	6615 (21)	0.13
Hepatic encephalopathy			0.01
None	373 (27.3)	8409 (26.7)	
Grade 1-2	925 (67.7)	20790 (66.1)	
Grade 3-4	68 (4.9)	2218 (7)	
Arterial hypertension	68 (14.8)	2254 (19.7)	0.01
Age	53.5 $\pm$ 8.5	53.6 $\pm$ 9.3	0.65
MELD score at listing	16.6 $\pm$ 6.6	19.66 $\pm$ 8.8	< 0.001
Creatinine	1.2 $\pm$ 0.8	1.4 $\pm$ 1.2	< 0.001
Bilirubin	4.1 $\pm$ 6.3	6.61 $\pm$ 9.0	< 0.001
INR	1.5 $\pm$ 0.4	1.7 $\pm$ 0.8	< 0.001
Albumin	2.9 $\pm$ 0.6	2.9 $\pm$ 0.6	0.66
BMI at list entry			
Continuous (kg/m <sup>2</sup> )	28.8 $\pm$ 5.6	28.8 $\pm$ 5.7	0.89
Dichotomous ( $\geq$ 26 kg/m <sup>2</sup> )	905 (66.4)	20747 (66.2)	0.85
Cold ischemia time			
Continuous (h)	7.1 $\pm$ 3.7	6.9 $\pm$ 3.5	0.03
Dichotomous (> 12 h)	66 (5.0)	1360 (4.5)	0.38
MELD score at transplantation	23.1 $\pm$ 9.1	22.6 $\pm$ 9.7	0.03

TIPS: Transjugular intrahepatic porto-systemic shunt; ALD: Alcoholic liver disease; MELD: Model for end-stage liver disease; INR: International normalized ratio; BMI: Body mass index.

**Table 2** Comparison of various outcomes on univariate analysis on waitlist and post liver transplant

	TIPS on waitlist ( <i>n</i> = 1366) % or mean $\pm$ SD	No TIPS on waitlist ( <i>n</i> = 31417) % or mean $\pm$ SD	<i>P</i> -values
Days on LT waitlist	408 $\pm$ 552.6	183 $\pm$ 330.5	< 0.001
Mortality within 30 d	46 (3.5)	915 (3.0)	0.344
Length of hospital stay	17.58 $\pm$ 22.4	16.62 $\pm$ 22.1	0.118
Re-LT at 30 d	15 (1.1)	560 (1.8)	0.06

TIPS: Transjugular intrahepatic porto-systemic shunt; LT: Liver transplantation.

low serum albumin (OR = 0.88; 95%CI: 0.79-0.98, *P* = 0.029), and increasing CIT (OR = 1.04; 95%CI: 1.02-1.05, *P* < 0.001). Another predictor of 30-d mortality was bilirubin (OR = 1.014; 95%CI: 1.004-1.024; *P* = 0.008 (Table 3).

### TIPS and re-LT at 30 d

On logistic regression, TIPS was not associated with re-LT at 30 d (OR = 0.61; 95%CI: 0.36-1.05). Predictors of re-LT at 30 d included advanced age (OR = 0.97; 95%CI: 0.96-0.98; *P* < 0.001), creatinine (OR = 0.87; 95%CI: 0.77-0.99; *P* = 0.032) and CIT (OR = 1.05; 95%CI: 1.03-1.07; *P* < 0.001) (Table 4).

**Table 3** Multivariable logistic regression analysis to assess the impact of transjugular intrahepatic porto-systemic shunt on 30-d mortality after liver transplant

Variable	OR (95%CI)	<i>P</i> -values
TIPS	1.26 (0.90-1.75)	0.17
Male candidate	0.83 (0.71-0.95)	0.01
Candidate race		
White	Ref.	
Black	1.08 (0.85-1.37)	0.54
Other	1.08 (0.90-1.29)	0.40
Diabetes mellitus	1.12 (0.95-1.31)	0.17
ALD	0.89 (0.74-1.07)	0.22
Hepatic encephalopathy		
None	Ref.	
Grade 1-2	0.86 (0.73-1.01)	0.06
Grade 3-4	1.12 (0.85-1.47)	0.41
Age	1.02 (1.01-1.03)	< 0.001
MELD score	1.02 (1.00-1.04)	0.05
Creatinine	1.03 (0.97-1.10)	0.33
Bilirubin	1.01 (1.00-1.02)	0.008
INR	0.97 (0.86-1.09)	0.59
Albumin	0.88 (0.79-0.98)	0.03
BMI	1.00 (0.99-1.02)	0.86
Cold ischemia time	1.04 (1.02-1.05)	< 0.001

TIPS: Transjugular intrahepatic porto-systemic shunt; ALD: Alcoholic liver disease; MELD: Model for end-stage liver disease; INR: International normalized ratio; BMI: Body mass index.

### TIPS and LOS

Advanced HE (grade 3-4) on waitlist contributed most days to LOS ( $\beta$  = 3.46; 95%CI: 2.37-4.55, *P* < 0.001), followed by TIPS ( $\beta$  = 2.16; 95%CI: 0.92-3.38, *P* = 0.001). Other factors that contributed to LOS were black race ( $\beta$  = -1.58; 95%CI: -2.46 to -0.69, *P* < 0.001) and advanced age ( $\beta$  = 0.09; 95%CI: 0.06-0.11, *P* < 0.001). High MELD score, INR, albumin, BMI and CIT also significantly contributed to LOS after LT (Table 5).

## DISCUSSION

The most important finding of the current study is that TIPS for the treatment of ascites in the MELD era for LT is not associated with heightened 30-d mortality or the need for re-transplantation. However, hospital LOS was increased in patients with TIPS which may point to post-operative morbidity. TIPS was found to increase time on waitlist in patients with ascites.

Our findings of safety of TIPS in terms of short term mortality and need for re-LT is in line with multiple other studies, as these also did not find any difference in operative time, transfusion and LOS<sup>[9,12,14,17]</sup>. One of the largest retrospective studies of 207 patients explored the impact of TIPS on post-transplant survival and graft loss and found no significant difference<sup>[12]</sup>. In fact, a recent study went even further to find lower risk of mortality in TIPS group at 1 year after LT<sup>[15]</sup>.

Our study holds many advantages to prior studies including the use of a national database and large sample size. Furthermore, our study had increased homogeneity as it was limited to those undergoing TIPS for refractory

**Table 4** Multivariable logistic regression analysis to assess the impact of transjugular intrahepatic porto-systemic shunt on retransplantation

Variable	OR (95%CI)	P-values
TIPS	0.61 (0.36-1.05)	0.07
Male candidate	1.02 (0.85-1.24)	0.81
Candidate race		
White	Ref.	
Black	1.22 (0.91-1.64)	0.18
Other	1.05 (0.83-1.32)	0.69
Diabetes mellitus	0.98 (0.78-1.21)	0.83
ALD	0.98 (0.78-1.24)	0.90
Hepatic encephalopathy		
None	Ref.	
Grade 1-2	0.92 (0.76-1.13)	0.44
Grade 3-4	1.02 (0.69-1.51)	0.91
Age	0.97 (0.96-0.98)	< 0.001
MELD score	0.99 (0.97-1.02)	0.51
Creatinine	0.87 (0.77-0.99)	0.03
Bilirubin	0.99 (0.98-1.02)	0.54
INR	1.01 (0.86-1.18)	0.9
Albumin	1.03 (0.89-1.19)	0.65
BMI	1.005 (0.98-1.02)	0.54
Cold ischemia time	1.05 (1.03-1.07)	< 0.001

TIPS: Transjugular intrahepatic porto-systemic shunt; ALD: Alcoholic liver disease; MELD: Model for end-stage liver disease; INR: International normalized ratio; BMI: Body mass index.

ascites and was limited to a study period in the post-MELD era and with more homogeneity in shunt type (*i.e.*, ePTFE covered).

Existing literature on LOS is variable with certain studies describing intra-operative complications in patients who have undergone TIPS<sup>[16]</sup>. On the other hand, additional studies have not found TIPS to affect the LOS in post LT setting<sup>[14,18]</sup>. It has been shown in our study that advanced HE (grade 3-4) on waitlist cirrhotics contributes the most to LOS adding 3.5 d followed by TIPS insertion which prolonged stay by an average of 2.16 d. This finding is remarkable given encephalopathy is a known complication of TIPS<sup>[7,8]</sup>. We can hypothesize that TIPS insertion may contribute to ongoing encephalopathy and therefore increase length of hospital stay.

Among other predictors of increased LOS were advanced age, high MELD score and CIT. All these factors are recognized predictors of increased LOS and reported in literature<sup>[19,20]</sup>. Of note, the TIPS group in our study began with a lower MELD score at the time of listing but had higher MELD scores at the time of LT. This finding suggests patients undergoing TIPS were able to survive longer on the wait list with continued progression of liver disease at the time of LT. More advanced disease among TIPS patients would explain increased LOS post-LT.

We found that increased time on the waitlist in the TIPS group was consistent with findings from single center studies<sup>[18]</sup>. Several randomized controlled trials and a meta-analysis of individual patient data also found TIPS superior to repeated paracentesis in increasing time on waitlist and therefore transplant free survival<sup>[2,5,6]</sup>. The increased time on LT wait list may be explained by

**Table 5** Ordinary least squares regression to assess the impact of transjugular intrahepatic porto-systemic shunt on length of hospital stay after liver transplant

Variable	$\beta$ (95%CI)	P-values
TIPS	2.16 (0.92-3.38)	0.001
Male candidate	-1.99 (-2.52-1.46)	< 0.001
Candidate race		
White	Ref.	
Black	-1.58 (-2.46-0.69)	< 0.001
Other	0.11 (-0.53-0.77)	0.72
Diabetes mellitus	0.52 (-0.05-1.10)	0.07
ALD	0.17 (-0.44-0.79)	0.57
Hepatic encephalopathy		
None	Ref.	
Grade 1-2	-0.10 (-0.66-0.46)	0.73
Grade 3-4	3.46 (2.37-4.55)	< 0.001
Age	0.09 (0.06-0.11)	< 0.001
MELD score	0.37 (0.31-0.44)	< 0.001
Creatinine	0.05 (-0.21-0.31)	0.71
Bilirubin	0.03 (-0.008-0.08)	0.1
INR	-1.06 (-1.50-0.61)	< 0.001
Albumin	-0.63 (-1.01-0.24)	0.001
BMI	-0.05 (-0.100-0.01)	0.01
Cold ischemia time	0.36 (0.29-0.43)	< 0.001
Constant	7.83 (5.38-10.27)	< 0.001

TIPS: Transjugular intrahepatic porto-systemic shunt; ALD: Alcoholic liver disease; MELD: Model for end-stage liver disease; INR: International normalized ratio; BMI: Body mass index.

decreased portal hypertension produced by the TIPS and mortality associated with complications of portal hypertension. One study found that TIPS lowered mortality rate while on waitlist and decreased need for transplantation<sup>[21]</sup>. Hence, it is possible TIPS can be utilized as a bridge to transplant and even to improve waitlist survival of listed patients.

Our findings demonstrate the challenge of using TIPS in patients who need to undergo LT. Following TIPS placement, this patient population has an increased wait time for LT, yet suffers comparable immediate post procedural mortality as their non-TIPS counterparts. This longer time on the waitlist may allow for other decompensated non-TIPS patients with higher MELD scores to undergo LT first. Thus, it appears that a disparity is created where the patient population requiring more advanced treatment of ascites (*i.e.*, TIPS) have increased time on waitlist through improvement of the MELD score and therefore experience a delay in transplantation. Based on our findings, we propose an idea to potentially provide special circumstances to patients requiring TIPS on the waitlist for LT as their outcomes after transplantation are not influenced by placement of the shunt. An example of special circumstances could be exceptional MELD points to avoid further delay in LT.

Limitations of our study are mainly related to availability of variables in the UNOS database. This database only lists TIPS status at the time of LT recipient registration and does not provide information on control and recurrence of tense ascites, post TIPS encephalopathy, intra- and post-LT information such as operative time and blood



product transfusion requirements. Waitlist mortality, intensive care unit stay, and complications of TIPS placement such as TIPS migration and endovascular stenting were also not available to us. Due to these database limitations we cannot directly measure the number of patients on waitlist undergoing TIPS or the waitlist mortality. As a result, days on waitlist had to be used as a surrogate measure for waitlist mortality and transplant free survival.

In conclusion, we found that TIPS had no effect on the 30-d mortality after LT and the need for re-LT. TIPS increased time on LT waitlist while also increasing length of hospital stay post-LT. It was found that TIPS is not a commonly used intervention for the management of ascites in patients on the waitlist for LT. With TIPS not influencing 30-d mortality and need for re-LT, it appears that more patients may benefit from its use. However, one of the downsides of using TIPS could be a potential delay in LT due to improvement in MELD score. These important factors must be considered and discussed with patients before pursuing TIPS procedure.

## COMMENTS

### Background

Prior studies exploring the role of transjugular intrahepatic porto-systemic shunt (TIPS) with regards to cirrhotic patients being evaluated for liver transplant were limited by small sample sizes, single center studies, and heterogeneous study groups that resulted in poor generalizability. Further, these studies were completed prior to advent of expanded-polytetrafluoroethylene covered stents and introduction of model for end-stage liver disease allocation system. Here the authors would like to utilize the United Network for Organ Sharing (UNOS) database to address the effect of TIPS on waitlist times, liver transplantation (LT) morbidity and mortality, and hospital length of stay.

### Research frontiers

Since its inception, TIPS has been touted as a potential bridge to LT by possibly improving transplant free survival. Studies such as that performed by Berry *et al* have recently used the UNOS data base to confirm TIPS' role in improving transplant free survival and support the notion that TIPS is a bridge to LT.

### Innovations and breakthroughs

To our knowledge no study has utilized the UNOS database in exploring post-LT outcomes in the TIPS population. The study confirmed findings of prior single center studies that TIPS does not significantly affect post-LT outcomes. Of note, their large study group size adds power and improves generalizability of these findings. Short term outcomes were their primary focus given the concern for potential for intra-operative LT complications in patients who have undergone TIPS.

### Applications

The authors' findings support prior single center and more recent meta-analyses and database reviews in confirming increased transplant free survival while not affecting post-LT outcomes. The study supports the notion that TIPS can be utilized as a bridge to transplantation. Prospective studies will be necessary to further elucidate the influence of TIPS on LT outcomes and the potential detriments resulting from prolonged waitlist times.

### Terminology

CIT: Cold ischemia time; ePTFE: Expanded-polytetrafluoroethylene; HE: Hepatic encephalopathy; LOS: Length of hospital stay; LT: Liver transplantation; LVP: Large volume paracentesis; MELD: Model for end-stage liver disease; TIPS: Transjugular intrahepatic porto-systemic shunt; UNOS: United Network

for Organ Sharing.

### Peer-review

The paper is well written and the design is good.

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Retrospective Study

## Vasopressin use in critically ill cirrhosis patients with catecholamine-resistant septic shock: The CVICU cohort

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### Abstract

#### AIM

To examine patient-centered outcomes with vasopressin (AVP) use in patients with cirrhosis with catecholamine-refractory septic shock.

#### METHODS

We conducted a single center, retrospective cohort study enrolling adult patients with cirrhosis treated for catecholamine-resistant septic shock in the intensive care unit (ICU) from March 2011 through December 2013. Other etiologies of shock were excluded. Multivariable regression models were constructed for seven and 28-d mortality comparing AVP as a second-line therapy to a group of all other vasoactive agents.

#### RESULTS

Forty-five consecutive patients with cirrhosis were treated for catecholamine-resistant septic shock; 21 received AVP while the remaining 24 received another agent [phenylephrine (10), dopamine (6), norepinephrine (4), dobutamine (2), milrinone (2)]. In general,

no significant differences in baseline demographics, etiology of cirrhosis, laboratory values, vital signs or ICU mortality/severity of illness scores were observed with the exception of higher MELD scores in the AVP group (32.4, 95%CI: 28.6-36.2 *vs* 27.1, 95%CI: 23.6-30.6,  $P = 0.041$ ). No statistically significant difference was observed in unadjusted 7-d (52.4% AVP *vs* 58.3% and  $P = 0.408$ ) or 28-d mortality (81.0% AVP *vs* 87.5% non-AVP,  $P = 0.371$ ). Corticosteroid administration was associated with lower 28-d mortality (HR = 0.37, 95%CI: 0.16-0.86,  $P = 0.021$ ) independent of AVP use.

### CONCLUSION

AVP is similar in terms of patient centered outcomes of seven and 28-d mortality, in comparison to all other vasopressors when used as a second line vasoactive agent in catecholamine resistant septic shock. Large-scale prospective study would help to refine current consensus standards and provide further support to our findings.

**Key words:** Portal hypertension; Vasopressor; Liver; Intensive care unit; Hepatology

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**Core tip:** Although the management of septic shock has evolved dramatically in recent decades, data regarding optimal vasopressor therapy in critically-ill patients with cirrhosis is less robust and is based largely on consensus expert opinion. We found no difference in 7-d or 28-d mortality with vasopressin use when compared to all other vasoactive agents as a second line agent in catecholamine-resistant septic shock. Further large-scale studies are needed to refine current consensus standards and provide further support to our findings.

Myc LA, Stine JG, Chakrapani R, Kadl A, Argo CK. Vasopressin use in critically ill cirrhosis patients with catecholamine-resistant septic shock: The CVICU cohort. *World J Hepatol* 2017; 9(2): 106-113 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i2/106.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i2.106>

### INTRODUCTION

The management of septic shock has evolved since the inception of the Surviving Sepsis Campaign and the adoption of early goal-directed therapy, with short-term mortality rates improving markedly over the past decade<sup>[1]</sup>. Improved outcomes appear to have extended to special populations as well, including patients with cirrhosis of the liver, a population in which sepsis has traditionally been characterized by extremely high mortality rates of nearly 100% in some studies, well above those of the general population which approximate 40% at 28-d<sup>[2-4]</sup>. Concurrent with the development of bundled care protocols, the incorporation of arginine

vasopressin (AVP) into the management of septic shock has generated significant clinical and research interest. Based on reports of inappropriately low levels of circulating AVP coupled with apparent AVP-hypersensitivity in patients with cirrhosis and septic shock, exogenous AVP was seen as potentially restorative of both vascular tone and catecholamine-sensitivity in septic states<sup>[5-7]</sup>.

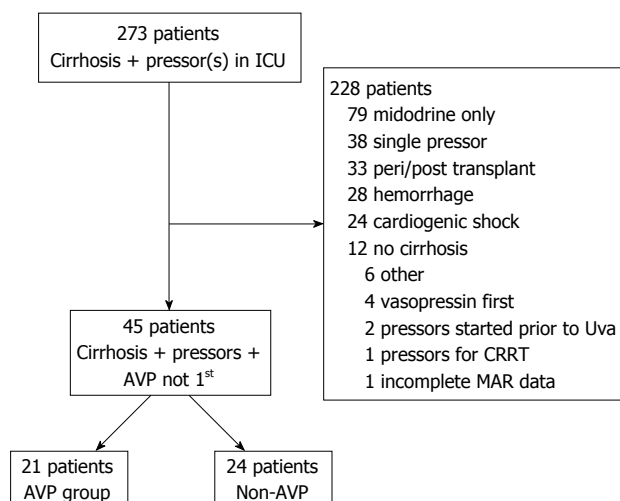
Current recommendations for AVP use in managing septic shock largely derive from the published results of the Vasopressin and Septic Shock Trial (VASST) which reported no significant difference in 28-d mortality in patients with septic shock treated with vasopressin *vs* norepinephrine<sup>[4]</sup>. Nevertheless, the authors did report improved 28-d mortality in a pre-specified subgroup of patients with less severe septic shock as well as decreased norepinephrine requirements in patients receiving AVP, leading to the adoption of exogenous AVP use as an ungraded recommendation into the Surviving Sepsis Guidelines.

Appreciating these general recommendations, it remains unclear what role exogenous AVP may serve in patients with cirrhosis given the unique characteristics of septic shock in this population. Although low levels of AVP coinciding with AVP-vasosensitivity have been reported in patients with cirrhosis, the distinctive features of septic shock in this population including hyperdynamic circulation, relative adrenal insufficiency, blood volume sequestration in the splanchnic venous plexus, and hypothermia together with underlying thrombocytopenia and varying degrees of hepatic dysfunction introduce ambiguity as to whether the generic Surviving Sepsis guidelines ought to be applied to patients with cirrhosis<sup>[2,3,8-10]</sup>. Data regarding AVP and AVP analogue use in patients with cirrhosis and septic shock are sparse.

Recently published guidelines addressing management of critically ill patients with cirrhosis do incorporate AVP use for treatment of persistent hypotension, however this recommendation relies largely on studies of terlipressin in non-cirrhotic populations<sup>[11]</sup>. In this respect, it should be noted that only 11.3% of the patients enrolled in the VASST study had any liver disease at all. While AVP may have salient effects in this population relating to improved hemodynamics, mobilization of large splanchnic blood volume, norepinephrine sparing, and improved catecholamine resistance, potential adverse effects specific to the cirrhotic state cannot be excluded and may include acute-on-chronic liver failure, worsening thrombocytopenia and hyponatremia, and decreased cardiac output<sup>[4,12-17]</sup>. Decreased cardiac output may be particularly significant in this population, which may be more dependent on oxygen delivery for oxygen consumption<sup>[18]</sup>. Together, such hepatic, renal and hematologic effects of AVP may be disproportionately detrimental in a vulnerable cirrhotic population often characterized by baseline hyponatremia and thrombocytopenia complicating underlying hepatic dysfunction.

In this single center retrospective cohort study,





**Figure 1 Study enrollment.** CRRT: Crrtcontinuous renal replacement therapy; ICU: Intensive care unit; AVP: Arginine vasopressin.

we aimed to characterize 7-d and 28-d mortality outcomes of AVP use in patients with cirrhosis and catecholamine-refractory septic shock (CRSS). Secondly, we aimed to investigate the effect of AVP on 24-h changes in important laboratory parameters including aminotransferases, total bilirubin and platelet concentrations as well as heart rate. We hypothesized that use of AVP as a second vasopressor in cirrhosis patients with catecholamine-resistant septic shock would be associated with increased mortality when compared with cirrhosis patients receiving an alternate adjunct vasoactive agent (e.g., norepinephrine, phenylephrine, dopamine).

## MATERIALS AND METHODS

### Cohort selection

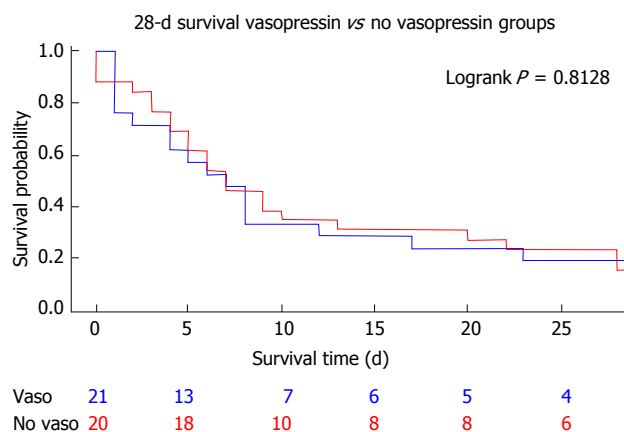
All adult patients with cirrhosis treated for CRSS shock requiring medical intensive care unit (ICU) care between March 4, 2011 and December 31, 2013 were identified through the University of Virginia Clinical Data Repository using billing and administrative codes in conjunction with data derived from medication administration reports. Cirrhosis of the liver was confirmed by direct histological examination of liver biopsy or by biochemical and imaging findings suggesting advanced liver disease with portal hypertension. Catecholamine-resistant septic shock was defined as a clinical requirement for  $\geq 2$  vasopressors (the first of which had to be a catecholaminergic agent) for hypotension attributable to an infectious origin on the basis of either culture data or clear clinical suspicion. Patients with cirrhosis meeting this definition of CRSS were included in our analysis. Patients with other etiologies of shock (e.g., hemorrhagic, obstructive, etc.) were excluded, as were patients who received AVP as the first vasopressor agent, patients who received vasopressors in the peri-transplant setting or for purposes of tolerating renal replacement therapy, or patients who were initiated on vasopressor therapy at an undetermined time prior to

interhospital transfer to our facility (Figure 1).

Baseline patient characteristics were reviewed, including demographics, medical comorbidities (coronary artery disease, congestive heart failure, chronic obstructive pulmonary disease, chronic kidney disease, diabetes, hypertension), smoking and alcohol use, etiology of liver disease with portal hypertensive complications (ascites, and hepatic encephalopathy), vital signs (heart rate, minimum mean arterial pressure, temperature, maximum respiratory rate) and laboratory values. MELD score was calculated using the standard formula:  $11.2 \times \ln(\text{INR}) + 9.57 \times \ln[\text{creatinine (mg/dL)}] + 3.78 \times \ln[\text{bilirubin (mg/dL)}] + 6.43$  with a lower limit of 1.0 for all variables<sup>[19]</sup>. ICU severity of illness variables were also collected including fraction of inspired oxygen, partial pressure of arterial carbon dioxide, partial pressure of arterial oxygen, pH, mean number of vasopressors, days on vasopressors, need for continuous renal replacement therapy, intubation, urine output over the first 24 h, new hemorrhage and new diagnosis of venous thrombosis. Illness severity scores were calculated [acute physiology and chronic health evaluation II (APACHE II), simplified acute physiology score (SAPS II), sequential organ failure assessment (SOFA)]. ICU medications were reviewed (volume of intravenous fluid, octreotide, antibiotic administration, albumin administration, proton pump inhibitor, corticosteroids and first vasopressor use). Captured outcomes included mean survival, hospital and ICU length of stay, ventilator free days, mortality (7-d, 28-d and 90-d), in-hospital mortality, in-ICU mortality and withdrawal of care. The 24-h changes in laboratory parameters (platelets, liver associated enzymes, heart rate, total bilirubin) were also extracted on the basis of the first available value of the parameter of interest available 24-48 h following vasopressor initiation.

### Statistical analysis

Subjects were sorted into two groups, those patients who received AVP as the second-line agent and those patients where another vasopressor was utilized as the second-line agent. The AVP group was compared to the non-AVP group in multiple factors including baseline patient demographics, medical comorbidities, smoking and alcohol use, etiology of liver disease, portal hypertensive complications, vital signs, laboratory values, severity of illness variables, ICU medications administered and patient-centered outcomes of mortality and withdrawal of care. Multivariable models were constructed to assess statistical associations and risk factors for 7-d and 28-d mortality. Individual factors were included in the multivariable model if they were statistically significant to  $P < 0.10$  in the univariate analysis, were clinically important, or have been shown in the literature to be of clinical significance. Univariate comparisons were performed using the Student-*t* test, Wilcoxon sign rank test,  $\chi^2$  test, or Fisher exact test as appropriate. Multivariable models were constructed using Cox proportional hazards models and analysis of maximum likelihood estimates. Modeling both with composite MELD score and



**Figure 2** Twenty-eight-day survival comparing second line vasopressors in catecholamine-resistant septic shock.

examining each variable in the MELD score independently were performed to ensure no one variable was dominant. Unadjusted, stratified Kaplan-Meier survival curves were constructed for 7-d and 28-d survival utilizing the log-rank test to determine statistical significance ( $P \leq 0.05$ ). All statistical tests for significance were two-sided and a significance level  $p$  less than or equal to 0.05 was considered statistically significant. All data set manipulation and statistical analyses were performed using SAS (version 9.4, Cary, NC). Institutional review board approval was obtained for this study.

## RESULTS

Forty-five consecutive patients with cirrhosis were treated for catecholamine-resistant septic shock; 21 received AVP as the second-line vasopressor while the remaining 24 received some other agent [phenylephrine (10), dopamine (6), norepinephrine (4), dobutamine (2), milrinone (2)]. Mean age was  $57.2 \pm 14.0$  years. The cohort was 53.3% male and nearly  $\frac{3}{4}$  had either alcoholic liver disease or chronic hepatitis C as the underlying etiology of cirrhosis (alcoholic alone 35.6%, chronic hepatitis C alone 26.7%, concomitant alcohol and hepatitis C 8.9%). All patients had either Child-Turcotte-Pugh Class B ( $n = 8$ , 14.5%) or Class C ( $n = 37$ , 85.5%) liver disease. Mean MELD score was  $29.0 \pm 9.0$ . Overall 7-d and 28-d mortality were 55.6% and 84.4% respectively, with two patients eventually undergoing liver transplantation at 34 and 67 d out from diagnosis of CRSS, respectively.

In general, no significant differences in baseline demographics, etiology of cirrhosis, laboratory values, vital signs or ICU mortality/severity of illness scores were observed when comparing those subjects who received AVP to those who received any other vasoactive agent, with the exception of higher MELD scores in the AVP group (32.4, 95%CI: 28.6-36.2 vs 27.1, 95%CI: 23.6-30.6,  $P = 0.041$ ) (Table 1). Glomerular filtration rates were also different between the two groups (23.9 mL/min, 95%CI: 18.6-29.2 in the AVP group vs 40.0 mL/min, 95%CI: 29.1-51.0 in the non-AVP group,  $P =$

0.013). Mean APACHE II scores were statistically similar (33.5, 95%CI: 30.6-36.5 in the AVP group vs 31.8, 95%CI: 29.4-34.2) as were SAPS II (72.6, 95%CI: 63.5-81.7 in the AVP group vs 70.3, 95%CI: 64.5-76.1 in the non-AVP group) and SOFA (17.6, 95%CI: 15.9-19.3 AVP vs 16.9, 95%CI: 15.9-18.0 non-AVP). Corticosteroid administration was also statistically similar (76.2% AVP vs 79.2% non-AVP) as was time to first vasopressor initiation (6.8, 95%CI: 4.9-8.7 h AVP vs 7.4, 95%CI: 5.7-9.3 h non-AVP). No statistically significant difference was observed in unadjusted 7-d mortality (52.4% AVP vs 58.3% and  $P = 0.408$ ) or 28-d mortality (81.0% AVP vs 87.5% non-AVP,  $P = 0.813$ ) (Figure 2). There was also no significant change in any recorded laboratory value of interest as measured 24-48 h after vasopressor initiation (Table 2).

On adjusted multivariable analysis, AVP use was not associated with increased 28-d mortality (HR = 0.77, 95%CI: 0.39-1.52,  $P = 0.771$ ). Age in years (HR = 1.05, 95%CI: 1.01-1.08,  $P = 0.004$ ) was associated with increased 28-d mortality (Table 3). In other words, for each addition year of age from the baseline cohort average, the mortality rate was increased 5%. Corticosteroid administration was a significant predictor of improved 28-d mortality (HR = 0.37, 95%CI: 0.16-0.86,  $P = 0.021$ ). The initiation of renal replacement therapy was associated with lower mortality (HR = 0.40, 95%CI: 0.19-0.85,  $P = 0.017$ ). No significant difference was found for MELD score.

## DISCUSSION

After adjusting for multiple confounding factors, we report that AVP is not associated with disparate outcomes when compared to all other vasoactive agents in terms of 7-d and 28-d mortality when used as a second line vasopressor in catecholamine-resistant septic shock. These results are particularly notable considering the extent to which our AVP group was comprised of patients with a higher severity of illness as reflected by statistically higher baseline MELD scores as well as severity of illness scores which, while not individually differing statistically between the two groups, nevertheless all tended to be higher in the AVP group. Estimated glomerular filtration rates were also significantly lower in the AVP group, however these data need to be interpreted with caution as several of these patients were already receiving some form of renal replacement therapy at the time of vasopressor initiation. Additionally, we report no statistically significant difference in the total number of vasoactive agents used among the groups with both groups receiving approximately three such agents during the study period, a surrogate outcome which may indicate that AVP did not impair attainment of target mean-arterial pressures when compared with other agents. We do acknowledge that, due to the high rate of transition to comfort care measures, these data should also be interpreted cautiously, nevertheless rates of changes in goals of care were essentially equivalent

Table 1 Baseline patient characteristics

	Vasopressin ( <i>n</i> = 21)	No Vasopressin ( <i>n</i> = 24)	<i>P</i> value
Patient demographics			
Age, yr (95%CI)	56.2 (50.2-62.3)	57.0 (50.7-63.3)	0.681
Male gender	10 (47.6)	14 (53.9)	0.672
Body mass index, kg/m <sup>2</sup> , (95%CI)	34.2 (30.5-37.9)	31.2 (28.0-34.3)	0.150
Comorbidities, <i>n</i> (%)			
CAD	3 (14.2)	4 (16.7)	0.985
CHF	1 (5.3)	6 (23.1)	0.103
COPD	3 (16.7)	4 (16.7)	1.00
CKD	6 (28.6)	7 (29.2)	0.956
DM	7 (35.0)	8 (30.8)	0.762
HTN	13 (61.3)	16 (66.7)	0.916
Smoking, <i>n</i> (%)	5 (23.8)	5 (23.8)	0.756
Alcohol use (active), <i>n</i> (%)	9 (42.9)	8 (33.3)	0.392
Liver disease etiology, <i>n</i> (%)			
Alcohol	6 (28.6)	10 (41.7)	0.477
NASH/crypto	5 (23.4)	7 (29.2)	0.240
HBV	0 (0.0)	0 (0.0)	1.00
HCV	3 (14.2)	3 (12.5)	0.566
Cardiac	1 (4.8)	1 (4.2)	0.947
Cholestatic	2 (9.5)	1 (4.2)	0.445
AIH	0 (0.0)	1 (4.2)	0.497
HCV/alcohol	3 (14.3)	1 (4.2)	0.329
PSE	14 (66.7)	15 (62.5)	0.927
Laboratory values and vital signs			
MELD, (95%CI)	32.4 (28.6-36.2)	27.1 (23.6-30.6)	0.041
CTP, <i>n</i> (%)			
A	0 (0.0)	0 (0.0)	1.00
B	2 (9.5)	6 (25.0)	0.074
C	19 (90.5)	18 (75.0)	0.162
AST, U/L, (95%CI)	429 (283-1141)	289 (90-667)	0.763
ALT, U/L, (95%CI)	180 (79-438)	133 (24-290)	0.795
Alk phos, U/L, (95%CI)	155 (109-200)	138 (90-185)	0.740
Bilirubin, mg/dL, (95%CI)	15.4 (9.0-21.9)	10.0 (5.3-14.6)	0.109
BUN, mg/dL, (95%CI)	58.0 (45.0-70.9)	48.7 (36.5-60.9)	0.222
Platelets, k/uL, (95%CI)	84.5 (66.2-102.8)	88.8 (68.9-108.8)	0.402
Creatinine, mg/dL, (95%CI)	3.02 (2.16-3.88)	2.50 (1.59-3.41)	0.37
GFR, mL/min per 1.73 m <sup>2</sup> , (95%CI)	23.9 (18.6-29.2)	40.0 (29.1-51.0)	0.013
Sodium, mmol/L, (95%CI)	135.8 (131.8-139.8)	134.1 (130.8-137.5)	0.553
INR, (95%CI)	2.63 (1.79-3.48)	2.15 (1.82-2.47)	0.176
Hematocrit, %, (95%CI)	25.7 (22.9-28.6)	28.0 (26.1-30.0)	0.200
Lactate, mmol/L, (95%CI)	3.90 (2.58-5.21)	3.60 (2.52-4.68)	0.669
WBC (max), k/uL, (95%CI)	16.1 (12.8-19.5)	16.7 (12.7-20.6)	0.607
Heart rate, (95%CI)	106 (96-115)	110 (102-118)	0.591
MAP (min), (95%CI)	45.1 (34.2-56.1)	50.5 (46.9-54.0)	0.197
Temperature, C, (95%CI)	36.3 (35.5-37.1)	36.7 (35.9-37.4)	0.125
RR (max), breaths/min, (95%CI)	35.7 (30.5-40.8)	31.8 (25.4-38.3)	0.145
ICU level of illness, (95%CI)			
FiO <sub>2</sub>	0.48 (0.36-0.59)	0.44 (0.34-0.54)	0.953
PaCO <sub>2</sub>	35.6 (32.7-38.5)	35.7 (32.1-39.3)	0.856
PaO <sub>2</sub>	100.2 (49.1-151.4)	70.4 (60.2-80.6)	0.235
pH	7.30 (7.24-7.35)	7.34 (7.30-7.37)	0.149
APACHE II	33.5 (30.6-36.5)	31.8 (29.4-34.2)	0.306
GCS	7.1 (5.0-9.3)	6.9 (5.2-8.6)	0.547
SAPS II	72.6 (63.5-81.7)	70.3 (64.5-76.1)	0.975
SOFA	17.6 (15.9-19.3)	16.9 (15.9-18.0)	0.173
Average number of vasopressors	2.9 (2.4-3.3)	3.3 (2.9-3.6)	0.357
Days on vasopressors	6.3 (3.7-8.9)	6.3 (3.6-9.0)	0.756
CRRT/HD, <i>n</i> (%)	13 (65.0)	17 (70.8)	0.762
Intubated, <i>n</i> (%)	18 (85.7)	22 (91.7)	0.466
UOP first 24 h, mL, (95%CI)	459.9 (225.8-694.0)	698.1 (383.9-1012.3)	0.067
GI bleed, <i>n</i> (%)	1 (20.0)	5 (20.8)	0.948
New VTE, <i>n</i> (%)	4 (20.0)	3 (12.5)	0.635
ICU medications			
Volume of IVF (L), (95%CI)	4.02 (2.52-5.53)	4.44 (2.62-6.26)	0.891
Octreotide, <i>n</i> (%)	14 (66.7)	12 (52.2)	0.329
Antibiotics, <i>n</i> (%)	21 (100.0)	24 (100.0)	0.790
Choice of first vasopressor, <i>n</i> (%)			

Norepinephrine	18 (85.7)	17 (70.8)	0.412
Dopamine	1 (4.8)	3 (12.5)	0.398
Phenylephrine	2 (9.5)	4 (16.7)	0.207
Albumin given, <i>n</i> (%)	18 (85.7)	21 (95.5)	0.954
PPI, <i>n</i> (%)	18 (90.0)	19 (79.2)	0.388
Corticosteroids, <i>n</i> (%)	16 (76.2)	19 (79.2)	0.701
Outcomes, (95%CI)			
Days to death	8.9 (5.2-11.4)	7.8 (4.4-11.1)	0.672
ICU LOS, d	13.5 (8.1-18.8)	12.3 (4.4-20.3)	0.114
Vent free days	22.6 (20.1-25.1)	15.8 (4.1-27.6)	0.633
Mortality, <i>n</i> (%)			
7 d	11 (52.4)	14 (58.3)	0.408
28 d	17 (81.0)	21 (87.5)	0.371
90 d	18 (85.7)	21 (87.5)	0.303
In hospital	18 (85.7)	20 (83.3)	0.654
ICU	17 (81.0)	18 (75.0)	0.360
Transition to comfort care	16 (76.2)	18 (75.0)	0.808

CAD: Coronary heart disease; CHF: Congestive heart failure; COPD: Chronic obstructive pulmonary diseases; CKD: Chronic kidney diseases; DM: Diabetes mellitus; HTN: Hypertension; NASH: Non-alcoholic steatohepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AIH: Autoimmune hepatitis; PSE: Portosystemic encephalopathy; CTP: Child-Turcotte-Pugh score; AST: Aspartate aminotransferase; ALT: Alanine transaminase; BUN: Blood urea nitrogen; WBC: White blood cell; ICU: Intensive care unit; APACHE II: Acute physiology and chronic health evaluation II; SAPS II: Simplified acute physiology score; SOFA: Sequential organ failure assessment; CRRT: Continuous renal replacement therapy; HD: Hemodialysis; GI: Gastrointestinal; PPI: Proton pump inhibitors.

**Table 2** Change in laboratory parameters with vasopressor support as measured 24 h after vasopressor initiation

	Vasopressin ( <i>n</i> = 21)	No Vasopressin ( <i>n</i> = 24)	<i>P</i> value
Platelets, k/uL, (95%CI)	-18.7 (-42.3, 4.9)	-13.6 (-31.6, 4.4)	NS
ALT, U/L, (95%CI)	47.2 (-12.1, 106.6)	206.3 (-113.3, 525.9)	NS
AST, U/L (95%CI)	236.7 (74.0, 399.4)	292.4 (-247.0, 831.8)	NS
Alkaline phosphatase, U/L, (95%CI)	-10.5 (-48.7, 27.8)	-19.6 (-39.5, 0.3)	NS
Heart rate, (95%CI)	-6.7 (-12.3, -1.0)	0.6 (-11.8, 13.0)	NS
Bilirubin, mg/dL, (95%CI)	0.45 (-0.99, 1.89)	0.87 (-0.64, 2.38)	NS

AST: Aspartate aminotransferase; ALT: Alanine transaminase; NS: No statistical significance.

**Table 3** Adjusted multivariable analysis for predictors of 28-d all-cause mortality

	Hazard ratio	95%CI	<i>P</i> value
Vasopressin <sup>1</sup>	0.77	0.39-1.52	NS
Age (yr)	1.05	1.01-1.08	0.004
CRRT	0.40	0.19-0.85	0.017
Corticosteroids	0.37	0.16-0.86	0.021
Sodium (mmol/L)	1.00	0.96-1.04	NS
Platelets (k/uL)	0.99	0.98-1.00	NS
MELD	1.04	0.98-1.09	NS

<sup>1</sup>Compared to reference of non-vasopressin group (*P* = 0.553). CRRT: Continuous renal replacement therapy; NS: No statistical significance.

in the 2 groups. Well-designed, prospective, randomized studies are needed to clarify whether AVP should be preferred as the second-line vasopressor in this patient population.

Potential adverse effects of AVP administration were not different when compared to all other vasoactive agents. While others have published reports suggesting acute-on-chronic liver failure, worsening thrombocytopenia and a decline in cardiac output with AVP use<sup>[4,12-17]</sup> our results do not lend support to these concerns during early treatment, as we did not find any significant laboratory

changes in these parameter between the two groups as measured 24-48 h after vasopressor initiation. Consonant with these findings, we report similar rates of *de novo* venous thromboembolic disease among the two groups. While direct measurement of cardiac output or cardiac index was not obtainable in our retrospective analysis, heart rate did not decline significantly after one-day of vasopressor therapy in the AVP group when compared with the non-AVP group, lessening concerns regarding clinically significant negative chronotropy affecting cardiac output in this population. Although some reports suggest mortality benefit with attenuation of tachycardia in patients with septic shock, a decline in cardiac output mediated by decreased heart rate may have a disparate and adverse effect in cirrhosis patients when compared to the general population given the possible underlying dependence of oxygen consumption on oxygen delivery in this population<sup>[18,20]</sup>.

From a safety and efficacy standpoint, our findings confirm a salient role for AVP use in cirrhosis patients with CRSS and strengthen the current level of evidence provided in support of recent consensus guidelines for critical care in patients with cirrhosis which are based largely on data extrapolated from studies of terlipressin administration<sup>[11]</sup>.



On adjusted multivariable analysis, corticosteroid use emerged as a marked predictor of improved 28-d mortality with a 63% reduction in death with corticosteroid administration. Current Surviving Sepsis guidelines do recommend low-dose hydrocortisone for patients with septic shock unresponsive to fluid resuscitation and 60 min of vasopressors support. However, while the prevalence of adrenal insufficiency among patients with cirrhosis and sepsis has been generally reported as higher than expected, upwards of some 76% of this population, a recent randomized-controlled trial did not evidence a mortality benefit when stress-dosed steroids were employed in the ICU management of these patients<sup>[10]</sup>. In a randomized, placebo-controlled trial of 75 cirrhosis patients admitted to an intensive care unit with septic shock that was stopped early due to futility, Arabi *et al.*<sup>[10]</sup> reported a 28-d mortality of 85% in the group of patients randomized to receive low-dose corticosteroids compared with 72% in the placebo-allocated group. While our mortality rates approximate those in the steroid-receiving group reported by Russell *et al.*<sup>[21]</sup> it is clear that our patients suffering CRSS represented a more critically ill population as evidenced not only by a pre-specified requirement for 2 or more vasopressors, but also by the higher APACHE II and SOFA scores which characterized our patients. While the discrepancy regarding steroid-benefit may be real and attributable to the differing populations under study, another intriguing hypothesis which emerged from a post-hoc substudy of VASST relates to a possible beneficial synergy between AVP and corticosteroid, with the authors of this substudy reporting a decrease in 28-d mortality from 44.7% to 35.9% in patients receiving corticosteroids plus AVP when compared with patients receiving corticosteroids in addition to norepinephrine.

Finally, rates of gastrointestinal hemorrhage, including that from gastroesophageal varices, were also similar between the AVP and non-AVP groups.

Our study has several limitations. First, it is retrospective in nature and suffers from missing data, a deficiency common to most retrospective analyses. Second, ours is a single center study with a relatively small sample size constraining analysis of additional variables. Third, we acknowledge the heterogeneity of the comparative group regarding the variety of second-line agents used. However, on the other hand, a salient feature of this study is that the 2<sup>nd</sup> vasoactive agent used in the comparator group was almost exclusively a catecholaminergic agent, which in effect resulted in a study comparing second-line vasopressin use vs second-line catecholaminergic augmentation.

Fourthly, an additional limitation relates to “cross-over” analysis, as we did not analyze our cohort of patients on the basis of whether or not they received AVP at any time during their course. Furthermore, we did not investigate the possible interaction between AVP and corticosteroids as discussed earlier. Our study is also relatively underpowered given the high 28-d mortality rates observed and the low-even rate of

patient survival. Other limitations include a lack of direct measurement of cardiac output or index with right heart catheterization in order to better characterize changes in hemodynamics following AVP administration.

Nevertheless, we provide more methodologically robust evidence for AVP use as a second-line vasopressor in catecholamine resistant septic shock and for attention to vasopressor selection in patients with cirrhosis. While further, large-scale multicenter prospective studies would be of benefit to refine current consensus standards, all potential lifesaving interventions, as long as the potential for iatrogenic harm is minimal, should be considered in this extremely sick patient population with 28-d mortality rates approaching 85%. Ultimately, the goal of correcting catecholamine-resistant septic shock in these patients involves both recovery from their immediate, life-threatening illness as well as providing for relative convalescence which may enable the individual patient to recover and receive a liver transplantation.

## COMMENTS

### Background

Cirrhosis patients with septic-shock requiring intensive care unit medical care have an exceedingly high mortality rate and are excluded from many existing clinical trials. Recent consensus guidelines suggest a role for vasopressin use in this patient population; however, this is based largely on expert opinion.

### Research frontiers

With the increasing prevalence of cirrhosis globally and improved access to tertiary medical care, the care of the critically ill patient with cirrhosis of the liver cannot be ignored. Current research and clinical care focuses largely on keeping the critically ill patient with cirrhosis alive in order to eventually receive a life-saving liver transplantation. The role of vasopressin in this population remains unknown.

### Innovations and breakthroughs

In the present study, the authors found that vasopressin is similar to all other vasopressors in terms of 7-d and 28-d mortality and in the absence of significantly more deleterious effects suggest a role for vasopressin use in patients with cirrhosis admitted to the intensive care unit with septic shock.

### Applications

The present report provides further evidence on the safety and efficacy of vasopressin use in patients with cirrhosis, and may suggest revisiting the currently available critical care guidelines.

### Peer-review

This retrospective cohort adds useful information for both clinical practice and further academic research with the goal of impacting common patient centered outcomes for critically ill patients with extremely high mortality rates.

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Observational Study

# Percutaneous drainage as a first therapeutic step prior to surgery in liver hydatid cyst abscess: Is it worth it?

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**Author contributions:** Lopez-Marcano AJ designed research; Ramia JM performed research; Lopez-Marcano AJ, Arteaga V, Gonzales JD and Medina A analyzed data; Lopez-Marcano AJ, Ramia JM and De la Plaza R wrote the manuscript.

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## Abstract

### AIM

To delay surgery until the patient is in a better condition, and thus to decrease postoperative morbidity.

### METHODS

Using this algorithm we treated three patients aged 55, 75 and 80 years. In all three patients the clinical presentation was fever without a clear source of infection; all had nonspecific symptoms such as general malaise, dyspnea, and abdominal discomfort in the previous 15 d. They came to the emergency room at our hospital due to deterioration of their general condition. Analytical tests showed leukocytosis, neutrophilia and increased polymerase chain reaction. In all cases an abdominal computed tomography (CT) was performed and liver hydatid abscess (LHA) was detected. The mean size of the LHA was 12 cm.

### RESULTS

All patients underwent CT-guided percutaneous drainage. The purulent material obtained was cultured, and *Klebsiella pneumoniae*, *Streptococcus viridans* and *Streptococcus salivarius* were identified. Antibiotic treatment was given adapted to antibiotic sensitivity testing. Surgery was performed two weeks after admission, once the patient's condition had improved. All three patients underwent an almost total cystectomy, cholecystectomy and omentoplasty in the residual cavity. Complications were: Clavien I (atelectasis and pleural effusion) and Clavien II (transfusion). The average length of stay (pre and postoperative) was 23 d. At the follow-up, no

relapses were recorded.

## CONCLUSION

LHA management is not standardized. Emergency surgery offers suboptimal results. Percutaneous drainage plus antibiotics allows improving patient's general condition. This enables treating patients in greater safety and also reduces complications.

**Key words:** Hydatidosis; Review; Surgery; Abscess; Liver

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**Core tip:** Liver hydatid abscess (LHA) management is not standardized. The traditional treatment is emergency surgery but the results are usually suboptimal because the patients are in poor medical condition. The initial treatment of LHA in septic patients with percutaneous drainage in combination with antibiotic therapy and supportive measures allows control of the infection and improves the patient's general condition. This enables the physician to treat the patient in greater safety and also reduces complications.

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## INTRODUCTION

Cystic echinococcosis is a zoonotic disease that is found worldwide. It is caused by larvae of the genus *Echinococcus*, and is endemic in certain areas of the planet<sup>[1]</sup>. The liver is the most common location for cyst development<sup>[2]</sup>. Infection of the liver hydatid cyst (LHC) and pyogenic abscess formation is a rare but highly severe complication. The clinical course is insidious and it is usually diagnosed when the infection has progressed, affecting the patient's overall condition and possibly even causing septic shock<sup>[3]</sup>. The treatment of liver hydatid abscess (LHA) is not yet standardized. Several options are available with the dual purpose of draining the LHA and treating the LHC, including simple surgical drainage, or surgical drainage associated with total or subtotal pericystectomy and percutaneous drainage<sup>[3]</sup>. We propose percutaneous drainage of the LHA as a first therapeutic step, and later, when the patient's general condition improves, surgical treatment of the LHC.

## MATERIALS AND METHODS

From May 1, 2007 to March 1, 2016 we treated 135

patients with LHC, of whom 72 underwent surgery. Three of these patients debuted with a severe septic condition caused by LHA. These patients were initially treated with computed tomography (CT)-guided percutaneous drainage of the abscess, and then underwent scheduled surgery when their condition had improved. Their data are included in Table 1.

We also conducted an unlimited literature search in PubMed, updated on 1 January 2016, with the following strategy: [(echinococcosis hepatic complications) and (liver abscess)], which yielded 136 papers. Review of the abstracts found three papers related to the topic of the current paper, and their references were analyzed. The aim of this review was to assess the literature on the value of percutaneous drainage in LHA for delaying surgery until the patient is in a better overall condition, in order to reduce postoperative morbidity and to perform definitive treatment of LHC.

## RESULTS

### Patients 1

Male, 80 years old, came to the Emergency Department due to fever, dyspnea and general malaise of 15 d's duration with hypotension, tachypnea and tachycardia. Past medical history: Mild Alzheimer's disease. His analysis showed: 18610 leukocytes, 90.8% neutrophils, hemoglobin 8.4 g/dL, INR 1.13, Cr 0.75 mg/dL, GGT 433 U/L, AST 35 U/L. Abdominal ultrasound showed a right liver lesion with calcified wall and echoes inside, probably detritus, compatible with LHA. Abdominal CT revealed a 13 cm liver mass with hypodense fluid level suggestive of LHA (Figure 1). CT-guided percutaneous drainage was performed and obtained purulent material. In the microbiology cultures, *Klebsiella pneumoniae* was identified. The patient received antibiotic therapy adjusted to antibiogram (piperacillin-tazobactam 4 g-0.5 g/8 h). Sixteen days later, with the patient in a satisfactory clinical and analytical condition, a subtotal cystectomy was performed after extensive cleaning of the cyst, cholecystectomy, bile duct exploration, closure of small cystobiliary communications and omentoplasty. Histopathology study showed the typical pericystic wall of LHC. After surgery, the patient suffered atelectasis and pleural effusion, and fungaemia (*Candida Albicans*) treated by fluconazole and requiring transfusion. He was discharged on postoperative day 34 (total stay: 50 d). He died 14 mo later of other medical causes, with no evidence of LHC recurrence at the CT performed one year after surgery.

### Patients 2

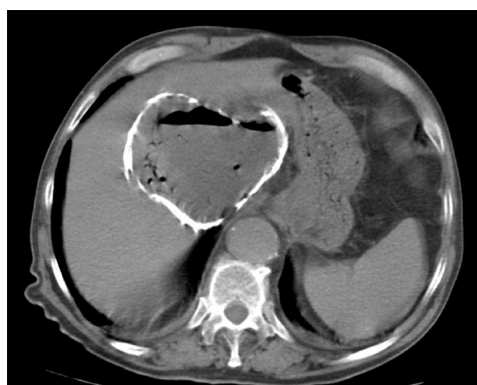
Female, 75 years old. Past medical history: Hypertension and diabetes mellitus. She came to the Emergency Department due to fever and malaise of several days, severe, with hypotension, tachypnea and tachycardia. Analysis: 24610 leukocytes (95% neutrophils), Hb 10.9 g/dL, INR 1.24, Cr 1.56 mg/dL, polymerase chain



**Table 1 Clinical debut, analysis, diagnostic methods, surgery, morbidity and follow-up of our cases**

	Case 1	Case 2	Case 3
Sex	Male	Female	Female
Age (yr)	80	75	55
Age	80	75	55
Clinic	Fever, dyspnoea and malaise last 15 d duration Poor general condition	Fever and malaise for several days Poor general condition	High fever (> 39 °C) accompanied by discomfort in right hypochondrium Poor general condition
Analytics	18610 leukocytes, 90.8% neutrophils, Hgb 8.4 g/dL INR 1.13, Cr 0.75 mg/dL, GGT 433 U/L, AST 35 U/L	24610 leukocytes (95% neutrophils), Hgb 10.9 g/dL, INR 1.24, Cr 1.56 mg/dL, PCR 315 mg/L, GGT 70 U/L and AST 47 U/L	18666 leukocytes, 84.8% neutrophils, Hgb 10.6 g/dL, INR 1.14, PCR 19.4 mg/dL, GGT 270 U/L, AST 379 U/L
Radiography/ultrasound	A right liver lesion with calcified wall and echoes inside, probably detritus, compatible with LHA	-	An abdominal mass with fluid level in right hypochondrium was seen
Abdominal CT	An abdominal mass with fluid level in right hypochondrium	A 12 cm abscess in the liver compatible with LHA	An 11.5 cm liver mass located in segments VI and VII with fluid level, communicating with bile duct and causing inferior vena cava compression
Size	13 cm	12 cm	11.5 cm
Culture	<i>Klebsiella pneumoniae</i>	<i>Streptococcus viridans</i>	<i>Streptococcus salivarius</i>
Time from pair to surgery	16 d	12 d	15 d
Surgery	Subtotal cystectomy, cholecystectomy, bile duct exploration, closure of small cystobiliary communications and omentoplasty	Subtotal cystectomy, cholecystectomy and bile duct clearance	Subtotal cystectomy and bile duct clearance
Morbidity	Atelectasis and pleural effusion, fungaemia ( <i>Candida Albicans</i> ) and transfusion	No	Red blood cell transfusion
Postsurgical stay	34 d	5 d	4 d
Total stay	50 d	17 d	19 d
Follow-up	No recurrence 14 mo	No recurrence 6 yr	No recurrence 2.5 yr

CT: Computed tomography; LHA: Liver hydatid abscess; PCR: Polymerase chain reaction.

**Figure 1** Abdominal computed tomography: Liver hydatid abscess.**Figure 2** Abdominal computed tomography: Percutaneous drainage inside liver hydatid abscess.

reaction (PCR) 315 mg/L, GGT 70 U/L and AST 47 U/L. Abdominal CT revealed a 12 cm abscess in the left liver compatible with LHA. CT percutaneous drainage was performed, obtaining purulent material (Figure 2). In microbiological cultures *Streptococcus viridans* was identified. She received empiric antibiotic treatment adjusted later to amoxicillin/clavulanic acid (1 g/8 h) as a result of the antibiogram. She was admitted to the intensive care unit due to severe SIRS and finally underwent surgery after 12 d when her clinical condition had improved. A right subcostal laparotomy was performed, revealing a LHA located in segments III, IVb, V

and VI. Subtotal cystectomy, cholecystectomy and bile duct clearance were performed. Postoperative course was uneventful and the patient was discharged after 5 d (total stay: 17 d). Histopathology showed chronic cholecystitis and hydatid cyst wall. No recurrence was seen at follow-up sessions over a 6-year period.

### **Patients 3**

Female, 55 years old. Past medical history: Human immunodeficiency virus infection and pulmonary fibrosis. She came to the Emergency Department for high

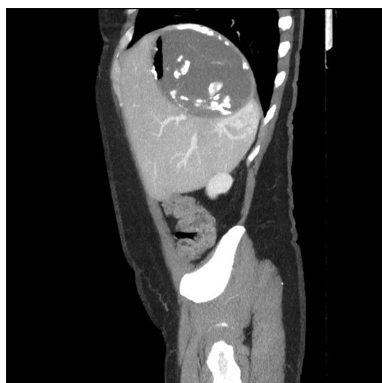


Figure 3 Abdominal computed tomography: Liver hydatid abscess.

fever ( $>39^{\circ}\text{C}$ ) accompanied by discomfort in right hypochondrium, with hypotension, tachypnea and tachycardia. Analytical results: 18666 leukocytes, 84.8% neutrophils, Hgb 10.6 g/dL, INR 1.14, PCR 19.4 mg/dL, GGT 270 U/L, AST 379 U/L. Abdominal radiography revealed an abdominal mass with fluid level in right hypochondrium. Abdominal CT showed a 11.5 cm liver mass located in segments VI and VII with fluid level (Figure 3), communicating with the bile duct and causing inferior vena cava compression. Empirical broad spectrum antibiotic therapy (piperacillin-tazobactam) was given. CT-guided percutaneous drainage was performed obtaining purulent material. *Streptococcus salivarius* was identified in microbiological cultures. Antibiotic therapy was changed to amoxicillin/clavulanic acid (1 g/8 h) as a result of the antibiogram. ERCP plus sphincterotomy was performed because of a frank intrabiliary rupture identified on CT. She was scheduled for surgery 15 d after coming to our center. After right subcostal incision, an 11-cm LHC was found in segments VII and VIII attached to the diaphragm, right hepatic vein and inferior cava vein. A subtotal cystectomy was performed. Postoperatively, the patient required red blood cell transfusion and was discharged on the fourth day (total stay: 19 d). Histopathology showed a pericystic wall with fibrosis, inflammation and calcification. No recurrence was seen at the last follow-up visit 2.5 years later.

## DISCUSSION

The most severe complications of LHC are rupture, biliary fistula and infection of the cyst, evolving into a liver hydatid abscess<sup>[4]</sup>. LHA has a prevalence of about 25%. In Manterola's series it was the most frequent complication (24.6%), but in ours it accounted for only 4.1%<sup>[3]</sup>. We attribute this huge difference to the lack of a generally accepted worldwide definition of LHA. Some authors define LHA as any hydatid cyst which presents purulent content if opened during surgery, but others require bacterial growth in microbiological cultures in both cases with or without infectious symptoms. Our idea is that LHA should be defined not only in the presence of pus or positive cultures but always with

severe infectious symptoms such as high fever, malaise, or even septic shock. The infection that provokes LHA may be primary, due to the invasion of bacteria from small bile ducts communicating into the cyst or rarely through the hematogenous route, or secondary, due to a communication through a fistula with the peritoneal cavity, bronchi, digestive tract, or skin, and after conservative surgery or incomplete PAIR<sup>[4]</sup>.

LHA patients are generally asymptomatic or have nonspecific clinical manifestations. Diagnosis is often made due to the clinical manifestations of other complications such as acute cholangitis, peritonitis, pericarditis or bronchobiliary fistula<sup>[5]</sup>. In the days prior to diagnosis all our patients reported nonspecific and insidious symptoms such as fever, malaise, dyspnea, abdominal discomfort and a progressive and significant deterioration in their general condition. The scarcity of symptoms of LHA (compared with pyogenic liver or intra-abdominal abscess) is probably due to the action of the pericystic wall offering theoretical protection against infectious dissemination<sup>[6]</sup>.

Usually, the first tool for diagnosing LHA is ultrasound. The ultrasound image of the LHA may not be characteristic, and differential diagnosis should include uncomplicated cyst type I Gharbi, liver abscess from another origin, or infected simple cyst<sup>[7]</sup>. In one of the cases reported here abdominal radiography provided important clues for diagnosis. CT was the best diagnostic method in our short series, but no evidence-based medicine information can be drawn from only three cases.

The management of LHA is not standardized. Simple surgical drainage of the cyst has been described, but this technique may need subsequent additional surgical procedures; if cyst surgery is not performed, relapse and chronic complications due to the persistence of residual cyst cavity are frequent<sup>[6]</sup>. The most widely accepted approach is non-scheduled conservative surgery, usually subtotal pericystectomy including opening of the cavity, exhaustive cleaning of the cyst, eradication of the parasite and closing of the cystobiliary fistulas. But this type of surgery could be suboptimal because the patient is often in a poor clinical condition, and in fact LHA is a risk factor for postoperative complications (especially infections) in patients undergoing surgery for LHC<sup>[8]</sup>. To our knowledge, percutaneous drainage of the cyst, supportive measures and intravenous antibiotics as a therapeutic bridge to more radical and safer surgery have not been described previously. Here we present three patients treated with this approach in whom we were able to control the infection and improve the patients' clinical condition prior to scheduled surgery two weeks later. What is more, we were able to perform an ERCP in one of our patients with a frank intrabiliary rupture. The improved medical condition allowed us the possibility of resecting a greater quantity of cyst, thus reducing the risk of possible relapse.

To conclude, percutaneous drainage of LHA as a bridge to surgery may be a valid procedure especially

in patients at high surgical risk due to septic conditions. With this approach we were able to control the infection with antibiotics and perform surgery once the patient's overall condition had improved.

## COMMENTS

### Background

Cystic echinococcosis is a zoonotic disease that is found worldwide. It is caused by larvae of the genus *Echinococcus*, and is endemic in certain areas of the planet. The liver is the most common location for cyst development. Infection of the liver hydatid cyst (LHC) and pyogenic abscess formation is a rare but highly severe complication.

### Research frontiers

The clinical course is insidious and it is usually diagnosed when the infection has progressed. The treatment of liver hydatid abscess (LHA) is not yet standardized. Several options are available with the dual purpose of draining the LHA and treating the LHC, including simple surgical drainage, or surgical drainage associated with total or subtotal pericystectomy and percutaneous drainage.

### Innovations and breakthroughs

The authors propose percutaneous drainage of the LHA as a first therapeutic step, and later, when the patient's general condition improves, surgical treatment of the LHC.

### Applications

The authors present a new therapeutical option that could let surgeons to obtain better results.

### Peer-review

Interesting case series of three cases. Detailed description and very helpful Table 1.

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## Dietary factors can protect against liver cancer development

Lemonica Koumbi

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### Abstract

Liver cancer is the third leading cause of cancer mortality worldwide with hepatocellular carcinoma (HCC) representing more than 90% of primary liver cancers. Most HCC patients are also suffering from chronic liver disease (CLD). Evidence is emerging that the composition

of diet plays an important role in HCC and CLD development and may also have a chemoprotective role. In contrast to other types of cancer, there are few studies investigating the role of diet in hepatocarcinogenesis. From the available data it is evident that high intakes of red meat and dietary sugar positively correlate with HCC occurrence. On the contrary, high consumption of white meat, fish, vegetables, fruits and cereals are inversely associated with HCC risk. This letter discusses the potential role of dietary interventions in the prevention of hepatocarcinogenesis. The increasing HCC incidence and its high fatality are making HCC prevention an urgent matter. Dietary modifications are found to offer protection against HCC, however, new studies from well-designed and large prospective trials are required to confirm these results.

**Key words:** Cancer prevention; Diet; Hepatitis virus; Meat; Hepatocellular carcinoma

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**Core tip:** Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality. Evidence shows that diet relates to HCC risk and may also have a protective role. Several dietary factors such as vegetables, cereals, fruits, white meat and fish have been found to be inversely associated with HCC risk, whereas a positive correlation has been found with red meat and dietary sugar intakes. The increasing HCC incidence makes its prevention an urgent matter and diet intervention represent an attractive potential. Dietary modifications are found to protect against HCC, however, new studies from well-designed and large prospective trials are required to confirm these results.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is third leading cause of cancer mortality worldwide and accounts for about 90% of primary liver cancers. The major risk factors for HCC occurrence include chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), excess of alcohol consumption, non-alcoholic fatty liver disease (NAFLD), dietary aflatoxin exposure, obesity, smoking and diabetes mellitus<sup>[1]</sup>. Exposure to these factors can injure the liver leading to chronic liver disease (CLD) and patients with CLD are at high risk of developing HCC. A substantial proportion of HCC, however, occurs in patients without exposure to these risk factors<sup>[2]</sup>, suggesting the existence of additional factors.

For the past few decades, epidemiological evidence has shown that diet-related factors are closely related with cancer. A healthy diet is known to reduce the development of some types of cancer, while a poor diet increases cancer risk<sup>[3]</sup>. However, there is no current definition of healthy eating. Observational studies have indicated a protective role of vegetables, fruits and cereals in cancer prevention. In contrast to other types of cancer, there are relatively few studies that investigated the association of diet and HCC risk. Although studies have reached conflicting results, consistent evidence suggests that high intakes of red meat<sup>[4,5]</sup> and dietary sugar<sup>[6]</sup> should be avoided in at-risk populations. Higher intakes of white meat or fish<sup>[7-10]</sup>, vegetables<sup>[10-16]</sup>, fruits<sup>[10,14,17,18]</sup>, cereals<sup>[6]</sup>, eggs<sup>[10,17,18]</sup>, milk<sup>[18]</sup> and yogurt<sup>[10]</sup> have been reported to decrease HCC development. Dietary patterns can capture interactions between dietary components and other risk factors providing a better understanding of the association between dietary intakes and HCC risk. Here the impact of dietary patterns on the prevention of HCC is being discussed.

## RED MEAT

Red meat is an important dietary source of saturated and monounsaturated fatty acids and iron. A number of studies associated meat, especially red and processed meat, with gastrointestinal cancers, including HCC<sup>[5,10,13,17-23]</sup>. Nanji *et al*<sup>[24]</sup> was the first to report in 1985 that high pork intake correlated with liver cancer mortality and since then significant associations between total red meat and an increased risk of CLD and HCC have been found<sup>[4,25,26]</sup> (Table 1). A large prospective study by Freedman *et al*<sup>[4]</sup> with a United States cohort revealed an association between total fat, monounsaturated fat, and saturated fat with both CLD and HCC incident and another smaller study from Greece observed that that high saturated fat intake correlated with liver cirrhosis and HCC<sup>[26]</sup>. One study of daily beef, pork and poultry intake found a statistically significant positive association between red meat and HCC risk in an age- and sex-adjusted analysis<sup>[5]</sup>. On the contrary, Polesel *et al*<sup>[27]</sup> reported no direct association of HCC risk with saturated fat intake and results from a Greek case-control study showed no

association with any fat type<sup>[20]</sup>. Notably, Polesel *et al*<sup>[27]</sup> did report a positive association between iron intake and HCC. Furthermore, a recent meta-analysis study by Luo *et al*<sup>[28]</sup> as well as the multicenter prospective EPIC cohort study, which associates diet with various types of cancer, reported no association between different kinds of meats (red and processed meats or poultry) and increased HCC risk<sup>[7,28,29]</sup>. The EPIC study, however, found that a 20 g/d substitution of fish with meat results in a 16% decrease in HCC risk.

The link between red meat and liver cancer is biological plausible, since red meat contains high amounts of known carcinogens including heme iron, N-nitroso compounds (NOC) and heterocyclic amines (HCA) that are produced when meat is cooked in high temperatures<sup>[30]</sup>. Red meat contains high amounts of bioavailable heme iron while reactive oxygen species are being formed when iron undergoes reduction. Interestingly, individuals with hereditary hemochromatosis, an iron overload disease, have substantially increased HCC occurrence<sup>[31]</sup>. Also excess dietary iron has been shown to contribute to HCC risk in several parts of Africa, and treatment with chelating agents, repeated phlebotomy and low iron diet appear to reduce the HCC incident<sup>[32]</sup>. Freedman *et al*<sup>[4]</sup> 2010 observed that meat processing, its heme iron and NOCs associated with CLD but not with HCC. A case-control study with an Italian cohort revealed a significant positive association between dietary iron intake and HCC risk but did not investigate the role of heme iron<sup>[27]</sup>. HCA and polycyclic aromatic hydrocarbons, carcinogens are generated during high-temperature cooking, and NOCs compounds have been shown to induce liver tumour development<sup>[30]</sup> while high doses of HCAs cause liver tumours in primates<sup>[30]</sup>. The higher fat content of red meat could also explain the harmful effect. Red meat and processed meat contain high levels of cholesterol and saturated fat, and correlate with high risk of obesity and diabetes, which are known as cancer risk factors. In addition, fat intake may play a role in insulin resistance, which relates with liver disease and cancer. Fatty acid deposition in the liver can result in NAFLD therefore increasing the risk of CLD and HCC<sup>[33]</sup>.

Although conflicting results, it can be suggested that red meat intake positively associates with CLD and HCC risk. The observed discrepancies between the studies can be attributed to the limitations of the studies, including differences in the dietary patterns of the various countries studied. However, further large prospective randomized trials investigating the relationship between meat intake and HCC risk are required to reach conclusive results.

## WHITE MEAT AND FISH

Evidence from case control and prospective studies from the NIH-AARP Diet and Health study, Italy and Japan reported an inverse association of white meat, including chicken, turkey and fish, with HCC development<sup>[4,7,8,10,29]</sup> (Table 1). In the EPIC study, the subgroup analyses revealed that lean fish, fatty fish, crustaceans and

**Table 1 Main characteristics of studies on dietary factors and hepatocellular carcinoma risk**

Conclusions	Study details	Location	Ref.
Inverse association of vegetable and fruit intake with HCC and upper digestive cancers risk	Study design: Case-control Cases: 285 Controls: 6147 Duration: 1983-1990 Intake: Vegetables and fruit	Italy	Negri <i>et al</i> <sup>[14]</sup> , 1991
No association between meat and vegetable intake and HCC risk	Study design: Case-control Cases: 97 Controls: 128 Duration: 1995-1998 Intake: Total meat, fruit and vegetables	Greece	Kuper <i>et al</i> <sup>[44]</sup> , 2000
Association of red meat intake and HCC risk NAT2 gene polymorphisms play a role in the effect of meat in HCC development	Study design: Case-control Cases: 185 Controls: 185 Duration: 1999-2001 Intake: Red and white meat, vegetables and fruits	China	Huang <i>et al</i> <sup>[23]</sup> , 2003
Inverse association of white meat, coffee and vegetables with HCC mortality Association of egg intake with HCC mortality	Study design: Cohort Cases: 401 Controls: 110688 Duration: 1988-1999 Intake: Fish, red meat, processed meat, chicken, vegetables	Japan	Kurozawa <i>et al</i> <sup>[17]</sup> , 2004
Inverse association of white meat, milk, yogurt, eggs, and fruits with HCC risk	Study design: Population based case-control Cases: 185 Controls: 412 Duration: 1999-2002 Intake: Milk, yogurt, white meats, eggs, fruits, vegetables	Italy	Talamini <i>et al</i> <sup>[10]</sup> , 2006
Association of red and processed meat intake with HCC risk	Study design: Case-control Cases: 403 Controls: 567169 Duration: 1995-2006 Type of meat: Processed meat	United States	Cross <i>et al</i> <sup>[25]</sup> , 2007
Association of dietary iron intake and HCC risk Inverse association of linoleic acid (white meat) intake and HCC risk	Study design: Case-control Cases: 185 Controls: 412 Duration: 1999-2002 Intake: Total meat	Italy	Polesel <i>et al</i> <sup>[27]</sup> , 2007
Association of red meat and saturated fat intake with CLD and HCC risk Inverse association of white meat with HCC and CLD risk	Study design: Case-control Cases: 338 Controls: 495006 Duration: 1995-2006 Intake: White and red meat	United States	Freedman <i>et al</i> <sup>[4]</sup> , 2010
Inverse association of fish or n-3 PUFAs intake and HCC risk	Study design: Cohort Cases: 398 Controls: 90296 Duration: 1990-2008 Intake: Fish	Japan	Sawada <i>et al</i> <sup>[9]</sup> , 2012
Inverse association of vegetables intake and HCC risk	Study design: Case control Cases: 267 Controls: 132837 Duration: 1997-2006 Intake: Meat	China	Zhang <i>et al</i> <sup>[41]</sup> , 2013
Inverse association of fish intake and HCC risk HCC risk decreases by 16% for 20 g/d substitution of fish with meat	Study design: Cohort Cases: 157 Controls: 35628 Duration: 1992-2000 Intake: Dietary flavonoids	World-wide	Zamora-Ros <i>et al</i> <sup>[46]</sup> , 2013
Inverse association of fish intake and HCC risk No association of meat and poultry intake and HCC development	Study design: Cohort Cases: 191 Controls: 477206 Duration: 1992-2010 Intake: Total meat, fish, red and white meat	Europe	Fedirko <i>et al</i> <sup>[7]</sup> , 2013
HCC risk decreases by 8% for every 100 g/d increase in vegetable intake	Study design: Meta-analysis Cases: 3912 Controls: 1290045 Duration: 1956-2014 Intake: Vegetables and fruits	World-wide	Yang <i>et al</i> <sup>[43]</sup> , 2014

HCC: Hepatocellular carcinoma; CLD: Chronic liver disease.

molluscs independently associated with low HCC risk<sup>[7]</sup>. In a large population-based prospective Japanese cohort, Sawada *et al*<sup>[9]</sup> revealed that the consumption of fish or n-3 polyunsaturated fatty acids (n-3 PUFA) protects against the HCC development even among subjects with HBV and/or HCV infection, and Freedman *et al*<sup>[4]</sup> reported an inverse association between fish intake and CLD risk.

The finding that both fish and white meat reduce HCC risk is unforeseen. Nutritionally, fish and white meat are a rich source of PUFA and have less cholesterol and saturated fat compared with red meat. Substantial evidence indicates that n-3 PUFA possess anti-inflammatory activity by inhibiting IL-1 and TNF synthesis<sup>[34]</sup>, which can contribute in HCC prevention, considering that chronic inflammation plays a central role in HCC development. PUFA might exert anticancer effects also through their ability to induce apoptosis, to modulate cell cycle and eicosanoid production<sup>[35]</sup>. In particular, n-3 PUFAs have been shown to inhibit HCC growth *in vitro* through the blockage of  $\beta$ -catenin and cyclooxygenase-2<sup>[36]</sup>. It is observed that n-3 PUFA supplementation can improve hepatic steatosis in patients with NAFLD in a pilot study<sup>[37]</sup>. All of this evidence support a possible chemoprotective effect for fish and white meat on HCC development and suggest a molecular mechanism of n-3 PUFA in HCC prevention. However, some fatty acids themselves can also have harmful effects, particularly the saturated fats and trans fatty acids since their increased consumption is strongly linked with the development of non-alcoholic steatohepatitis and its progression to cirrhosis and fibrosis<sup>[38]</sup>. As there is significant heterogeneity in fat subtypes within most foods, increasing fatty acid consumption should not be encouraged in at-risk populations at least until more studies prove the potential benefits of specific PUFA supplements.

## MILK AND EGGS

High intake of milk, yogurt and eggs was found to reduce liver cancer risk in a case control study<sup>[10]</sup>. Decreased risk of HCC with highest milk intakes was also reported in another case-control study from Italy<sup>[18]</sup>, while a Japanese case-control study revealed a higher risk with greater than average milk consumption<sup>[19]</sup>. Saturated fat from dairy products was also independently associated with CLD and HCC risk<sup>[4]</sup>. However, two other studies from China and Japan did not find such associations<sup>[17,39]</sup>. An inverse correlation between egg and HCC has been observed in two Italian case-control studies<sup>[10,18]</sup>, while a Japanese study reported an increased risk of HCC for high egg consumption in men only<sup>[19]</sup>. Such discrepancies, however, may be attributed to different dietary habits between the studied populations, such as the use of fat for cooking. Notably eggs are a different diet indicator in Italy and Japan. The inverse association with dairy products and eggs could be explained by their retinol content, since serum retinol levels have been

inversely related to HCC risk in a case-control study from China<sup>[40]</sup>.

## VEGETABLE, FRUIT AND CEREALS

The association of vegetable and fruit intakes with HCC incidence has been investigated by a number of observational studies since Negri *et al*<sup>[14]</sup> revealed in a case-control study in the 1990s that high intake of vegetables and fruit was inversely associated with risk of upper digestive cancers, including HCC. The majority of studies reported inverse associations between high vegetable consumption and liver cancer risk<sup>[1,10,11,13-15,17,41]</sup> (Table 1). In the large prospective study of a US cohort and two European cohorts it was revealed that adherence to the high vegetable content diets of the dietary recommendations and the Mediterranean diet decreases HCC risk<sup>[42,43]</sup>. This observation was confirmed by Yang *et al*<sup>[43]</sup> in a meta-analysis on 19 published studies where it was reported that HCC risk decreases by 8% for every 100 g/d increase in vegetable intake. On the contrary, two case-control studies from Greece reported no association, although they involved a small number of cases<sup>[20,44]</sup>. The role of fruit consumption in HCC risk is more controversial. Three case-control studies reported a decreased liver cancer risk with higher fruit intake<sup>[10,13,18]</sup>, while other four studies found no such association<sup>[19,20,28,41]</sup>. Another case-control study in northern Italy found that the population attributable-risk for liver cancer was as high as 40% for low vegetable and fruit consumptions<sup>[11]</sup>.

Vegetables and fruits are major sources of vitamins, minerals, dietary fibres, and other bioactive compounds, including flavonoids. Several *in vitro* studies have shown an anti-tumour effect of flavonoids in some hepatocarcinoma cell lines<sup>[45,46]</sup> while in animal models, flavonoids have been shown to modulate mechanisms involved in proliferation, invasion, angiogenesis, survival and metastasis<sup>[47]</sup>. According to the EPIC study and a case-control study from Greece an inverse association exists between the flavonoids subclass, flavones, and HCC occurrence<sup>[46,48]</sup>. Therefore, flavonoids may explain the favorable effects of vegetables and fruits against liver cancer. In addition, evidence is emerging from cell culture and animal model experiments that phytochemicals and other bioactive components found in vegetables, such as diallyl sulphides, lentinan, apigenin and luteolin, have cancer-inhibitory effects through their anti-oxidative properties, stimulation of the immune system, or inhibition of mutagenesis<sup>[41]</sup>. An animal study on effects of dietary dry bean on hepatic gene expression in rats, found that the expression of six genes was significantly altered after high bean intakes suggesting that these genes may exert cancer preventive effects in liver<sup>[49]</sup>.

A protective role of dietary fiber has been also suggested in HCC development<sup>[6]</sup>. High intake of fiber from cereals and cereal derivatives was found to be statistically significantly inverse associated with HCC risk. Consumption of fiber from vegetables or other sources (but not fruits)

was also revealed to have a chemoprotective role in HCC development although a statistical significance was not reached<sup>[6]</sup>. Diets with a high fiber content, like cereals, could lower HCC occurrence by decreasing subjective appetite and energy intake and hence contributing to the maintenance of normal body weight as well as exerting beneficial effects on postprandial glucose level and blood lipid profile. Further research is needed to understand the mechanisms underlying these associations.

## DIETARY SUGAR

Dietary glycemic load (GL) estimates how much the food will raise a person's blood glucose level after eating it and is therefore the extent to which carbohydrate-rich foods increase the concentration of glucose in the blood to represent the total glycemic effect of a diet. GL has been associated with diabetes mellitus and with several types of cancer. The mechanism for the role of high GL in carcinogenesis is thought to be *via* increased insulin concentrations, glucose intolerance and insulin resistance, even in the absence of diabetes mellitus. Foods such as added sugars, syrups, sweets, white bread and soft drinks are the main culprits. One case control study from Italy showed a positive association between GL and HCC overall and interestingly a stronger association was observed in patients with HBV and HCV<sup>[50]</sup>. On the contrary, the EPIC study reported that GL and total carbohydrate intakes did not correlate with HCC risk<sup>[6]</sup>. However, when specific carbohydrates were analysed, a positive association was found for total sugar. Increased fructose intake is known to underlie NAFLD and may therefore provide a possible explanation for the positive association seen in HCC<sup>[51]</sup>.

## DIET IN VIRAL HEPATITIS-INDUCED HCC

HBV and HCV infections are major risk factors for the development of CLD and HCC. Three studies in the United States and Italy agree that the risk estimates for meat and fish consumption were similar between subjects with or without HBV and/or HCV infection<sup>[4,7,10,27]</sup>. The association of n-3 PUFA, fibers and flavonoids with HCC risk has also been found to exist independently of the HBV and/or HCV status<sup>[6,9,46,48]</sup>. However, the positive correlation of GL and HCC was observed to be stronger in patients with HBV and HCV in one case control study from Italy<sup>[50]</sup>. This evidence indicates that the dietary patterns, possibly except the GL, do not appear to affect HCC outcome in chronically HBV or HCV infected individuals. However, the possibility that dietary intakes can protect against the HCC progression in viral hepatitis infection cannot be excluded.

## CONCLUSION

Considering the increasing trend of HCC incidence and its high fatality, prevention of HCC is an urgent matter. At present attempts to prevent HCC mainly include the

control of HBV or HCV infection, reduction of alcohol consumption, and reducing the prevalence of obesity and diabetes. It is of great importance to discover novel strategies to prevent HCC and dietary factors represent an attractive potential.

Up to date the results from studies investigating the impact of dietary factors in HCC development are conflicting and often inconclusive. Notably, there are several limitations in the methodology of most studies, including selection bias, errors in diet assessment and insufficient adjustment for potential confounders such as HBV/HCV status, diabetes, alcohol and energy intake. Since both case control and prospective studies include data that comes from questionnaires, which are based on self-reported food intakes it is important to consider the dietary habits. Food consumption categories are different across the studies and this can contribute to the heterogeneity of the results. In addition, the case-control studies assessed diet after HCC diagnosis, at which stage the health of individuals has already been compromised, affecting the accuracy of dietary recall. Indeed, a major problem of the case-control studies on HCC is reverse causation because HCC precedes chronic hepatitis and cirrhosis. Furthermore, in the large prospective EPIC study, the diet was assessed only at baseline without considering any potential dietary changes during the follow-up, the period of exposure to cancer initiation was not taken into account and also that dietary patterns of different European countries may have not been fully accounted. It is therefore possible that dietary errors may have occurred that might underestimate the true associations described in the EPIC study.

Nevertheless from the available data it is evident that dietary factors play an important role in CLD and HCC occurrence and their identification can be used for the development of new public health diet recommendations. In particular, consistent associations indicate that higher consumption of vegetables, white meat, fish, milk and cereals have beneficiary effects in liver cancer development. It should be noted that increased consumption of fatty acids correlates with the progression of cirrhosis and fibrosis. The potential benefits of fat subtypes, deriving from white meat, fish and milk, should not be encouraged until more well designed studies prove their chemoprotective effect. Furthermore, it can be inferred that red meat and dietary sugar consumption intake associates with CLD mortality and HCC risk and hence their intake should be monitored and controlled in at-risk populations to attempt to slow down HCC development. Flavonoids appear to reduce the risk of HCC, but pharmacological doses might be required in order to effectively protect against carcinogenesis. Although current studies on infections with HBV and HCV suggest that the effect of diet is independent of viral hepatitis infection, an association between diet and hepatitis virus related HCC progression is possible.

To prevent disease progression to CLD or HCC it is crucial to investigate the impact of diet and subsequently to lead to the development of clinical trials using new



dietary patterns. Evidence from well designed prospective interventional studies with large sample sizes and long-term follow-up are required to develop diet modifications to lower HCC incidence or to prolong survival in HCC patients. Additional experimental and molecular research is also needed to explore the possible mechanisms involved.

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## Iatrogenic amyloid polyneuropathy after domino liver transplantation

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### Abstract

Liver transplantation has been used in treatment of transthyretin amyloidosis, and some patients undergo domino liver transplantation (DLT) with explanted liver

being transplanted to another patient with liver failure as the liver is otherwise usually functionally normal. Until end of 2015, there were 1154 DLT performed worldwide. DLT for transthyretin amyloidosis is associated with the risk of developing *de novo* systemic amyloidosis and amyloid neuropathy, and the risk may be greater with some non-Val30Met mutations. *De novo* amyloid neuropathy has been described in up to 23% of transplant recipients. Neuropathy may be preceded by asymptomatic amyloid deposition in various tissues and symptoms of neuropathy started after a median of 7 years following DLT ( $5.7 \pm 3.2$  years; range 2 mo to 10 years). Typical initial symptoms include neuropathic pain and sensory loss, while dysautonomia usually starts later. Progression of neuropathy may necessitate liver re-transplantation, and subsequent improvement of neuropathy has been reported in some patients. Explant allograft recipients need close monitoring for signs of systemic amyloidosis, neuropathy and dysautonomia as progressive symptoms may require re-transplantation.

**Key words:** Transthyretin; Familial amyloid neuropathy; Domino liver transplantation; Systemic amyloidosis; Acquired amyloid neuropathy

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**Core tip:** Domino liver transplantation (DLT) has been used in treatment of transthyretin amyloidosis, with explanted liver being transplanted to another patient with liver failure as the liver is otherwise usually functionally normal. Domino liver explant recipients are at risk of developing *de novo* systemic amyloidosis and amyloid neuropathy has been described in up to 23% of transplant recipients after a median of 7 years following DLT. Typical initial symptoms include neuropathic pain and sensory loss, while dysautonomia usually starts later. Explant allograft recipients need close monitoring for signs of systemic amyloidosis, neuropathy and dysautonomia as progressive symptoms may require re-transplantation.

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## INTRODUCTION

Transthyretin familial amyloidosis is an autosomal dominant multisystem disorder caused by deposition of insoluble transthyretin (TTR) amyloid deposits in various tissues<sup>[1]</sup>. Amyloid deposits are found in peripheral nerve, liver, skin, heart and other organs, and peripheral neuropathy is one of the major clinical manifestations. Three main phenotypes of transthyretin familial amyloidosis include familial amyloid polyneuropathy (TTR-FAP), cardiomyopathy and leptomeningeal amyloidosis. Additionally, wild type TTR may be also deposited in senile systemic amyloidosis which more commonly presents with cardiomyopathy, and only rarely as neuropathy. TTR-FAP is a progressive sensorimotor neuropathy with dysautonomia which results in a severe disability. The neuropathy usually starts with distal sensory loss and dysesthesias, followed by autonomic dysfunction, while motor function is usually affected only later<sup>[2]</sup>. Carpal tunnel syndrome is usually an early feature, but there is a significant variability of symptoms, even among the patients with the same TTR mutation. Overall prevalence of TTR-FAP is estimated at 0.9-1.1 per 1000000 people, with expected survival of 7-12 years after the onset of symptoms<sup>[3-5]</sup>. To date more than 120 mutations of TTR have been reported to cause TTR-FAP, and high prevalence is found in endemic regions in Portugal, Japan and Sweden (up to 1 in 1000 to 1 in 10000), and the most common genotype with predominant neuropathy is Val30Met TTR mutation<sup>[6]</sup>.

Transthyretin is mainly synthesized in the liver and liver transplantation has been used to ameliorate the progression of systemic amyloidosis by decreasing the synthesis of abnormal TTR<sup>[7,8]</sup>. There is a shortage of liver grafts available for transplantation, and explanted liver of the patient with TTR amyloidosis is sometimes transplanted to another patient with liver failure as the liver is otherwise usually functionally normal Domino liver transplantation (DLT)<sup>[8]</sup>. Until end of 2015, there were 1154 DLTs performed worldwide<sup>[9]</sup>. Best outcomes for liver transplantation have been reported in young Val30Met patients with mild symptoms, and the overall 20-year survival after liver transplantation for TTR-amyloidosis has reached 55%<sup>[8,10,11]</sup>. In transplant patients (DLT donors) with non-Val30Met genotype, there is a wide spectrum of survival rates which vary a lot depending on the underlying mutations<sup>[12]</sup>. The overall risk of amyloid production by transplanted domino liver was thought to be low with potential delayed manifestations of amyloidosis in patients with otherwise very short survival if they are not transplanted. Nevertheless, amyloid deposition in the tissue may start soon after domino

transplantation and iatrogenic amyloid neuropathy may affect up to 8%-24% of DLT recipients<sup>[13,14]</sup>.

In this manuscript, we review clinical features of acquired amyloid TTR neuropathy after DLT.

## LITERATURE

We have identified 16 case reports with detailed description of acquired TTR-FAP in recipients of DLT in the literature search (Table 1)<sup>[14-23]</sup>. The patients were 73% men with age of  $60.7 \pm 10.4$  years. The most common TTR mutation was Val30Met ( $n = 10$ ; 61.3%), and others included Ser23Asn, Ser77Tyr, Leu58His, Thr49Ala, Gly47Glu, Glu54Gly ( $n = 1$  each; 6.7%). Underlying causes of liver failure include hepatitis C infection ( $n = 8$ ), hepatocellular carcinoma ( $n = 6$ ), hepatitis B infection ( $n = 5$ ), primary sclerosing cholangitis, primary biliary cirrhosis and nonalcoholic liver steatosis ( $n = 1$  each; some patients had more than 1 cause).

*De novo* amyloid neuropathy presented at after a median of 7 years following DLT ( $5.7 \pm 3.2$  years; range 2 mo to 10 years). Initial symptoms included neuropathic pain ( $n = 14$ ), sensory loss ( $n = 5$ ), erectile dysfunction ( $n = 2$ ), weakness, diarrhea and orthostatic hypotension ( $n = 1$ , each). Nerve biopsies showed amyloid deposits in 3 reported cases<sup>[16,18,23]</sup>. Other abnormal tests included positive rectal and abdominal fat ( $n = 2$  each), duodenal and endomyocardial biopsies ( $n = 1$  each) showing amyloid deposits. Three patients were treated with retransplantation of the liver, with improved outcome in two patients (outcome not reported in the third case).

The age of donors and recipients of domino liver allografts was not associated with an earlier onset of amyloid neuropathy. Non-Val30Met TTR mutations were overall associated with earlier onset of *de novo* TTR-FAP neuropathy (latency 3.95 years vs 6.83 years after transplantation; range 2 mo to 10 years).

## CONCLUSION

DLT has been advocated to alleviate shortage of liver allografts for patients in liver failure who would not have survived without such procedure. Initial reports estimated a low risk of systemic amyloidosis from synthesis of abnormal transthyretin liver allografts. Nevertheless, subsequent studies demonstrated that recipients of DLT are indeed at risk from systemic amyloidosis and some patients may develop *de novo* amyloid neuropathy after a median of 7 years following transplantation ( $5.7 \pm 3.2$  years; range 2 mo to 10 years). Similarly, less favorable survival of patients with non-Val30Met after liver transplantations (domino liver donors) was paralleled by shorter latency of *de novo* amyloid neuropathy in domino liver recipients with allografts with non-Val30Met TTR mutations (3.95 years vs 6.83 years)<sup>[12]</sup>. Nevertheless, there seems to be a marked variability of clinical course, depending on the underlying TTR mutation in domino liver donors (and allografts). Exceptionally short latency of *de novo* amyloid neuropathy in DLT recipients was reported with Ser23Asn and Leu58His TTR mutations



**Table 1** *De novo* transthyretin amyloid neuropathy cases after liver transplantation

Ref.	TTR mutation	Age at transplant	Latency (yr)	Cause of liver failure	Initial Neuropathy symptoms
[14]	Val30Met	61	8	HCV EtOH	Pain
[14]	Val30Met	35	5	HBV	Pain
[14]	Val30Met	26	3.5	HCC	Pain, erectile dysfunction
[14]	Val30Met	28	5	HBV	Pain
[14]	Ser77Tyr	57	2	HCC HBV HCV	Pain
[14]	Leu58Hys	60	0.17	HCV HIV	Pain, weakness
[14]	Thr49Ala	49	2	HCC HCV	Pain, orthostatic hypotension
[15]	Glu54Gly	43	9	HCC	Diarrhea, pain, sensory loss
[16]	Val30Met	60	7	HBV cirr	Pain
[17]	Gly 47Glu	65	10	HCC	Pain
[18]	Val30Met	54	9	HCV	Pain, erectile dysfunction
[19]	Ser23Asn	72	0.5	NASH	Sensory loss, pain
[20]	Val30Met	50	7	PBC	Sensory loss
[21]	Val30Met	35	10	PSC	Pain, sensory loss
[22]	Val30Met	59	12	HBV HCV cirr	Pain, sensory loss
[23]	Val30Met	47	8	HCV cirr	Pain

PBC: Primary biliary cirrhosis; NASH: Nonalcoholic hepatic steatosis; EtOH: Alcoholic liver cirrhosis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; cirr: Cirrhosis of the liver; PSC: Primary sclerosing cholangitis; HCC: Hepatocellular carcinoma.

(< 6 mo)<sup>[14,19]</sup>. These series did not show an association with donor or recipient age with *de novo* systemic TTR amyloidosis, although other studies suggest that recipient aging may accelerate tissue deposition of amyloid<sup>[24]</sup>.

Clinically, these patients typically present with sensory loss and neuropathic pain, while dysautonomia is often not the initial symptom and follows later (Table 1). Tissue deposition of amyloid in the gastrointestinal tract and skin may precede clinical symptoms of neuropathy by several years<sup>[20,25]</sup>, and prospective study showed deposition of amyloid in salivary glands in up to 48% of DLT recipients<sup>[14]</sup>. Additionally, amyloid deposits were also found on sural nerve biopsies in the absence of clinical signs of neuropathy (research study and autopsy)<sup>[13,26]</sup>. Transthyretin amyloid neuropathy in DLT recipients presents in the context of systemic amyloidosis and *de novo* amyloid produced by transplanted liver is also found in myocardium, gastrointestinal tract, skin and fatty tissues<sup>[15,17,19-22]</sup>. Autopsy case of a DLT patient with asymptomatic amyloidosis at 8 years after transplantation (died from lymphoma) showed amyloid deposits in the heart, lungs, gastrointestinal tract (upper and lower), pancreas, spleen, testes, epididymis, prostate, skeletal muscle, thoracic sympathetic ganglia and median nerve (carpal tunnel syndrome)<sup>[26]</sup>. Amyloid deposition in the myocardium after DLT is usually asymptomatic, and so far only two cases of *de novo* cardiomyopathy after DLT have been reported with Val71Ala and Val30Met mutations<sup>[27,28]</sup>. One of the patients developed cardiac amyloidosis 5 years after retransplantation for systemic *de novo* amyloidosis after DLT, and it is unclear whether cardiac amyloidosis was related to wild-type or mutant transthyretin<sup>[28]</sup>. Similarly, DLT donors continue to have deposition of amyloid in different tissues of FAP-TTR patients after transplantation, which is at least partly related to wild-type ("senile") amyloid<sup>[29]</sup>. Worsening of peripheral neuropathy after transplantation has been reported in 24% of DLT liver allograft donors<sup>[5]</sup>, and ocular deposition of amyloid is

not abated after liver transplantation, as mutant TTR continues to be synthesized in the retinal pigment epithelium<sup>[30]</sup>.

While *de novo* amyloid neuropathy is associated with significant morbidity, DLT recipients may also have other potential causes of neuropathy and nerve biopsy may be needed to establish the etiology and exclude alternative etiologies<sup>[14]</sup>. Nevertheless, nerve biopsy may be false negative with a reported sensitivity of 33%-80%, and this may be attributed to uneven distribution of amyloid along peripheral nerves<sup>[31-33]</sup>. Biopsies of gastrointestinal tract, myocardium and fat aspirate may also demonstrate systemic deposition of amyloid in DLT recipients<sup>[15,17,19-22]</sup>.

*De novo* amyloid neuropathy presents in the context of systemic amyloidosis and careful monitoring of clinical manifestations of amyloidosis is needed after DLT (Table 2)<sup>[34]</sup>. Once iatrogenic amyloid neuropathy and systemic amyloidosis are diagnosed, treatment options are limited and retransplantation has been reported to stabilize or improve neuropathy in some recipients<sup>[15,16]</sup>. Tafamidis, a tetramer stabilizer, is approved for treatment of FAP-TTR in Europe, Japan, Mexico and Argentina and is used as first-line treatment for early FAP-TTR<sup>[35]</sup>. In countries where tafamidis is available, liver transplantation is often considered as a second-line option for patients who progressed on tafamidis or did not tolerate the treatment. Liver-retransplantation typically carries worse prognosis and more significant morbidity than the initial transplantation, but the survival after liver retransplantation in DLT recipients seems to be greater than in other subgroups of liver retransplant recipients<sup>[36]</sup>. Despite a single report of benefits with deflunisal<sup>[37]</sup>, potential benefits of treatment with tetramer stabilizers in DLT recipients remain uncertain. There are also no reports on efficacy of inhibition of expression of *TTR* gene in this setting. At this time, tafamidis remains available in a limited number of countries, and new experimental treatments are still not ready to substitute the role of DLT in treatment of systemic amyloidosis. In conclusion, DLT

**Table 2** Evaluation and monitoring of possible *de novo* amyloidosis after domino liver transplantation

Clinical presentation	Signs and symptoms	Testing
Dysautonomia (small fiber neuropathy)	Orthostatic hypotension Sweating abnormalities (anhidrosis) Constipation/diarrhea Erectile dysfunction Neuropathic pain Arrhythmia Neurogenic bladder	Tilt table testing QSART with autonomic battery ECG Neurologic examination
Large fiber polyneuropathy	Sensory loss Weakness Neuropathic pain Ataxia Areflexia	EMG/NCS Nerve (and muscle) biopsy Neurologic examination
Cardiac amyloidosis	Fatigue Arrhythmia Syncope Orthostatic hypotension	ECG Transthoracic echo Radionuclide imaging Cardiac MRI Endomyocardial biopsy BNP/troponin GI tract biopsy
Gastrointestinal amyloidosis	Constipation/diarrhea Nausea/vomiting	
Ocular amyloidosis	Dry eye Vitreous opacity Glaucoma	Ophthalmologic evaluation
Leptomeningeal amyloidosis	Cerebral infarction/hemorrhage Hydrocephalus Ataxia Spastic paresis Seizures Dementia	MRI of brain and spinal cord Meningeal biopsy
Other system involvement	Coldness Weight loss Peripheral edema Anemia Dry mouth	Rectal and abdominal fat biopsy Salivary gland biopsy TSH Urinalysis/urine protein collection

QSART: Quantitative sudomotor axon reflex test; ECG: Electrocardiograph; EMG/NCS: Electromyography and nerve conduction studies; MRI: Magnetic resonance imaging; BNP: Brain natriuretic peptide; GI: Gastrointestinal; TSH: Thyroid stimulating hormone.

is associated with the risk of developing *de novo* systemic amyloidosis and amyloid neuropathy, and the risk may be greater with some non-Val30Met mutations. Explant allograft recipients need close monitoring for signs of systemic amyloidosis, neuropathy and dysautonomia as progressive symptoms may require re-transplantation.

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## Cockcroft-Gault revisited: New de-liver-ance on recommendations for use in cirrhosis

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### Abstract

The Cockcroft-Gault (CG) equation has become perhaps the most popular practical approach for estimating renal function among health care professionals. Despite its widespread use, clinicians often overlook not only the limitations of the original serum creatinine (SCr) based equation, but also may not appreciate the validity of the many variations used to compensate for these limitations. For cirrhotic patients in particular, the underlying pathophysiology of the disease contributes to a falsely low SCr, thereby overestimating renal function with use of the CG equation in this population. We reviewed the original CG trial from 1976 along with data surrounding clinician specific alterations to the CG equation that followed through time. These alterations included different formulas for body weight in obese patients and the "rounding up" approach in patients with low SCr. Additionally, we described the pathophysiology and hemodynamic changes that occur in cirrhosis; and reviewed several studies that attempted to estimate renal function in this population. The evidence we reviewed regarding the most accurate manipulation of the original CG equation to estimate creatinine clearance (CrCl) was inconclusive. Unfortunately, the homogeneity of the patient population in the original CG trial limited its external validity. Elimination of body weight in the CG equation actually produced the estimate closest to the measure CrCl. Furthermore, "rounding up" of SCr values often underestimated CrCl. This approach could lead to suboptimal dosing of drug therapies in patients with low SCr. In cirrhotic patients, utilization of SCr based methods overestimated true renal function by about 50% in the literature we reviewed.

**Key words:** Cockcroft-Gault; Cirrhosis; Renal function; Pharmacokinetics; Creatinine clearance

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**Core tip:** For many health care professionals in the



United States, the Cockcroft-Gault (CG) equation has become perhaps the most popular practical approach for estimating renal function. Despite its widespread use, clinicians often overlook not only the limitations of the original serum creatinine (SCr) based equation, but also may not appreciate the validity of variations used to compensate for these limitations. For cirrhotic patients in particular, the underlying disease pathophysiology contributes to a falsely low SCr, thereby overestimating renal function with use of the CG equation in this population.

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## INTRODUCTION

In order to optimize efficacy and minimize potential toxicity of pharmacologic agents, appropriate patient-specific dosing of medications remains an inherent responsibility of all healthcare providers. Proper assessment of a patient's renal function is essential when managing medications that are primarily renally excreted<sup>[1]</sup>. With an estimated 14% of adults in the United States experiencing varying degrees of chronic kidney disease (CKD), optimization of drug therapies poses a frequent challenge to clinicians<sup>[2]</sup>. Renal impairment may significantly alters the pharmacokinetic (PK) properties of many medications<sup>[3]</sup>. Therefore, reasonably accurate yet convenient quantification of the degree of renal impairment is an essential tool for clinicians implementing renal dose adjustments<sup>[3]</sup>.

In the majority of clinical settings, calculating the creatinine clearance (CrCl) using the Cockcroft-Gault (CG) equation has become the most popular and practical approach for estimating renal function<sup>[4,5]</sup>. Many institutions provide dosing recommendations based on calculated CrCl, and even often utilize electronic health record (EHR) software that automatically calculates CrCl based on the CG equation. Unfortunately, some clinicians fail to realize the inherent limitations of a serum creatinine (SCr) based equation and the subsequent variations that stem from many of these limitations<sup>[1,4,6]</sup>.

SCr concentrations can be altered by patient specific factors including age, sex, weight, muscle mass, disease state, diet, and certain drug therapies, thus limiting the generalizability of the CG equation<sup>[1,4-6]</sup>. For example, patients with hepatic impairment not only experience altered drug metabolism, but also have secondarily reduced creatinine production. If not taken into consideration, these SCr-based formulas can lead to an overestimation of GFR in cirrhotic patients<sup>[7]</sup>.

To help clarify the true applicability of the CG equation, we will discuss the origins of the CG equation and

the evidence and reasoning behind specific alterations to the equation used in current practice.

## DATA SOURCES AND SELECTION

Data included in this review were identified from a PubMed search of publications starting in 1970 through June of 2016. Searches included the keywords "Cockcroft-Gault", "serum creatinine", "creatinine clearance", "renal function", "cirrhosis" and related search terms. Publications were considered for review if they were designed as meta-analyses, retrospective, or prospective studies that compared different methods of estimating CrCl using the CG equation.

## THE ORIGINS OF THE COCKCROFT-GAULT EQUATION

The CG equation (Table 1A; equation I) was derived from 236 patients (96% male), aged 18-92 years old in 1976 at the Queen Mary Veterans' Hospital in Canada. SCr values used in the equation were the mean values calculated from two 24-h SCr levels obtained from blood for each patient at steady state. The CrCl was calculated using 4 different formulas (Table 1A; equations I-IV) that were compared against each other and with each patient's 24-h urine creatinine excretion. The CG equation was found to provide an estimated CrCl that was  $80\% \pm 30\%$  of the actual creatinine clearance calculated from the 24-h urine creatinine excretion test<sup>[5]</sup>.

Limitations acknowledged at the end of this trial included requirements for SCr to be at steady state, the need for normal relationship between muscle mass and total body weight, and factors related to age, sex, and height. In addition to this, the formula was tested in a patient population that was 96% male, which obviously limits the external validity of the results in female cohorts. To compensate for females having different relative amounts of fat and muscle compared to males, a somewhat arbitrary 15% reduction of predicted CrCl was considered appropriate based on previous study estimations<sup>[8-10]</sup>. Furthermore, it was noted that certain patients had predictably low creatinine excretion for age and body weight. Examples included paraplegics and patients with marked obesity or ascites. To correct for these patients, although no data were presented to support this decision, the authors suggested using ideal body weight (IBW).

Finally, due to the delay in SCr fluctuations and the time needed to establish a new steady state, the authors acknowledged that CrCl can be significantly overestimated in early phases of acute renal failure<sup>[5]</sup>. This is an extremely important concept for clinicians to grasp. In patients with excellent renal function, the  $t_{1/2}$  is on the order of 4 h, and a new steady state could be reached in about 1 d. However, a 75% reduction in GFR would increase the half-life to about 15 h, and the time to steady state would increase to about  $2 \frac{1}{2}$  d<sup>[11]</sup>. In the

**Table 1** Different methods of estimating creatinine-clearance (A): Equations I-IV were evaluated in the original Cockcroft-Gault study, Equation V is a modified Cockcroft-Gault that only incorporates age and serum creatinine into the equation; B: Different body weight equations tested in the Cockcroft-Gault equation to compensate for various body types

A: Formula = CrCl (mL/min)		B: Formula = weight (kg)	
I <sup>1</sup>	$[(140 - \text{age})(\text{weight in kg})] / (72 \times \text{SCr})$	IBW <sub>male</sub>	$50 + (2.3 \text{ kg} \times \text{inches} > 60)$
II	$(100/\text{SCr}) - 12$	IBW <sub>female</sub>	$45.5 + (2.3 \text{ kg} \times \text{inches} > 60)$
III	$98 - 16 \times [(age - 20)/20] \times \text{SCr}$	AdjBW	$\text{IBW} + [(\text{TBW} - \text{IBW}) \times C^2]$
IV	$(94.3/\text{SCr}) - 1.8$	LBW <sub>male</sub>	$9270 \times \text{TBW}$ $6680 + (216 \times \text{BMI})$
V	$(140 - \text{age}) / \text{SCr}$	LBW <sub>female</sub>	$9270 \times \text{TBW}$
VI	$100 / \text{SCr}$	FFW	Calculated using BIA <sup>[15]</sup>

<sup>1</sup>CrCl  $\times (1.73 \text{ m}^2/\text{BSA})$  to normalize to body surface area (BSA) of  $1.73 \text{ m}^2$ ;

<sup>2</sup>0.3 for 30% ABW and 0.4 for 40% ABW. AdjBW: Adjusted body weight; BMI: Body mass index; CrCl: Creatinine clearance; FFW: Fat free weight; IBW: Ideal body weight; LBW: Lean body weight; SCr: Serum creatinine; TBW: Total body weight; BIA: Bioelectrical impedance analysis.

case of oliguric or anuric renal failure, it may take several days to reach a new steady state for the SCr<sup>[11,12]</sup>.

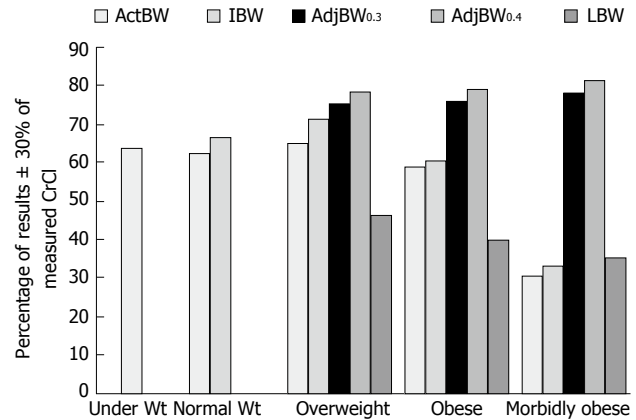
Despite the above acknowledged limitations of the CG equation, it has become the most popular renal function prediction method used for renal dosing by clinicians<sup>[1,13,14]</sup>. Attempts to validate CrCl calculated using the CG equation have produced mixed results<sup>[6,13]</sup>. In their 2010 Guidance for Industry, the Food and Drug Administration (FDA) advocated for the use of the CG equation in drug development because it has been widely used in PK studies<sup>[14]</sup>. For instance, where CrCl may be inaccurate (muscle wasting, malnutrition, amputation, etc.), alternative methods of calculating CrCl are suggested but not required<sup>[14]</sup>.

As clinicians, it is important to understand that attempts to modify the equation to compensate for some of these patient-specific factors often lead to variable results. In the sections that follow, we discuss the rationale and results that these various adjustments yield in predicting actual CrCl.

## WEIGHING YOUR OPTIONS

Once again, recall that the CG equation was derived based on the assumption that SCr represents muscle mass as a definite percentage of the patient's body weight, and that both of these values decline in a linear manner as patients age<sup>[13]</sup>. In obese patients, these assumptions may not be true, as body fat becomes the major contributor to body mass<sup>[13]</sup>. Given that over 50% of the United States population > 20 years old are overweight or obese, reviewing available literature comparing accuracy of different weights used in the CG equation may help clinicians optimize dose selection<sup>[1,15]</sup>.

The CG equation was derived from a population of



**Figure 1** Impact of various body weights used in estimating creatinine clearance from Winter *et al.*<sup>[1]</sup>. In patients with a BMI  $\geq 25 \text{ kg/m}^2$ , using AdjBW<sub>0.4</sub> was the most accurate weight to estimate CrCl when compared to a 24-h urine CrCl. Under Wt: BMI  $< 18.5 \text{ kg/m}^2$ ; Normal Wt: BMI  $18.5\text{--}24.9 \text{ kg/m}^2$ ; Overweight: BMI  $25\text{--}29.9 \text{ kg/m}^2$ ; Obese: BMI  $30\text{--}39.9 \text{ kg/m}^2$ ; Morbidly obese: BMI  $\geq 40 \text{ kg/m}^2$ ; CrCl: Creatinine clearance; ActBW: Actual body weight; IBW: Ideal body weight; AdjBW: Adjusted body weight; LBW: Lean body weight; BMI: Body mass index.

normal weight individuals (mean = 72 kg) using actual body weight; and therefore, its use in obese patients may lead to significant estimation errors<sup>[5,16]</sup>. Despite 40 years of clinical experience and numerous studies evaluating different weight calculations in obese patients (Table 1B), no uniform consensus appears to exist for estimating CrCl using the CG equation in this patient population<sup>[1,15-17]</sup>.

Winter *et al.*<sup>[1]</sup> studied the impact of various body weights used when calculating CrCl in obese and non-obese patients. They estimated CrCl using the CG equation with actual body weight (actBW) for body mass index (BMI)  $< 18.5 \text{ kg/m}^2$ ; IBW and actBW for BMI  $18.5\text{--}24.9 \text{ kg/m}^2$ ; and actBW, IBW, adjusted body weight (adjBW<sub>0.3</sub>), adjBW<sub>0.4</sub>, and lean body weight (LBW) for all patients with BMI  $> 25 \text{ kg/m}^2$ . The calculated CrCl was compared to a CrCl derived from a measured 24-h urine collection for all 952 patients in the study. ActBW was shown to underestimate CrCl by 0.221 mL/min in underweight patients (BMI  $< 18.5 \text{ kg/m}^2$ ); in normal weight patients (BMI:  $18.5\text{--}24.9 \text{ kg/m}^2$ ), IBW was shown to be more accurate than actBW (IBW underestimated CrCl by 1.3 mL/min vs actBW overestimated by 4.7 mL/min); and in patients with a BMI  $> 25 \text{ kg/m}^2$ , adjBW<sub>0.4</sub> was shown to be the most accurate method of predicting CrCl (BMI  $25\text{--}29.9 \text{ kg/m}^2$  -2.4 mL/min; BMI  $30\text{--}39.9 \text{ kg/m}^2$  -6.2 mL/min; BMI  $> 40 \text{ kg/m}^2$  -5.9 mL/min) (Figure 1).

In a similar study, Demirovic *et al.*<sup>[15]</sup> prospectively evaluated the impact different body-size descriptors would have on the accuracy of the CG equation when compared to a timed 24-h urine collection. They estimated the CrCl in only obese patients with a BMI  $\geq 40 \text{ kg/m}^2$  and used ActBW, IBW, AdjBW<sub>0.3</sub>, AdjBW<sub>0.4</sub>, fat free weight (FFW), and LBW in the CG equation. Bioelectric impedance analysis (BIA) was used to estimate the FFW in patients. The calculated CrCl was compared to

**Table 2** Estimating creatinine clearance in morbidly obese patients by Demirovic *et al.*<sup>[15]</sup> showed that using fat free weight and lean body weight provided the closest estimate to the control 24-h urine creatinine clearance

Method <sup>1</sup>	Mean estimated CrCl ± SD	Mean bias (mL/min)	± 30% of measured CrCl	± 50% of measured CrCl
Measured CrCl	109.5 ± 44.4			
ActBW	217 ± 113	-107	13%	30%
IBW	85 ± 29	+24	48%	89%
AdjBW <sub>0.3</sub>	129 ± 55	-20	54%	76%
AdjBW <sub>0.4</sub>	142 ± 63	-33	52%	67%
FFW	103 ± 48	+7	61%	83%
LBW	102 ± 43	+8	56%	87%
MDRD4	96.3 ± 29.4	+13.3	51.90%	87%
Salazar-Corcoran	155.2 ± 65.1	-45.7	46.20%	55.60%

<sup>1</sup>All weight variables used in CG equation only. MDRD4<sup>[18]</sup>:  $186 \times \text{SCr}^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$ ; Salazar-Corcoran<sup>[19]</sup>: Male:  $(137 - \text{age}) \times [(0.285 \times \text{Wt}) + (12.1 \times \text{height meters}^2)]$ ,  $51 \times \text{SCr}$ ; Female:  $(146 - \text{age}) \times [(0.287 \times \text{Wt}) + (9.74 \times \text{height meters}^2)]$ ,  $60 \times \text{SCr}$ ; ActBW: Actual body weight; AdjBW<sub>0.3</sub>: 30% adjusted body weight; AdjBW<sub>0.4</sub>: 40% adjusted body weight; CG: Cockcroft-Gault; CrCl: Creatinine clearance; FFW: Fat free weight; IBW: Ideal body weight; LBW: Lean body weight; MDRD: Modification of diet in renal disease study equation; SD: Standard deviation; Wt: Weight.

a CrCl derived from a measured 24-h urine collection for all 54 patients in the study (Table 2). On average, the CG equation using a patient's actBW overestimated the CrCl by 107.4 mL/min; using IBW underestimated CrCl by 24.3 mL/min; using AdjBW<sub>0.3</sub> and AdjBW<sub>0.4</sub> both overestimated the CrCl by 19.8 and 32.3 mL/min, respectively; FFW and LBW were found to be the most accurate estimate of the measured CrCl, as the FFW underestimated CrCl by 6.8 mL/min and LBW underestimated by 8.1 mL/min (Table 2)<sup>[15,18,19]</sup>.

A meta-analysis by Wilhelm *et al.*<sup>[17]</sup> analyzed a total of 1197 patients from 13 different trials and compared CrCl calculated with CG using ActBW, IBW, AdjBW<sub>0.3</sub>, AdjBW<sub>0.4</sub>, and no body weight (NBW) with a measured 24-h urine collection. For NBW, the authors assumed the patient weight to be 72 kg, as this was the average weight from the original CG trial<sup>[5]</sup>. NBW slightly modified the CG equation as it only incorporates age and SCr (Table 1A; Equation V). When using actBW, the mean difference in the CG estimated CrCl was an overestimation of 15.91 mL/min; using IBW underestimated CrCl by 5.15 mL/min; using adjBW<sub>0.3</sub> slightly underestimated the CrCl by 4.55 mL/min whereas the adjBW<sub>0.4</sub> considerably underestimated the CrCl by 19.94 mL/min. The most accurate method of estimating CrCl was using the modified CG equation without a variable for body weight (NBW), which underestimated CrCl by 0.43 mL/min.

The studies presented above reiterate the challenges faced by many clinicians when estimating CrCl using the CG equation. The CG equation was not originally studied in obese patients, and therefore, has limited applicability in this population. The study by Winter *et al.*<sup>[1]</sup> showed that in patients with a BMI > 25 kg/m<sup>2</sup>, use of adjBW<sub>0.4</sub> was the most accurate method of estimating CrCl when

using the CG methods. Unfortunately, Demirovic *et al.*<sup>[15]</sup> did not come to the same conclusion with the results found by Winter *et al.*<sup>[1]</sup> and Demirovic *et al.*<sup>[15]</sup>. They found that FFW and LBW provided the most accurate estimate of CrCl when compared to a measured 24-h urine collection. These findings support what was originally assumed by CG. That is, that SCr can best be used as a surrogate marker for renal function when an accurate assessment of a patient's muscle mass is used to calculate CrCl<sup>[5]</sup>. Unfortunately, calculating FFW and LBW on a daily basis is not practical in most clinical settings. Finally, Wilhelm's study illustrated that in a large, heterogeneous sample, removing the weight variable from the CG equation actually produced the estimate closest to the measured CrCl<sup>[17]</sup>. Although body weight remains controversial, utilizing the NBW equation assumes SCr predictable declines with age. Like the original CG equation, the NBW equation may be of limited use in patients with low SCr or falsely low SCr due to muscle mass or underlying disease. Certainly, the evidence presented by the authors of this review reiterate the potential limitations of the CG equation, and why this equation cannot be used as the sole means of estimating renal function in all patients.

## SERUM CREATININE - HOW LOW CAN YOU GO?

It is well known that, due to a decrease in muscle mass beyond about age 40, SCr and CrCl decline as a patient ages. The CG equation assumes this decline is linear<sup>[5,20]</sup>. In patients with a SCr ≤ 0.6 mg/dL, CrCl estimation using the CG equation often overestimate CrCl, and may consequently lead to supratherapeutic dosing of renally excreted drugs<sup>[21]</sup>. To compensate for this, clinicians often arbitrarily round a SCr ≤ 0.6 mg/dL to a closer-to-normal value (0.8-1 mg/dL)<sup>[21]</sup>. Although "rounding up" of SCr is a widely used technique by many clinicians, it has not been robustly validated<sup>[21]</sup>.

Dooley *et al.*<sup>[21]</sup> performed a study comparing measured GFR using diethyl triamine penta-acetic acid (DTPA) to an estimated CrCl calculated using the CG equation in patients with low SCr levels (< 0.6 mg/dL), and determined the impact of rounding SCr to 0.6 mg/dL. This retrospective study analyzed 26 patients with an average age of 57 years old. When compared to the measured GFR, the CG equation, using actual SCr overestimated CrCl by 12.9%, whereas the rounded SCr of 0.6 mg/dL underestimated CrCl by 7% (Table 3). Although rounding of SCr to 0.6 mg/dL was more accurate when calculating CrCl in this study, it was noted by the authors that clinicians typically round to either 0.8 or 1 mg/dL which would increase the underestimation when calculating CrCl. Furthermore, in patients with a measured CrCl that was > 100 mL/min, the rounding of SCr to 0.6 mg/dL underestimated CrCl by 18.9% vs 0.1% using the actual SCr.

Smythe *et al.*<sup>[22]</sup> performed a prospective study in elderly patients, but chose to round SCr to 1.0 mg/dL

**Table 3** Results from Dooley *et al.*<sup>[21]</sup> illustrated that rounding of serum creatinine to 0.6 mg/dL underestimated creatinine clearance by 7%; of note, the majority of clinicians round low serum creatinine values to 0.8 or 1.0 mg/dL

		Mean $\pm$ SD (mL/min)	Range (mL/min)	Mean % error	P value
DTPA	All	111 $\pm$ 46	45-256		
	$\leq 100$ mL/min	77 $\pm$ 14	45-96		
	$> 100$ mL/min	140 $\pm$ 45	103-256		
CG (no rounding)	All	117 $\pm$ 38	55-207	12.9	0.352
	$\leq 100$ mL/min	98 $\pm$ 28	55-152	29.2	0.024
	$> 100$ mL/min	135 $\pm$ 38	86-207	-0.1	0.631
CG (rounding SCr to 0.6 mg/dL)	All	97 $\pm$ 30	46-172	-7.0	0.029
	$\leq 100$ mL/min	82 $\pm$ 23	46-127	7.9	0.543
	$> 100$ mL/min	110 $\pm$ 29	72-172	-18.9	0.003

CG: Cockcroft-Gault; DTPA: Diethyl triamine penta-acetic acid; SCr: Serum creatinine.

**Table 4** Results from Smythe *et al.*<sup>[22]</sup> showed that rounding of serum creatinine to 1.0 in elderly patients was less accurate than using the patients actual serum creatinine

Method	Bias = CrCl <sub>meas</sub> - CrCl <sub>calc</sub> (CI)	Precision
CG using IBW without gender adjustment		
Actual SCr	2.3 (-10.3-14.8)	22.5
Rounded SCr	28.8 (19.1-38.4)	17.4
CG using ActBW without gender adjustment		
Actual SCr	-13.6 [-26.8-(-0.43)]	23.6
Rounded SCr	16.3 (4.5-28.1)	21.2
CG using ActBW with gender adjustment		
Actual SCr	-5.2 (-17.2-7.1)	22.1
Rounded SCr	22.6 (11.5-33.7)	19.9

ActBW: Actual body weight; CG: Cockcroft-Gault; CrCl: Creatinine clearance; IBW: Ideal body weight; SCr: Serum creatinine.

when calculating CrCl using the CG. This study included 23 patients (age  $69.2 \pm 8.1$  years old) and compared the calculated CrCl using various body weights with or without rounding of SCr to 1 mg/dL with a 24-h measured CrCl. The results of this study showed that of all three examples of calculating CrCl, using the actual SCr values produced the most accurate estimate of CrCl (Table 4).

The inverse relationship between SCr and CrCl has lead clinicians to further deviate from the studied CG equation in order to broaden the applicability of the equation<sup>[22]</sup>. Rounding of low SCr values ( $\leq 0.6$  mg/dL) when calculating CrCl is often used by clinicians to prevent overestimation of renal function and over-dosing of renally excreted drugs<sup>[21]</sup>. The fact that "rounding up" of SCr has not been validated by strong evidence, clinicians who routinely round low SCr in patients may underestimate CrCl and consequently overcompensate for a perceived problem<sup>[21,22]</sup>. The studies presented in this section confirm the limitations of the CG equation in elderly patients and the use of SCr as a surrogate marker of GFR. Unfortunately, the limitations of SCr based equations extend to additional populations where SCr is falsely low due to underlying disease.

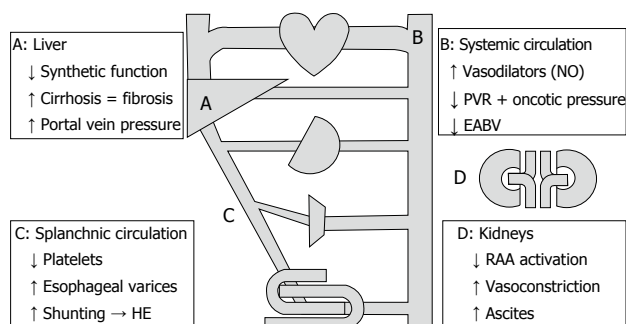
## CIRRHOTICS - THE EXCEPTION TO THE RULE

Among the many complications that arise in patients with liver cirrhosis, renal dysfunction has become a well-established predictor associated with poor prognosis and increased mortality<sup>[7,23]</sup>. The overall survival of patients with cirrhosis who develop hepatorenal syndrome (HRS) is approximately 50% at 1 mo and 20% at 6 mo<sup>[24]</sup>. Given the frequency at which cirrhotics demonstrate "cryptic" renal impairment and so often go on to develop HRS, it is critical that clinicians appropriately dose all drugs in cirrhotic patients, particularly those that are nephrotoxic<sup>[25]</sup>. Unfortunately, due to the underlying disease pathophysiology producing a falsely low SCr, SCr based calculations of CrCl are of limited use in cirrhotics<sup>[7]</sup>.

Underlying CKD in cirrhotics results from alterations in hemodynamics, renal autoregulatory mechanisms, and cardiac function (Figure 2)<sup>[26,27]</sup>. Hemodynamically, because of increased portal vein pressure, compensatory vasodilators such as nitrous oxide (NO) decrease peripheral vascular resistance and dilate the splanchnic circulation<sup>[26,27]</sup>. Progressive vasodilation in the presence of portal hypertension results in a decrease in effective arterial blood volume and activation of sodium retention mechanisms such as the renin-angiotensin-aldosterone system (RAAS)<sup>[26,27]</sup>. Unfortunately, these compensatory mechanisms lead to renal vasoconstriction and reduced GFR<sup>[26,27]</sup>.

In addition to the above hemodynamic changes, cirrhotics have falsely low-to-normal levels of SCr, thus further complicating a clinician's assessment of renal function. Creatine is originally produced in the liver before it is transferred to the skeletal muscles to be stored for energy. In the muscles, it is then phosphorylated, converted to creatinine, and then transferred back into the bloodstream<sup>[28]</sup>. As cirrhosis progresses, creatine production declines and becomes inconsistent<sup>[28]</sup>. Furthermore, due to malnutrition and low androgen levels, muscle wasting in cirrhotics limits the storage capacity and phosphorylation of creatine, thereby further decreasing the serum concentration of creatinine<sup>[28-30]</sup>. Finally,





**Figure 2 Systemic effects of cirrhosis.** Increased portal vein pressure results in vasodilation decreasing peripheral vascular resistance (PVR) and effective arterial blood volume (EABV). To compensate for this, increased renin-angiotensin-aldosterone (RAA) activation leads to sodium and fluid retention along with renal vasoconstriction and reduced glomerular filtration rate. Adopted with permission from Ho *et al*<sup>[27]</sup>. HE: Hepatic encephalopathy.

detecting early acute kidney injury (AKI) in cirrhotics using SCr is already tenuous, and may require far greater than 24 h given the pharmacokinetic properties of creatinine in patients with reduced GFRs<sup>[11]</sup>.

MacAulay *et al*<sup>[31]</sup> compared estimates of GFR using three SCr based formulas (CG, MDRD and SCr<sub>rec</sub>) with standard radionuclide measurements (DTPA) of GFR in patients with advanced liver disease. Of the 57 patients in their trial, the mean GFR *via* DTPA was 83 mL/min per 1.73 m<sup>2</sup> (range 28-173 mL/min per 1.73 m<sup>2</sup>). On average, estimation using the MDRD was most accurate (mean difference % = +4.0; CI = -5.73-20.39), followed by the CG equation (mean difference % = +18.56; CI = 8.48-22.83), and the SCr<sub>rec</sub> (mean difference % = +28.68; CI = 14.3-30.28). The authors concluded that using the CG and SCr<sub>rec</sub> (Table 1A equation VI) equations to estimate GFR in this population can lead to a significant overestimation of GFR.

A similar study by Rognant *et al*<sup>[32]</sup> compared GFR estimates using the CG and MDRD equations to a measured GFR using inulin. Estimating CrCl using the CG was normalized to 1.73 m<sup>2</sup> body surface area (Table 1A equation I). The 143 patients in this study all had decompensated alcoholic cirrhosis. The mean measured GFR using inulin was 76.9 ± 28 mL/min per 1.73 m<sup>2</sup>, and 30.4% of patients had a GFR ≥ 90 mL/min per 1.73 m<sup>2</sup> (group 1), 39.2% had a GFR between 60-89.9 mL/min per 1.73 m<sup>2</sup> (group 2), 26.3% had a GFR < 60 mL/min per 1.73 m<sup>2</sup> (group 3) with 4.1% of these patients having a GFR ≤ 30 mL/min per 1.73 m<sup>2</sup>. Mean GFR estimates using the CG and MDRD equations were 98.7 ± 32 mL/min and 99.4 ± 34 mL/min per 1.73 m<sup>2</sup>. The mean estimates using the CG and MDRD equations both overestimated the GFR mean by 21.8 mL/min (28.3%) and 22.5 mL/min (29.3%), respectively. For patients in group 1, the mean absolute bias for the CG was 20 ± 25 mL/min and 18 ± 23 mL/min per 1.73 m<sup>2</sup>. In group 2, the mean absolute bias for the CG was 25 ± 18 mL/min and 27 ± 19 mL/min per 1.73 m<sup>2</sup> using the MDRD. For those in group 3, the mean absolute bias using the CG was 21 ± 19 mL/min and 19 ± 25 mL/min per 1.73 m<sup>2</sup>.

The authors of the study concluded that although the differences between the CG and MDRD estimations were not statistically significant, their findings suggest both equations significantly overestimated renal function in cirrhotics, particularly in those with lower GFRs.

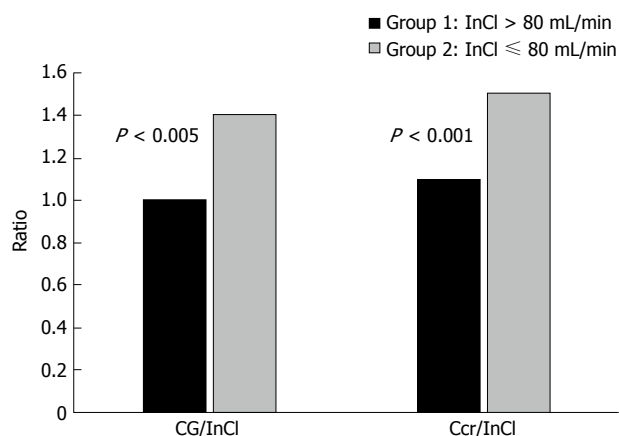
A third study assessing renal function in cirrhotics by Caregaro *et al*<sup>[33]</sup> was designed to evaluate the sensitivity of SCr and CrCl in detecting renal insufficiency and the magnitude of overestimation of GFR by CrCl. Estimation of CrCl was made using the CG equation and a 24-h urine collection. Estimates of CrCl were compared to measured GFR using inulin (Inulin Clearance = InCl). Patients in this study were divided into 2 groups based on measured GFR; group 1 (*n* = 29) had a GFR > 80 mL/min per 1.73 m<sup>2</sup> and group 2 (*n* = 27) had a GFR ≤ 80 mL/min per 1.73 m<sup>2</sup>. For the patients in groups 1 and 2, the mean measured GFR (InCl) was 113.5 ± 27.9 mL/min per 1.73 m<sup>2</sup> and 56.8 ± 19.8 mL/min per 1.73 m<sup>2</sup>, respectively. Estimating CrCl using the CG and 24-h urine collection provided an adequate assessment of measured GFR (InCl) in group 1 (CG = 106.3 ± 34.0 mL/min; 24-h = 121.5 ± 28.8 mL/min) but significantly overestimated measured GFR in group 2 (CG = 75.9 ± 40.1 mL/min; 24-h = 78.7 ± 39.2 mL/min) (Figure 3). Only 18.5% of patients in group 2 had a SCr level above normal limits and 81.5% of patients with a GFR ≤ 60 mL/min per 1.73 m<sup>2</sup> had normal SCr levels. Overall, the sensitivity of SCr, CrCl estimated using the CG equation and 24-h urine collection in detecting renal insufficiency was 18.5%, 51% and 74%, respectively.

The authors concluded renal failure in cirrhotic patients is greatly underestimated because of the low sensitivity and accuracy of SCr levels in this population (Figure 3). Based on the data presented in this study, utilization of SCr based methods overestimated true renal function by about 50% in cirrhotic patients with a GFR ≤ 80 mL/min per 1.73 m<sup>2</sup>.

## CONCLUSION

The importance of accurate and appropriate dosing of all medications remains a critical component of healthcare to maximize efficacy while limiting toxicity. For renally excreted medications, assessment and interpretation of renal function often dictates dosage selection. Due to the impractical nature of a 24-h urine collection, SCr has become a widely accepted surrogate marker of renal function used in several equations including the CG equation. With now over 40 years since its development, the CG equation remains one of the most widely used methods of assessing renal function. Unfortunately, because of its seemingly ubiquitous use and acceptance, many have forgotten its limitations and provider-specific variations used to compensate for these limitations.

Many of the limitations of the CG equation largely stem from the original study supporting its accuracy. The homogenous sample population limits the external validity and creates opportunity for the implementation of empiric correction factors that may or may not be



**Figure 3** In cirrhotics, as renal function declines, conventional methods of estimating renal function are no longer accurate. Both the Cockcroft-Gault (CG) equation and a 24-h urine creatinine clearance (Ccr) significantly overestimated true renal function as measured by inulin clearance (InCl) in cirrhotics with a baseline InCl ≤ 80 mL/min. The CG equation and Ccr were better estimates in those with a baseline InCl > 80 mL/min<sup>[33]</sup>.

supported by data<sup>[5]</sup>. As outlined in this review, selection of appropriate weight and rounding of low SCr levels are two examples of techniques used to broaden the applicability of the CG equation. These techniques vary among clinicians, largely because of a lack of evidence unanimously supporting one method over another. Unfortunately, additional limitations in the CG equation extend beyond body composition and habitus to include the subtle manifestations of the underlying disease process in a patient.

In cirrhotic patients, in addition to declining liver function, secondary physiological hemodynamic changes lead to a resultant reduction in GFR. Meanwhile, reductions in creatinine production and reduced muscle mass result in low SCr levels. Because of this, a sort of “cryptic renal failure” picture ensues, whereby SCr-based formulae will overestimate actual GFR by an average of about 50%<sup>[28-30]</sup>. Based on the evidence presented in this review and in the authors experience, multiplying the SCr by 1.5 in patients with decompensated cirrhosis provides a better CrCl estimate using the CG equation.

Utilization of the CG equation plays a significant role in the dosing decisions of many clinicians. In order to appropriately utilize this equation, clinicians have an inherent responsibility to understand its origins and limitations. True clinicians comprehensively assess each patient and consider SCr and CrCl as two variables that carry equal weight with several other parameters. Regardless of one’s approach to dosing medications, sole reliance on CrCl will undoubtedly lead to the ultimate realization that there is indeed fault in the CG.

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## Retrospective Cohort Study

# Advanced non-alcoholic steatohepatitis cirrhosis: A high-risk population for pre-liver transplant portal vein thrombosis

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**Author contributions:** Stine JG and Northup PG planned and conducted study, collected and/or interpreted data; Stine JG, Argo CK, Pelletier SJ, Maluf DG, Caldwell SH and Northup PG drafted the manuscript and approved final version.

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## Abstract

### AIM

To examine if liver transplant recipients with high-risk non-alcoholic steatohepatitis (NASH) are at increased risk for pre-transplant portal venous thrombosis.

### METHODS

Data on all liver transplants in the United States from February 2002 through September 2014 were analyzed. Recipients were sorted into three distinct groups: High-risk (age > 60, body mass index > 30 kg/m<sup>2</sup>, hypertension and diabetes), low-risk and non-NASH cirrhosis. Multivariable logistic regression models were constructed.

### RESULTS

Thirty-five thousand and seventy-two candidates underwent liver transplantation and of those organ recipients, 465 were transplanted for high-risk and 2775 for low-risk NASH. Two thousand six hundred and twenty-six (7.5%) recipients had pre-transplant portal vein thrombosis; 66 (14.2%) of the high-risk NASH group had portal vein thrombosis vs 328 (11.8%) of the low-risk NASH group. In general, all NASH recipients were less likely to be male or African American and more likely to be obese. In adjusted multivariable regression analyses, high-risk recipients had the greatest risk of



pre-transplant portal vein thrombosis with OR = 2.11 (95%CI: 1.60-2.76,  $P < 0.001$ ) when referenced to the non-NASH group.

## CONCLUSION

Liver transplant candidates with high-risk NASH are at the greatest risk for portal vein thrombosis development prior to transplantation. These candidates may benefit from interventions to decrease their likelihood of clot formation and resultant downstream hepatic decompensating events. Prospective study is needed.

**Key words:** Liver transplantation; Non-alcoholic fatty liver disease; Portal hypertension; Hepatology; Coagulopathy

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**Core tip:** Non-alcoholic steatohepatitis (NASH) is increasing in prevalence and is expected to be the leading indication for liver transplantation in the foreseeable future. There is a growing body of evidence supporting the clinical importance of a thrombophilic state in patients with NASH. In NASH patients, the most severe hypercoagulable environment is found in patients with NASH cirrhosis. High-risk NASH patients (concomitant age > 60 years, obesity, diabetes and hypertension) have inferior post transplantation outcomes, however, how this group's risk of clotting compares to other etiologies of liver disease is unknown. In a retrospective nationwide United States based cohort, we provide further evidence of coagulation derangement in NASH and identify a new high-risk subtype in the high-risk NASH population. Whether or not this high-risk group may benefit from preventative anticoagulation remains unknown.

Stine JG, Argo CK, Pelletier SJ, Maluf DG, Caldwell SH, Northup PG. Advanced non-alcoholic steatohepatitis cirrhosis: A high-risk population for pre-liver transplant portal vein thrombosis. *World J Hepatol* 2017; 9(3): 139-146 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i3/139.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i3.139>

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of hepatic disorders ranging from simple steatosis with or without mild inflammation to non-alcoholic steatohepatitis (NASH) which is diagnosed by the presence of inflammation, cellular injury with hepatocyte ballooning and accumulation of Mallory-Denk bodies and in the most advanced cases, fibrosis<sup>[1]</sup>. NAFLD is increasing in prevalence in Western society<sup>[2]</sup> with rates approaching 20%-30%<sup>[3]</sup> and more importantly, NASH is projected to become the number one indication for liver transplantation in the foreseeable future<sup>[4]</sup>, especially in light of the new all oral direct acting antiviral treatment regimens for

hepatitis C virus (HCV), and it is the most rapidly growing indication for simultaneous liver-kidney transplantation<sup>[5]</sup>. High-risk NASH (HR-NASH) is a subtype of NASH defined by the presence of the following: Age > 60 years, body mass index (BMI) > 30 kg/m<sup>2</sup>, hypertension and diabetes<sup>[6,7]</sup>. In general, liver transplant recipients with NASH have similar liver graft and overall one-, three- and five-year survival rates when compared to other etiologies<sup>[4-6,8,9]</sup>. Outcomes for HR-NASH recipients are less promising with single center experiences showing significantly lower one-<sup>[7]</sup> and five-year survival rates<sup>[6]</sup>. While the exact explanation for this remains relatively unexplored, post-transplant cardiovascular events<sup>[8]</sup>, some of which are attributable to macrovascular arterial thrombosis<sup>[7,9]</sup>, and chronic renal dysfunction<sup>[10]</sup> are more common in NASH patients and these could contribute to lesser outcomes.

Venous thromboembolism (VTE) including portal vein thrombosis (PVT) is a common affliction in patients with cirrhosis<sup>[11]</sup>. Incidence rates of PVT are reported to be as high as 16%<sup>[12]</sup> and 30-d mortality is increased in patients with pulmonary embolism (PE) or deep vein thrombosis (DVT)<sup>[11]</sup>. In a matched retrospective case-control study of 414 patients, Di Minno *et al.*<sup>[13]</sup> found that NAFLD was associated with VTE (PE or DVT) with an OR of 1.8 on adjusted multivariable analysis controlling for additional VTE and NAFLD risk factors. While the presence of PVT may mirror the degree of liver disease burden, it is nonetheless associated with adverse outcomes including increased pre- and post-liver transplant mortality and impaired quality of life, as well as technical challenges during the transplant procedure<sup>[14,15]</sup>. We have previously shown that liver transplant recipients with NASH are predisposed to pre-transplant PVT<sup>[16]</sup>. Patients with NASH and metabolic syndrome, which encompasses many of the features of the HR-NASH definition, are known to have increased degrees of fibrosis<sup>[17]</sup>, and presumably increased thrombotic risk. To date, there is a lack of data investigating the relationship between pre-transplant PVT and HR-NASH. We aim to explore this potential association and hypothesize that liver transplant recipients with HR-NASH are at increased risk for PVT when compared directly to other NASH patients and all other etiologies of liver disease.

## MATERIALS AND METHODS

### Study design and recipient characteristics

Data on all transplants in the United States during the model for end-stage liver disease (MELD era) through September 2014 were reviewed from the Organ Procurement and Transplantation Network (OPTN) with permission from the United Network for Organ Sharing (UNOS). Status 1a, multi-organ, living donor, re-transplants, pediatric recipients, donation after cardiac death, recipients with pre-transplantation transjugular intrahepatic portosystemic shunts and malignancy (hepatocellular carcinoma, hepatoblastoma, cholangiocarcinoma) were excluded. Recipients with cryptogenic

cirrhosis were also excluded due to the potential for misclassification of NASH. The conclusions of the model were not significantly changed with the exclusion of the cryptogenic recipients. Recipients were sorted into two distinct groups: Those with NASH and those without NASH (all other etiologies except cryptogenic cirrhosis, which was excluded due to the potential for misclassification of NASH). The NASH group was then subdivided into HR and low-risk (LR) subgroups. HR-NASH was based on the standard definition used in previous large-scale single center experiences and was defined as the presence of all of the following: Age > 60 years, BMI > 30 kg/m<sup>2</sup>, and pre-transplantation hypertension and diabetes<sup>[6,7]</sup>. Recipient characteristics (age at listing and at transplantation, ethnicity, gender, BMI, diabetes), severity of liver disease based on native laboratory MELD score at allocation, laboratory values [international normalized ratio (INR), bilirubin, creatinine, albumin], and clinically relevant manifestations of portal hypertension (ascites and hepatic encephalopathy) were reviewed in each of the three groups to compare baseline covariates.

### Outcomes analysis

Separate analyses were performed comparing recipients with NASH to non-NASH controls and comparing HR-NASH to LR-NASH. In the UNOS data set, PVT is categorized as "Present", "Not present", or "Unknown" and the data are based upon direct surgical evaluation of the veins at the time of hepatectomy. The degree of clot burden is not specified in the dataset nor is the chronicity. Based on previously validated methodology and due to the potential for misclassification bias<sup>[16]</sup>, 831 recipients with "Unknown" PVT status were excluded. In general, univariate comparisons of the excluded cases to the included cohort with known PVT status did not reveal any baseline differences with the exception that the included cohort had a greater percentage of patients with cholestatic liver disease (10.2% vs 5.5%,  $P < 0.001$ ). It was felt by the study team that this was not a significant clinical factor in the analysis. The dataset also does not contain information on treatment of PVT or testing for thrombophilia.

### Statistical analysis

Recipients were statistically evaluated in multiple factors including demographics, medical comorbidities, waiting list and transplantation characteristics. Univariate comparisons were performed using the Student-*t* test, Wilcoxon sign rank test,  $\chi^2$  test, or Fisher exact test as appropriate. Multivariable models were constructed using logistic regression and analysis of maximum likelihood estimates to test the primary hypothesis that patients with HR-NASH are at increased risk for the development of PVT and to assess statistical associations and risk factors for the development of PVT. Individual covariates were included in the multivariable model if they were statistically significant to  $P < 0.20$  in univariate analysis, have been shown in the literature to be important or

were deemed to be clinically important by the study team<sup>[18,19]</sup>. In separate models, individual components of the HR-NASH definition (hypertension, age, BMI and diabetes) were entered into the model as individual variables to ensure one of these did not dominate. Final variables included in the regression model included HR-NASH, LR-NASH, individual laboratory values at transplant (creatinine, bilirubin, INR, albumin, sodium), HCV, cholestatic liver disease, male gender, African American race, Hispanic race, encephalopathy (which was dichotomized into those with severe encephalopathy with score > 2), ascites (similarly dichotomized), pre-transplant dialysis treatment and autoimmune liver disease. No data imputation was performed. All statistical tests for significance were two sided and a significance level  $P$  less than or equal to 0.05 was considered statistically significant. All data set manipulation and statistical analyses were performed using SAS (version 9.4, Cary, NC). No transplants involving prisoners were included in this analysis. Institutional review board approval was not required for this study as the UNOS/OPTN dataset is de-identified.

## RESULTS

Thirty-five thousand and seventy-two candidates underwent liver transplantation and of those organ recipients, 3240 (9.2%) were transplanted for NASH of which 465 met criteria for HR-NASH (1.3%) and 2775 for LR-NASH (7.9%). Two thousand six hundred and twenty-six (7.5%) recipients had pre-transplant PVT, of which 394 (12.2%) were in the NASH group (Figure 1). The prevalence of PVT was not significantly different between HR-NASH and LR-NASH ( $n = 66$ , 14.2% vs  $n = 328$ , 11.8%,  $P = 0.145$ ). In general, NASH recipients were older, more likely to be female, less likely to be African American or Hispanic, had higher BMI values and were more likely to have diabetes, hypertension and renal dysfunction (Table 1). Severity of liver disease, while statistically significantly different, was not deemed to be clinically significantly different (e.g., MELD at listing of 20.0, 95%CI: 19.8-20.3 for NASH vs 19.6, 95%CI: 19.5-19.7 for non-NASH). The leading indication for transplantation in the non-NASH group was chronic HCV (46.6%) while alcoholic liver disease was the second leading indication (19.0%).

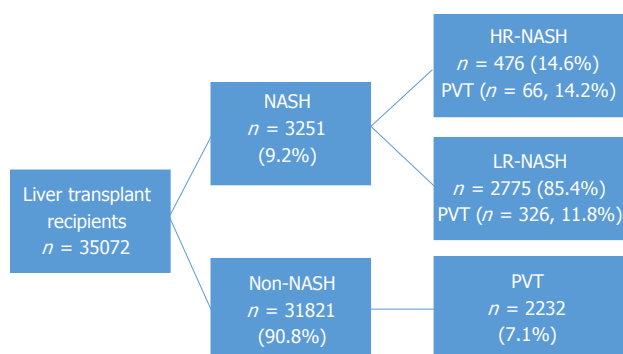
When comparing HR-NASH recipients to LR-NASH recipients, several differences were noted (Table 2). As expected by definition, HR-NASH recipients were older both at listing (64.0 years, 95%CI: 63.8-64.3 vs 56.7 years, 95%CI: 56.2-56.9,  $P < 0.001$ ) and at transplantation (64.5 years, 95%CI: 64.2-64.7 vs 57.0 years, 95%CI: 56.8-57.4,  $P < 0.001$ ). BMI values were greater for HR-NASH (35.1 kg/m<sup>2</sup>, 95%CI: 34.7-35.5 vs 31.8 kg/m<sup>2</sup>, 95%CI: 31.5-32.0,  $P < 0.001$ ) as was renal dysfunction (mean creatinine 1.98 g/dL, 95%CI: 1.85-2.11 vs 1.78 g/dL, 95%CI: 1.73-1.85,  $P = 0.003$ ).

Severity of liver disease based on MELD scores and portal hypertensive manifestations of ascites and

**Table 1** Baseline characteristics comparing non-alcoholic steatohepatitis recipients to all other etiologies of liver disease *n* (%)

	NASH ( <i>n</i> = 3240)	Other etiologies ( <i>n</i> = 31832)	<i>P</i> value
Recipient characteristics			
Age at listing, mean years (95%CI)	57.6 (57.3-57.9)	52.2 (52.1-52.3)	< 0.001
Age at transplant, mean years (95%CI)	58.1 (57.8-58.4)	52.7 (52.6-52.8)	< 0.001
Male gender	1747 (52.9)	22099 (67.3)	< 0.001
African American race	65 (2.0)	3,559 (10.9)	< 0.001
Hispanic race	348 (10.5)	4009 (12.2)	0.005
BMI at transplant, kg/m <sup>2</sup> , mean (95%CI)	32.3 (32.0-32.5)	27.8 (27.7-27.9)	< 0.001
Hypertension requiring medical treatment	160 (33.2)	2155 (19.2)	< 0.001
Diabetes	1698 (51.4)	6211 (18.9)	< 0.001
Portal vein thrombosis	394 (12.2)	2232 (7.1)	< 0.001
Etiology of liver disease			
Alcohol alone		6236 (19.0)	
Autoimmune disease		1,202 (3.7)	
Cholestatic disease		3638 (11.1)	
Hepatitis B		986 (3.0)	
Hepatitis C		15298 (46.6)	
Other		4472 (14.0)	
Severity of liver disease			
MELD score at listing, mean (95%CI)	20.0 (19.8-20.3)	19.6 (19.5-19.7)	0.014
MELD score at transplantation, mean (95%CI)	23.5 (23.2-23.8)	22.8 (22.7-22.9)	< 0.001
Laboratory values			
Serum bilirubin, mg/dL, mean (95%CI)	7.4 (7.1-7.8)	9.1 (9.0-9.2)	< 0.001
INR, mean (95%CI)	1.92 (1.90-1.95)	1.93 (1.92-1.94)	NS
Serum albumin, g/dL, mean (95%CI)	3.0 (3.0-3.1)	3.0 (2.9-3.0)	0.002
Creatinine, g/dL, mean (95%CI)	1.81 (1.76-1.86)	1.65 (1.63-1.67)	< 0.001
On dialysis at transplantation	488 (10.6)	4135 (12.2)	< 0.001
Portal hypertension manifestations			
Moderate-severe ascites at transplant	1210 (36.7)	10782 (32.9)	< 0.001
Moderate-severe hepatic encephalopathy at transplant	375 (11.7)	3708 (11.3)	NS

In general, NASH recipients were older, more likely to be female, less likely to be African American or Hispanic, had higher BMI values and were more likely to have diabetes, hypertension and renal dysfunction. BMI: Body mass index; NASH: Non-alcoholic steatohepatitis; NS: Not significant; INR: International normalized ratio; MELD: Model for end-stage liver disease.

**Figure 1** Study enrollment. HR: High-risk; LR: Low-risk; NASH: Non-alcoholic steatohepatitis; PVT: Portal vein thrombosis.

encephalopathy were similar between the two groups. Interestingly, non-NASH recipients with HR features were not at increased odds of PVT ( $P = 0.11$ ), albeit only 58 patients met criteria for this subgroup.

In adjusted multivariable analysis (Table 3), recipients with HR-NASH had the greatest risk of pre-transplant PVT with OR = 2.11 (95%CI: 1.60-2.76,  $P < 0.001$ ) when referenced to the non-NASH cohort and 30% increased odds when compared to LR-NASH recipients (OR = 1.71, 95%CI: 1.49-1.96,  $P < 0.001$ ). Other significant associations with pre-transplant PVT included male gender (OR = 1.18, 95%CI: 1.07-1.29,  $P$

< 0.001), Hispanic race (OR = 1.24, 95%CI: 1.10-1.39,  $P < 0.001$ ) moderate-to-severe ascites (OR = 1.14, 95%CI: 1.04-1.25,  $P = 0.007$ ) and autoimmune liver disease (OR = 1.43, 95%CI: 1.14-1.79,  $P = 0.002$ ). African Americans were less likely to have pre-transplant PVT with OR = 0.75 (95%CI: 0.64-0.89,  $P < 0.001$ ), similar to our previous findings<sup>[16]</sup>.

While MELD was significantly different on univariate analysis, the individual factors (bilirubin, INR, creatinine) involved in the MELD regression equation were not clinically important statistically significant predictors in adjusted multivariable analysis nor was pre-transplantation dialysis.

## DISCUSSION

Based on a large national liver transplant database and building on our previous work in transplant recipients with NASH<sup>[16]</sup>, we have shown an independent cross-sectional association documenting an increased risk of pre-transplant PVT in recipients undergoing transplantation for HR-NASH. This association was significant despite adjustment for multiple established risk factors for pre-transplant PVT. Given the technical difficulties associated with pre-transplant PVT, careful recipient selection is paramount in preventing post-transplant vascular complications.

**Table 2** Baseline characteristics comparing high-risk non-alcoholic steatohepatitis to low-risk non-alcoholic steatohepatitis recipients

	High-risk NASH (n = 465)	Low-risk NASH (n = 2775)	P value
Recipient characteristics			
Age at listing, mean years (95%CI)	64.0 (63.8-64.3)	56.7 (56.2-56.9)	< 0.001
Age at transplant, mean years (95%CI)	64.5 (64.2-64.7)	57.0 (56.8-57.4)	< 0.001
Male gender	247 (52.4)	1500 (53.0)	NS
African American race	7 (0.2)	58 (1.6)	NS
Hispanic race	43 (9.1)	305 (10.8)	NS
BMI at transplant, kg/m <sup>2</sup> , mean (95%CI)	35.1 (34.7-35.5)	31.8 (31.5-32.0)	< 0.001
Portal vein thrombosis	66 (14.2)	326 (11.8)	NS
Severity of liver disease			
MELD score at listing, mean (95%CI)	19.5 (18.7-20.3)	20.1 (19.8-20.4)	NS
MELD score at transplantation, mean (95%CI)	22.8 (21.9-23.6)	23.7 (23.3-24.0)	NS
Laboratory values			
Serum bilirubin, mg/dL, mean (95%CI)	6.2 (5.4-7.0)	7.7 (7.3-8.0)	0.002
INR, mean (95%CI)	1.81 (1.75-1.86)	1.94 (1.91-1.97)	0.002
Serum albumin, g/dL, mean (95%CI)	3.1 (3.0-3.2)	3.0 (2.9-3.1)	0.006
Creatinine, g/dL, mean (95%CI)	1.98 (1.85-2.11)	1.78 (1.73-1.83)	0.003
On dialysis at transplantation	73 (15.5)	415 (14.7)	NS
Portal hypertension manifestations			
Moderate-severe ascites at transplant	178 (37.8)	1032 (36.5)	NS
Moderate-severe hepatic encephalopathy at transplant	51 (10.8)	324 (11.5)	NS

HR-NASH recipients were older, had higher BMI values and were more likely to have renal dysfunction. BMI: Body mass index; HR: High-risk; NASH: Non-alcoholic steatohepatitis; NS: Not significant; INR: International normalized ratio.

**Table 3** Adjusted multivariable analysis for predictors of portal vein thrombosis at the time of liver transplantation

	Odds ratio	95%CI	P value
African American race	0.75	0.64-0.89	< 0.001
AIH	1.43	1.14-1.79	0.002
Hispanic race	1.24	1.10-1.39	< 0.001
HR-NASH	2.11	1.60-2.76	< 0.001
LR-NASH	1.71	1.49-1.96	< 0.001
Male gender	1.18	1.07-1.29	< 0.001
Moderate-severe ascites	1.14	1.04-1.25	0.007

Recipients with HR-NASH had the greatest risk of pre-transplant PVT when referenced both to the non-NASH cohort and to LR-NASH recipients. Variables that were not significant in the model: Albumin levels, bilirubin levels, cholestatic liver disease, creatinine levels, hepatitis C, international normalized ratio (INR); moderate-severe encephalopathy, pre-transplant dialysis treatment, sodium levels. AIH: Autoimmune hepatitis; HR: High-risk; LR: Low-risk; NASH: Non-alcoholic steatohepatitis.

Patients with NASH and in particular NASH cirrhosis, have *in vivo* abnormalities in primary, secondary and tertiary hemostasis<sup>[20,21]</sup>. The role of platelet dysfunction *via* increased activation, adherence and aggregation is the best described abnormality in primary hemostasis<sup>[22-24]</sup>, however multiple investigators have shown elevations in vonWillebrand factor as a surrogate for endothelial dysfunction as well<sup>[21,25]</sup>. Secondary hemostasis is impaired in NASH due to elevations in fibrinogen, factor VIII, IX, XI, XII, increased clotting activity of factor VII, and low levels of antithrombin III<sup>[20,21,25-27]</sup>. Protein C levels may be increased or decreased in patients with NASH<sup>[20]</sup>. Tertiary hemostasis is disrupted due to elevations in plasminogen activator inhibitor-1 and low levels in both thrombin activatable fibrinolysis inhibitor and tissue plasminogen activator<sup>[20,21,27]</sup>. The aggregate of these impaired mechanisms of coagulation leads to the hypercoagulable milieu

responsible in part for the development of PVT.

Whether or not the presence of pre-transplant PVT plays a role in the decreased survival of HR-NASH<sup>[6,7]</sup> remains unknown as our study did not investigate patient centered outcomes such as graft and overall patient survival. We did not attempt to do this due to the limitations of the UNOS/OPTN dataset, which does not contain information on the use of anticoagulants and data on post-transplant vascular complications is hindered by a large degree of missing data. The lack of this information introduces significant heterogeneity into the dataset and concrete post-transplant outcomes based conclusions for patients with PVT are problematic. However, what is clear is that the high-risk subgroup of NASH is the most at risk for PVT both in comparison to other NASH recipients not meeting the HR definition and also to all other etiologies of liver disease. We have previously shown that the independent factors of diabetes and obesity do not predispose to PVT on an individual basis<sup>[16]</sup>. It is only in combination with advanced age > 60 years and hypertension that these factors interact in a way to produce clinically meaningful thrombotic disease, perhaps due to increased physiologic endothelial dysfunction with advancing age<sup>[28]</sup>.

In general, treatment outcomes for patients with PVT are impaired by a lack of large-scale, randomized, placebo-controlled trials that are generalizable. Villa *et al.*<sup>[29]</sup> demonstrated in an un-blinded, single center randomized controlled trial that daily prophylactic dosing of low molecular weight heparin (40 mg daily) for twelve months prevented the development of PVT in patients with compensated cirrhosis at 48 wk, an effect that persisted through the 5-year follow-up period when compared to standard of care<sup>[29]</sup>. This study also demonstrated significantly less hepatic decompensation in the low



molecular weight heparin arm and a survival benefit in the absence of a single bleeding event<sup>[29]</sup>. Building on this, Cui *et al*<sup>[30]</sup> recently published a controlled trial evaluating the efficacy and safety of anticoagulation therapy with different doses of enoxaparin (1 mg/kg twice a day vs 1.5 mg/kg daily) for PVT in patients with cirrhosis secondary to chronic hepatitis B in 65 patients, the majority of which had partial thrombosis. Importantly, 79% of patients achieved partial or complete response with anticoagulation based on follow-up imaging, however, non-variceal bleeding was significantly greater in the daily group (23.5% vs 6.4%) and the authors concluded that dosing at 1 mg/kg of enoxaparin subcutaneously twice a day was the preferred anticoagulation regimen. While the inclusion criteria were stringent limiting generalizability and the imaging guided definition of PVT open for criticism in these studies, the findings are nonetheless intriguing. A recent meta-analysis by Qi *et al*<sup>[31]</sup> of 16 studies (the authors did not include either of the aforementioned studies) and 960 patients found a pooled OR of 4.16 (95%CI: 1.88-9.20,  $P < 0.001$ ) for complete portal vein recanalization with anticoagulation. Interestingly, the pooled rate of bleeding was only 3.3% (95%CI: 1.1%-6.7%). A recently published animal model found that 3 mo of dabigatran significantly reduced fibrin deposition, inflammation, hepatocellular injury, steatosis and weight gain<sup>[32]</sup> through mitigation of thrombin generation, the end-result of the coagulation abnormalities in NASH, suggesting a potential novel therapeutic approach. Newer data regarding the potential use of prothrombin complex concentrates in combination with antithrombin with or without concurrent fibrinogen administration to restore the delicate homeostasis of coagulation and normal thrombin and fibrinogen is emerging<sup>[33,34]</sup>. However, this combination of therapy has not been broadly studied in patients with chronic liver disease and concrete recommendations about the utility of this treatment cannot be made at this juncture.

In general, prospective, randomized, placebo-controlled studies are sorely needed in all patients with cirrhosis, however, targeting those most at risk including patients with HR-NASH, may provide the most substantial benefits including the potential to reduce disease burden from cerebrovascular accidents that this population is at risk for. If the reduction in inflammation and fibrosis with the direct thrombin inhibitors is validated in human subjects, these agents may provide an antifibrotic therapy which could alter the prognosis of liver disease.

Our study has several limitations. Despite containing a large number of transplant recipients in the MELD era, it is a retrospective study. Furthermore, missing data and correct diagnostic coding are potentially problematic with all large datasets. We attempted to control for missing data by excluding the small percentage of patients with unknown PVT status who were demographically similar to our included cohort to ensure bias towards or away from the null was not introduced. Although transplant

centers are gaining increased experience transplanting recipients with PVT, it is possible that small volume centers may not have the same surgical technique or experience and pre-transplant PVT may in fact preclude transplantation in a subset of patients that would go on to be excluded from this study. The PVT variable in the dataset also has inherent heterogeneity as there is no differentiating between partial and complete thrombus or the chronicity of the clot. Our analysis also could not account for thrombophilia disorders and therapy in the pre-transplant phase. Additionally, our study excluded HCC patients in the event that PVT was associated with HCC as tumor thrombus, which may limit generalizability.

In conclusion, as the *in vivo* evidence of a thrombophilic state in patients with NASH continues to grow, epidemiologic evidence continues to lag behind. Building on our previous work, we have shown that liver transplant candidates with HR-NASH are at the highest risk for PVT development when compared to other NASH patients and also to all other etiologies of liver disease. Prospective study enrolling HR-NASH patients in anticoagulation trials seems warranted in order to determine a direct benefit in improving patient centered outcomes including the potential for overall and post-transplantation graft survival.

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## COMMENTS

### Background

Non-alcoholic steatohepatitis (NASH) is increasing in prevalence and will soon be the leading indication for liver transplantation in western nations. Patients with NASH are at increased risk for thrombosis. Patients with high-risk NASH have inferior liver transplantation outcomes. Whether or not this high-risk group has an increased risk of portal vein thrombosis (PVT) remains unknown.

### Research frontiers

The field of coagulation disorders in chronic liver disease continues to grow. Much of the research focuses on PVT and/or venothromboembolic disease. Identifying high-risk groups for possible preventative intervention through clinical trials remains a goal of the liver and hematology fields alike.

### Innovations and breakthroughs

In the present study, the authors investigated the association between high-risk NASH and PVT in liver transplant recipients with cirrhosis. This is the first report of PVT risk in patients with high-risk NASH.

### Applications

The present report furthers understanding regarding the thrombophilic state of NASH and highlights a potential high-risk group who may benefit from further prospective study.

### Peer-review

This is an excellent very large retrospective review that clearly shows that high risk NASH patients are more thrombophilic than low risk NASH patients and much more thrombophilic than non-NASH cirrhotic patients.

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Retrospective Study

# Biliary complications following liver transplantation: Single-center experience over three decades and recent risk factors

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## Abstract

### AIM

To identify independent risk factors for biliary complications in a center with three decades of experience in liver transplantation.

### METHODS

A total of 1607 consecutive liver transplantations were analyzed in a retrospective study. Detailed subset analysis was performed in 417 patients, which have been transplanted since the introduction of Model of End-Stage Liver Disease (MELD)-based liver allocation. Risk factors for the onset of anastomotic biliary complications were identified with multivariable binary logistic



regression analyses. The identified risk factors in regression analyses were compiled into a prognostic model. The applicability was evaluated with receiver operating characteristic curve analyses. Furthermore, Kaplan-Meier analyses with the log rank test were applied where appropriate.

## RESULTS

Biliary complications were observed in 227 cases (14.1%). Four hundred and seventeen (26%) transplantations were performed after the introduction of MELD-based donor organ allocation. Since then, 21% ( $n = 89$ ) of the patients suffered from biliary complications, which are further categorized into anastomotic bile leaks [46% ( $n = 41$ )], anastomotic strictures [25% ( $n = 22$ )], cholangitis [8% ( $n = 7$ )] and non-anastomotic strictures [3% ( $n = 3$ )]. The remaining 18% ( $n = 16$ ) were not further classified. After adjustment for all univariably significant variables, the recipient MELD-score at transplantation ( $P = 0.006$ ; OR = 1.035; 95%CI: 1.010-1.060), the development of hepatic artery thrombosis post-operatively ( $P = 0.019$ ; OR = 3.543; 95%CI: 1.233-10.178), as well as the donor creatinine prior to explantation ( $P = 0.010$ ; OR = 1.003; 95%CI: 1.001-1.006) were revealed as independent risk factors for biliary complications. The compilation of these identified risk factors into a prognostic model was shown to have good prognostic abilities in the investigated cohort with an area under the receiver operating curve of 0.702.

## CONCLUSION

The parallel occurrence of high recipient MELD and impaired donor kidney function should be avoided. Risk is especially increased when post-transplant hepatic artery thrombosis occurs.

**Key words:** Biliary complications; Liver transplantation; Prognostic model; Risk factors; Multivariable analyses

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**Core tip:** This retrospective study investigates the occurrence of biliary complications in a total of 1607 consecutive liver transplant patients throughout three decades. Since introduction of Model of End-Stage Liver Disease (MELD)-based liver allocation, the recipient's MELD-score at transplantation, the development of hepatic artery thrombosis post-operatively, as well as the donor creatinine prior to explantation were identified as independent risk factors, thus a combination of high recipient MELD-score and impaired donor kidney function should be avoided. Risk is especially increased when post-transplant hepatic artery thrombosis occurs. A prognostic model for the prediction of anastomotic biliary complications was developed and successfully internally validated.

Kaltenborn A, Gutcke A, Gwiasda J, Klempnauer J, Schrem H. Biliary complications following liver transplantation: Single-

center experience over three decades and recent risk factors. *World J Hepatol* 2017; 9(3): 147-154 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i3/147.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i3.147>

## INTRODUCTION

Since its introduction as standard procedure in 1983, liver transplantation is nowadays widely accepted as the only live-saving treatment for end stage liver diseases. Nevertheless, several serious complications still endanger successful short- and long-term outcome. Biliary complications appear to be one of the most common issues during follow-up<sup>[1]</sup>. Diverse studies reveal their notable association with mortality and an overall incidence of 10%-40% is described<sup>[2]</sup>. Moreover, the socio-economic implications due to prolonged morbidity are of increasing relevance and represent a serious burden to health care systems<sup>[3]</sup>.

As the bile duct is supplied only arterially without the benefit of portal vein nourishment, there is a pre-determined breaking point to cause repercussions<sup>[4]</sup>. Furthermore, donor parameters, surgical aspects as well as the recipients' condition prior to transplant seem to affect the outcome<sup>[2]</sup>. In general, anastomotic lesions are distinguished from non-anastomotic stenosis or leakage<sup>[5]</sup>. The most common manifestations of biliary complications are strictures of the bile duct<sup>[5]</sup>. Anastomotic lesions are usually due to mechanical and surgical issues which alter the bile duct's arterial support and occur mainly within the first 90 d after transplantation, whereas non-anastomotic lesions show a predominantly manifestation period of about six to nine month after transplant<sup>[5]</sup>. Under the term of non-anastomotic lesions systemic complications such as post-thrombotic, inflammatory and immunological processes as well as the presence of cytotoxic hydrophobic bile salts are summarized, which all lead to damage of the biliary epithelium<sup>[6]</sup>.

In recent studies, inadequate surgical technique, arterial complications such as hepatic artery thrombosis, as well as donor age and macrovesicular graft steatosis could be identified as relevant risk factors for the occurrence of biliary complications in risk-adjusted multivariate analyses<sup>[5,7,8]</sup>.

This study has two aims. Firstly, a historical overview over three decades of biliary complications after liver transplantation should show the development of their incidence. Secondly, main focus was to identify independent risk factors in the most recent years, which contribute to the development of early biliary complications, occurring during the direct hospital stay after liver transplantation.

## MATERIALS AND METHODS

In this single-center, retrospective, observational study, the influence of pre-, inter- and post-transplant aspects as relevant risk factors for the occurrence of early biliary

complications, which occur during the direct hospital stay after liver transplantation, were investigated.

### **Follow-up period and exclusion criteria**

During follow-up patients were routinely seen in the transplant outpatient clinic at least once per year and follow-up visits consisted of physical examination, blood chemistry, as well as standardized abdominal ultrasound. Mean follow-up was 9.4 years (SD: 7.5 years). Included were all consecutive adult liver transplantations (pediatric was defined as younger than 18 years of age). Excluded from analysis were combined transplantations, split liver transplantations, and patients with re-transplantation during initial hospital stay. Since donation after cardiac death is not allowed in Germany by current law, there are no such transplantations included in this study.

### **Definition of eras in 30 years of liver transplantation**

In this study, the three decades of liver transplantation were divided into four eras. Era 1 reaches from 01.01.1983-31.12.1991, Era 2 from 01.01.1992-31.12.1999. Era 3 (Child-Pugh) includes the years 2000-2006 until the Model of End-Stage Liver Disease (MELD) allocation (Era 4) started in 2006.

### **Study endpoints**

Onset of biliary complications during the post-transplant hospital stay is defined as primary study endpoint. In the MELD-era, a more detailed analysis was performed. The further respective study endpoints are complications occurring at the bile duct anastomosis, defined as anastomotic biliary leak or stricture.

### **Regular operative procedure and clinical diagnosis scheme of biliary complications**

For the detection of early biliary complications after liver transplantation, daily ultrasound/Doppler investigations, daily laboratory works, and daily clinical rounds were applied. It is standard operating procedure to implant abdominal drainages during the transplant procedure, which are regularly pulled after the secretion is less than 100 mL post-transplant. The biliary anastomosis was usually performed as end- to end-anastomosis between donor common bile duct and recipient common hepatic duct using a 6/0 prolene suture in continuous manner. University of Wisconsin preservation solution was routinely used until recently, the application of HTK solution increased since its introduction in the early 1990s and is nowadays the mostly applied preservation solution. A more detailed analysis of preservation solutions and their application at the study center is given elsewhere<sup>[10]</sup>. Due to certain indications for liver transplantation, such as primary biliary diseases, a hepaticojejunostomy in Roux-Y-technique is implemented<sup>[11]</sup>. The implantation of T-tube was omitted as standard procedure at our center around 2004 and has not been applied since.

### **Treatment schemes for biliary complications**

There are various therapy options for biliary com-

plications. Early biliary complications, such as biliary leaks can be managed *via* endoscopic retrograde cholangiopancreatography with stent implantation, whereas late complications, such as biliary stenosis or a diffuse leakage often require a percutaneous transhepatic biliary drainage, surgical revision with a Y-Roux hepaticojejunostomy or at last resort a re-transplantation.

### **Ethics statement**

The study was reviewed and approved by the institutional review board of Hannover Medical School (application number 1683-2013).

### **Statistical analysis**

Risk factors for the onset of study endpoints after liver transplantation were identified with univariable and multivariable binary logistic regression analyses. The alpha-level for inclusion into multivariate modeling was set at 0.05. All variables which were significant in univariable binary logistic regression analysis were considered for the multivariable binary regression model. Variables which were included in the multivariable regression model were compiled as the prognostic score for the prediction of anastomotic biliary complications. The clinical usefulness of this score was assessed with receiver operating characteristic curve analysis. Areas under the receiver operating curve (AUROCs) larger than 0.700 indicate a clinically useful prognostic model<sup>[9]</sup>. For internal validation of the developed score, randomized backwards bootstrapping was applied. Kaplan-Meier analysis with the Log-Rank test was applied where appropriate. For all statistical tests a *P*-value < 0.05 was defined as significant. The SPSS statistics software version 21.0 (IBM, Somers, NY, United States) was used to perform statistical analysis.

## **RESULTS**

Descriptive statistics of the investigated study population of 1607 consecutive liver transplants is summarized in Tables 1-3. During 30 years of follow-up, 561 (35.1%) patients deceased. The documented causes of death are summarized in Table 4.

### **Biliary complications during 30 years of liver transplantation**

During 30 years of liver transplantation at a single center, biliary complications were observed in 227 cases (14.1%). The development of biliary complication incidence since 1983 is shown in Figure 1. Patient as well as graft survival were significantly associated to the occurrence of biliary complications during 30 years of follow-up, as shown in Kaplan Meier analysis (Figure 2).

### **Detailed analysis of biliary complications in the MELD era**

Of the 1607 included transplantations, 417 (26%) were performed after introduction of MELD-based donor

**Table 1 Clinical characteristics of recipient data**

Variable	Mean (SD)	Median (range)	n (% of cohort)
MELD	21 (11.5)	18 (6-40)	
BMI	24.2 (4.5)	24.7 (15.1-40)	
Days on the waiting list	277 (41.5)	143 (0-4299)	
Male: Female		915 (57%) to 692 (43%)	
Age	46.1 (12)	47.5 (18-73.6)	
ICU stay in days	23 (33)	9 (1-276)	
Pre-transplant PVT			143 (9)
Creatinine (μmol/L)	121 (92)	86 (38-707)	
Bilirubine (μmol/L)	177 (209)	72 (7-930)	
Indication HCC			244 (15)
Indication PSC			153 (9.5)
Indication ALF			137 (8.5)
Indication HCV cirrh.			101 (6.3)
Indication alc. cirrh.			104 (6.4)
Indication biliary dis.			326 (20.3)

MELD: Model of End-Stage Liver Disease; BMI: Body mass index; ICU: Intensive care unit; PVT: Portal vein thromboses; HCC: Hepatocellular carcinoma; PSC: Primary sclerosing cholangitis; ALF: Acute liver failure; HCV: Hepatitis C virus.

**Table 2 Clinical characteristics of transplant specific data**

Variable	Mean (SD)	Median (range)	n (% of cohort)
Gender mismatch			929 (58)
Cold ischemic time (min)	6.9 (220)	593 (152-1696)	
Era 1 of transplantation			303 (19)
Era 2 of transplantation			434 (27)
Era 3 of transplantation			453 (28)
Era 4 of transplantation			417 (26)
> 1 arterial anastom.			71 (4.5)
Aortal anastomosis			164 (10.8)
Portal vein interpos. Graft			14 (0.8)
Hepaticojunostomy			353 (22.4)
Post-transplant HAT			59 (3.7)

HAT: Hepatic artery thrombosis.

organ allocation in December 2006. During this MELD-era, 21% of patients ( $n = 89$ ) suffered from early biliary complications during the initial post-transplant hospital stay. The distribution of complication type in the MELD-era is shown in Figure 3. In 46% ( $n = 41$ ) of the patients an anastomotic bile leak occurred, whereas 25% ( $n = 22$ ) showed an anastomotic stricture. Cholangitis occurred in 8% ( $n = 7$ ), non-anastomotic strictures in 3% ( $n = 3$ ) of the cases. The remaining 18% ( $n = 16$ ) were not further classified.

Since the biliary anastomosis can be influenced the most by the operating surgeon, risk factors were evaluated for anastomotic biliary complications, which included biliary strictures and anastomotic leakage. In 63 patients (15.1%) anastomotic complications were observed. Table 5 shows the results of univariable and multivariable binary regression analysis for identification of significant, independent risk factors for the development of anastomotic biliary complications during the initial post-transplant hospital stay. After adjustment for all univariable significant variables, the recipient MELD-score at transplantation ( $P = 0.006$ ;

**Table 3 Clinical characteristics of donor data**

Variable	Mean (SD)	Median (range)	n (% of cohort)
Age	42 (16.9)	43 (15-88)	
Male: Female		969 (60%) to 624 (40%)	
BMI	24.8 (16.6)	24 (20-44)	
Pre-transplant ICU stay	8.7 (19.7)	6 (1-383)	
Body temperature °C	36.4 (1.0)	36 (33-39)	
Bilirubine (μmol/L)	12.5 (11.7)	9.9 (0.9-154)	
Creatinine (μmol/L)	96 (79)	80 (12-885)	
CRP (mg/L)	127 (108)	114 (0.1-818)	
ALT (u/L)	48 (190)	22 (0-1136)	
AST (u/L)	59 (2.67)	29 (0-1074)	
GGT (u/L)	51.2 (78.6)	24 (0-912)	
Urea (mg/dL)	7.2 (8.4)	5.2 (0.2-103)	
CMV positivity			805 (50)

BMI: Body mass index; ICU: Intensive care unit; ALT: Alanine aminotransferase; AST: Aspartate transaminase; GGT: Gamma glutamyltransferase; CMV: Cytomegalovirus; CRP: C-reactive protein.

**Table 4 Causes of death in transplanted patients over 30 years**

Cause of death	No. of patients (% of cohort)
Sepsis	109 (6.8)
Tumor recurrence	103 (6.4)
<i>De novo</i> malignancy	33 (2.1)
Pneumonia	31 (1.9)
Liver graft: Biliary complications	20 (1.3)
Cardiovascular event	18 (1.1)
Liver graft: Chronic rejection	15 (0.9)
Cerebral ischemia	12 (0.8)
Cerebral bleeding	11 (0.7)
Liver graft: HCV reinfection	11 (0.7)
Cerebral edema	9 (0.6%)
Liver graft: HBV reinfection	9 (0.6)
Gastrointestinal bleeding	7 (0.4)
Gastrointestinal perforation	6 (0.4)
Liver graft: Venous thrombosis	6 (0.4)
Lung: Acute respiratory distress syndrome	4 (0.3)
Polytrauma	4 (0.3)
Cerebral infection	3 (0.3)
Liver graft: HCV <i>de novo</i> infection	3 (0.3)
Liver graft: Initial non function	3 (0.3)
Pulmonary embolism	6 (0.4)
Gastrointestinal ischemia	2 (0.1)
Liver graft: Arterial thrombosis	2 (0.1)
Suicide	2 (0.1)
Liver graft: Portal vein thrombosis	1 (0.1)
Non-compliance to immunosuppression	1 (0.1)
Recurrent alcoholism	1 (0.1)
Unknown	129 (8.1)
Total	561 (35.1)

HCV: Hepatitis C virus; HBV: Hepatitis B virus.

OR = 1.035; 95%CI: 1.010-1.060), the development of HAT post-operatively ( $P = 0.019$ ; OR = 3.543; 95%CI: 1.233-10.178), as well as the donor creatinine prior to explantation ( $P = 0.010$ ; OR = 1.003; 95%CI: 1.001-1.006) were revealed as independent risk factors.

### Retrospective prediction of anastomotic biliary complications in the MELD-era

Compiling all variables which were included in multi-

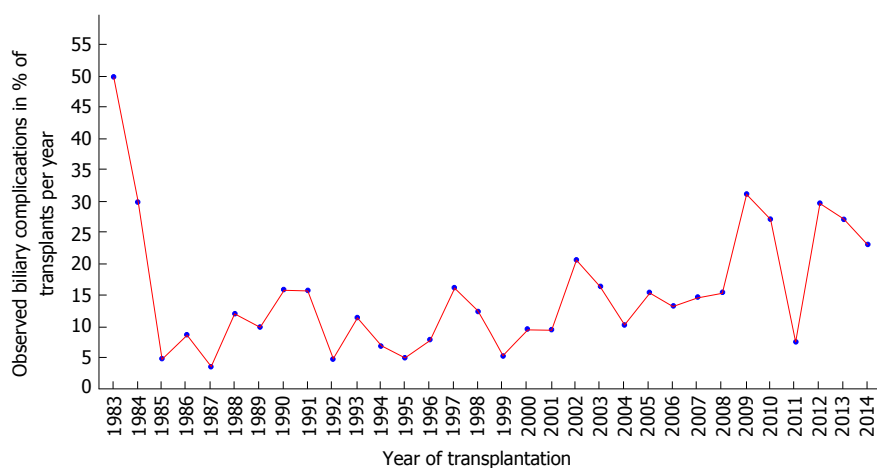


Figure 1 Observed biliary complications as a percent of transplant per year over 30 years.

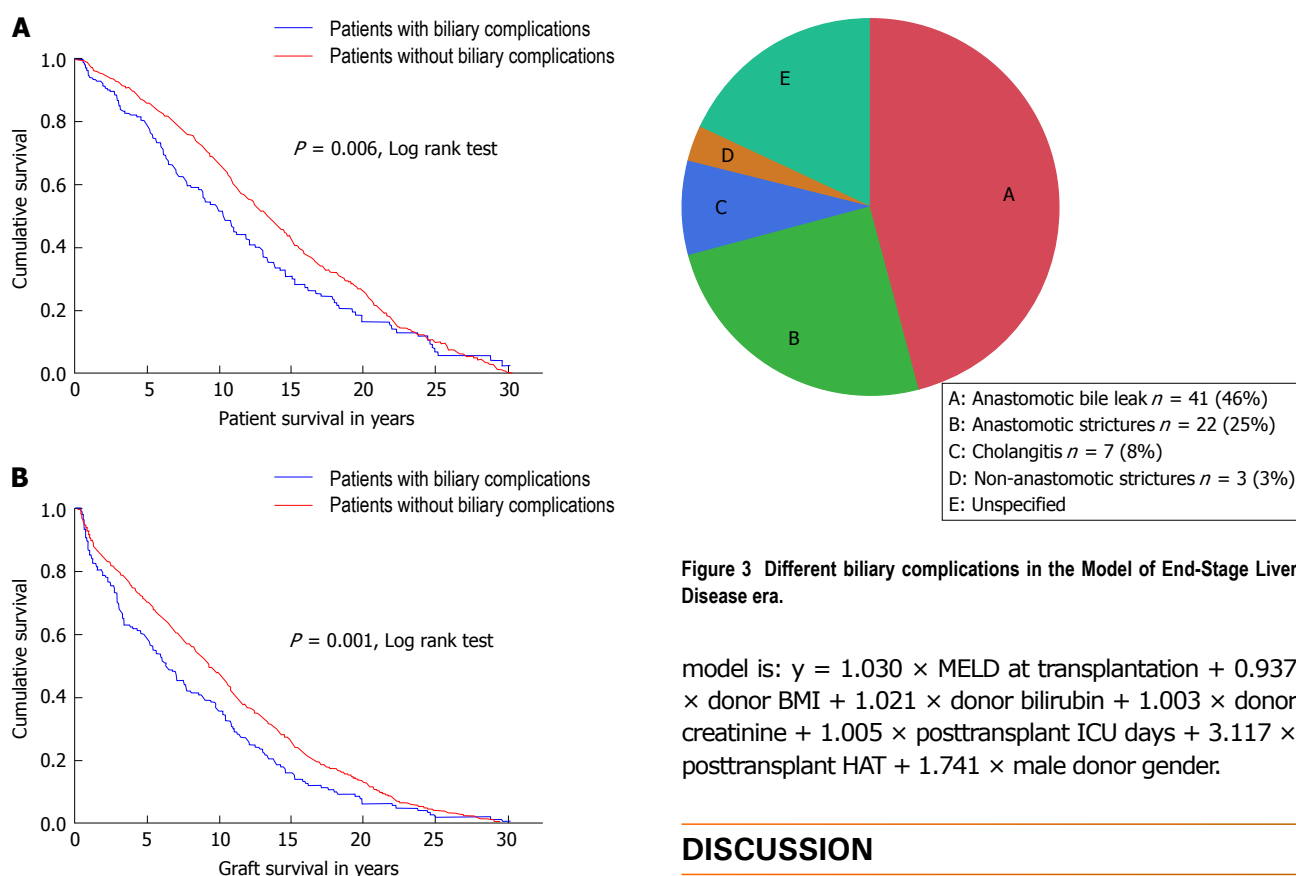


Figure 2 Kaplan Meier curve showing the cumulative survival of (A) patients with and without biliary complications (B) graft survival of both groups.

variable analysis [recipient MELD-score, post-operative HAT, donor creatinine, donor body mass index (BMI), recipient intensive care unit (ICU) stay in days, donor gender, donor bilirubin] in a regression equation, this model provides good prognostic abilities in the investigated cohort with an AUROC of 0.702 (Figure 4). The model was internally validated applying a backwards randomized bootstrap analysis in 100 cases [mean AUROC: 0.720 (SD: 0.040)]. The proposed prognostic

Figure 3 Different biliary complications in the Model of End-Stage Liver Disease era.

model is:  $y = 1.030 \times \text{MELD at transplantation} + 0.937 \times \text{donor BMI} + 1.021 \times \text{donor bilirubin} + 1.003 \times \text{donor creatinine} + 1.005 \times \text{posttransplant ICU days} + 3.117 \times \text{posttransplant HAT} + 1.741 \times \text{male donor gender}$ .

## DISCUSSION

Biliary complications are a common post-operative issue after liver transplantation. Liver transplantation has been established in the 1980s as the only life-saving standard treatment for many conditions leading to end-stage liver disease. Therefore, the number of performed liver transplantations has been increasing ever since. Biliary complications endanger early as well as long-term success of liver transplantation and are thus constantly in focus of research to improve care for transplant recipients. However, evidence from large single-center databases on the long-term follow-up of liver transplant recipients including risk-adjusted identification of probable risk factors for the development of biliary complications is still scarce. In the current study,



**Table 5 Univariable and multivariable analyses for identification of risk factors for anastomotic biliary complications since 2006**

	Variable	Univariable analysis		Multivariable analysis	
		P-value	OR (95%CI)	P-value	OR (95%CI)
Recipient data	MELD	0.029	1.036 (1.003-1.050)	0.006	1.035 (1.010-1.060)
	BMI	0.438			
	Days on the waiting list	0.594			
	Gender	0.494			
	Age	0.752			
	ICU stay in days	0.025	1.018 (1.001-1.012)	0.093	
	Pre-transplant PVT	0.056			
	Creatinine	0.042	1.003 (1.001-1.005)		
	Bilirubine	0.034	1.001 (1.001-1.002)		
	Indication HCC	0.328			
	Indication PSC	0.415			
	Indication ALF	0.620			
	Indication HCV cirrh.	0.685			
	Indication alc. cirrh.	0.769			
	Indication biliary dis.	0.115			
Transplant-specific data	Gender mismatch	0.620			
	Cold ischemic time	0.417			
	Era of transplantation	0.124			
	Preservation solution	0.746			
	> 1 arterial anastom.	0.396			
	Aortal anastomosis	0.331			
	Portal vein interpos. Graft	0.251			
	Hepaticojejunostomy	0.425			
	Post-transplant HAT	0.048	2.999 (1.010-8.056)	0.019	3.543 (1.283-10.178)
	Operative duration	0.624			
Donor-specific data	Age	0.738			
	Gender male	0.003	1.835 (1.050-3.303)	0.066	
	BMI	0.014	0.923 (0.861-0.990)	0.056	
	Pre-transplant ICU stay	0.115			
	Body temperature	0.921			
	Bilirubine	0.022	1.027 (1.005-1.050)	0.073	
	Creatinine	0.020	1.003 (1.001-1.005)	0.010	1.003 (1.001-1.006)
	CRP	0.406			
	ALT	0.765			
	AST	0.613			
	GGT	0.278			
	Urea	0.581			
	CMV positivity	0.100			

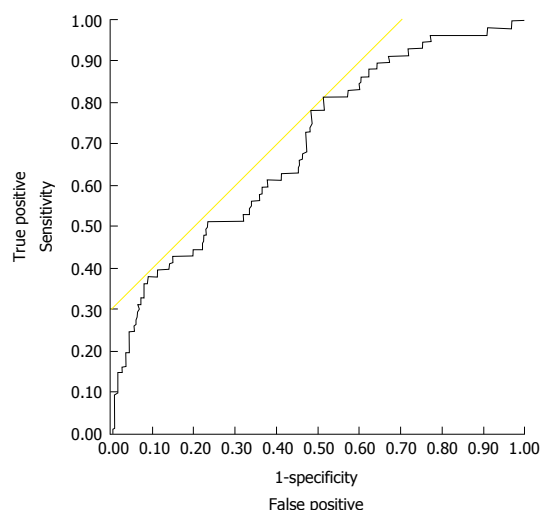
MELD: Model of End-Stage Liver Disease; BMI: Body mass index; ICU: Intensive care unit; ALT: Alanine aminotransferase; AST: Aspartate transaminase; GGT: Gamma glutamyltransferase; CMV: Cytomegalovirus; PVT: Portal vein thromboses; HCC: Hepatocellular carcinoma; PSC: Primary sclerosing cholangitis; ALF: Acute liver failure; HCV: Hepatitis C virus; CRP: C-reactive protein.

a large European center reports its results regarding biliary complications overlooking over three decades of transplant experience. Moreover, in the recent era with the introduction of MELD-based organ allocation in late 2006 as defined starting point, relevant independent risk factors were investigated.

It could be shown that the onset of post-transplant biliary complications endangers patient as well as graft survival even in the long run (Figure 2;  $P = 0.006$ ;  $P = 0.001$ , resp.). This also has serious implications for healthcare economy. A recent analysis of a large dataset with more than 12800 liver transplantations could show that biliary complications in recipients receiving a graft after brain death donation were responsible for an increment of cost of nearly 55000\$ in the first post-transplant year<sup>[3]</sup>. These findings could be confirmed in the following post-transplant years as well as in donation after cardiac death transplantations.

As shown in Figure 1, the number of patients suffering from biliary complications after liver transplantation decreased very early in the observed series to a minimum of closely over 5% in 1985. This early drop might well be a result of the early surgical learning curve. It can also be assumed that some biliary complications might not be detected in these years due to technically less developed diagnostic capabilities, such as computed tomography or ultrasound. Furthermore, early mortality was comparatively high and recipients might have died before developing detectable/treatable biliary complications.

In the years between 1985 and 2006, the incidence of biliary complications was ranging from 5% to 25% of performed transplantations per year, which is comparable to other reported data<sup>[3,5,6]</sup>. These years were characterized by introduction of the Child-Pugh score-based center allocation scheme in the year 2000, which represents a paradigm shift and is therefore regarded in



**Figure 4** Receiver operating characteristic-curve of the developed prognostic model for the prediction of anastomotic biliary complications in the Model of End-Stage Liver Disease-era ( $n = 417$  liver transplants). The area under the receiver operating-curve is 0.702 indicating a useful and clinical applicable prognostic model.

the analysis as a new era. After introduction of MELD-based organ allocation in late 2006, the rate of biliary complications starts to fluctuate in a wider range from 10% to as high as 30%. The notion that donor organ quality might have decreased as well as the general condition of the transplant recipients since the start of this recent era has led to a controversial discussion in the German transplant community about the usefulness of this current allocation policy.

As early as 2009, Weismüller *et al.*<sup>[12]</sup> reported of decreased short-term survival since the introduction of MELD-based liver allocation. An association with longer surgery duration and higher recipient morbidity could be revealed as possible underlying causes. As another example, it was reported recently that indications with high chances for successful long-term survival after liver transplantation such as primary sclerosing cholangitis did not show any improved outcome since MELD-introduction in Germany and that there is relevant outcome stagnation for this entity<sup>[13]</sup>.

Therefore, a more detailed analysis was applied on the MELD-era data, in which a categorization of the biliary complication into different subtypes was possible due to consequent and clear documentation, as well as electronic patient files. Since the biliary anastomosis seems to be the most susceptible part for surgical improvement measures, the anastomotic complications are in a special focus of this investigation. Multivariable, risk-adjusted analysis revealed that the MELD-score at transplantation, the donor creatinine at time of graft donation and the development of HAT after transplantation were statistically significant, independent risk factors for the onset of anastomotic biliary complications. The association of post-transplant HAT and the development of biliary complications has been observed several times and can be explained by an anatomical circumstance. The biliary tract tissue is

especially vulnerable to impaired arterial vascular supply. Whereas the liver parenchyma is nourished *via* a dual vascular supply *via* portal vein and hepatic artery, the bile ducts are supplied only arterially<sup>[5]</sup>. Therefore, the biliary epithelium is more susceptible to decreased perfusion than hepatocytes, which is the case in ischemic injury and severe hypotension, both occurring in the donor organ during transplantation and after HAT.

The finding that the recipient MELD score has influence on the onset of post-transplant biliary complications seems not to be surprising. The MELD-score was shown to accurately depict the recipients state of morbidity prior to transplantation<sup>[14,15]</sup>, thus identifying patients with a risk profile to have impaired healing capabilities at the bile duct anastomosis. This is further confirmed by the finding that impaired donor kidney function as depicted *via* increased donor creatinine levels contributes to the development of anastomotic bile duct lesions, since this further intensifies the unfavorable metabolic situation at the anastomosis, which is at risk for ischemic injury.

After a relevant drop of the incidence of biliary complications in 2011, the number of observed complications increased again in 2012. The data does not clearly provide insights into the root-causes of this observed development, thus, only assumptions can be discussed here. There was no notable change in allocation policies at that time, MELD-based allocation was introduced in late 2006 in Germany as mentioned above. Furthermore, the clinical setting did not change significantly, just a slight increase in the application of Histidine-Tryptophane-Ketoglutarat (HTK) preservation solution could be detected in 2012. HTK-solution was suspected to be associated to biliary complications previously and a trend towards this association was shown in our center in previously published research<sup>[10,16]</sup>.

The proposed prognostic model, which is basing on the results of regression analyses, was shown to have good predictive capabilities with an AUROC > 0.700. Furthermore, it could be internally validated successfully with 100 randomized backwards bootstraps. These promising results regarding this model warrant its validation in an external dataset, which is definitely necessary before a broad application in clinical transplantation seems useful. This validation is preferably performed in a prospective, multi-centric cohort or a large transplant registry, which contains all relevant data as described above.

This study is limited by its single center design and its retrospective character. Furthermore, the long observation period of three decades naturally includes changes in diagnostics and management of biliary complications. This circumstance was addressed with the categorization of the data into four eras, which was taken into account during statistical analysis.

Taken together, the results of the current study lead to the assumption that high recipient MELD scores in combination with impaired donor kidney function as depicted in donor creatinine levels at the time of transplantation should be avoided to protect the recipient from

the onset of early biliary complications. This is especially the case, when HAT occurs in the post-transplant setting, which further endangers the recipient to develop serious anastomotic bile duct issues.

## COMMENTS

### Background

Biliary complications account for a great part of early issues after liver transplantation leading to re-intervention, serious morbidity and even mortality. Moreover, they are responsible for a high amount of healthcare costs after transplantation.

### Research frontiers

In recent years, studies were outlined and published to identify relevant risk factors for the development of biliary complications. However, long-term follow-up data and large series overlooking decades of liver transplant experience are scarce. There is especially no prognostic model available so far, which helps to identify patients who are threatened by this serious complication.

### Applications

The results of this study should reduce the incidence of anastomotic biliary complications after liver transplantation. The proposed prognostic model for the prediction of anastomotic biliary complications can be a tool for the transplant clinician to identify patients at high risk for the development of biliary complications, which then can be included into a stricter diagnostic observation scheme, e.g., with repeating abdominal ultrasound examinations and blood works.

### Terminology

Biliary complications do regularly occur after liver transplantation. They can be classified into anastomotic (bile leak vs strictures) and non-anastomotic lesions. In many countries, liver grafts are currently allocated on the basis of the Model of End-Stage Liver Disease (MELD)-score, which is a score including the recipient's bilirubine levels, creatinine levels as well as the coagulation state via the international normalized ratio-value. The MELD-score is able to reliably predict short-term death on the liver transplant waiting lists in many transplantation systems.

### Peer-review

An interesting well-written study.

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## Primary mucosa-associated lymphoid tissue lymphoma of the liver: A report of two cases and review of the literature

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### Abstract

Mucosa-associated lymphoid tissue (MALT) lymphoma of the liver is a very rare condition and thus the diagnosis may be challenging. The clinical presentation is usually variable, ranging from minimal clinical symptoms to severe end stage liver disease. In this paper, we describe the clinicopathologic findings in two cases of primary hepatic MALT lymphoma. One case is an 80-year-old female with no underlying chronic liver disease and the second case is a 30-year-old female with autoimmune hepatitis complicated by MALT lymphoma. In both specimens, there was diffuse infiltration of atypical B-lymphocytes that were positive for CD20 and CD79a, but negative for CD5, CD43 and CD10. There were occasional lymphoepithelial lesions involving the hepatocytes or bile ducts. Polymerase chain reaction analysis showed monoclonal immunoglobulin heavy chain gene rearrangement in both cases. The first case was treated with surgery but developed pulmonary recurrence a year after complete resection but went into remission following treatment with rituximab. A second recurrence occurred in the right parotid gland 7 years later, which was treated with idelalisib. The second case was effectively treated with rituximab. To our knowledge, the second case is the first reported case linked to autoimmune hepatitis.

**Key words:** Extranodal; Mucosa-associated lymphoid tissue; Lymphoepithelial; Lymphoma; Polymerase chain reaction

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**Core tip:** The diagnosis and management of mucosa-associated lymphoid tissue lymphoma of the liver can be a clinical dilemma. Recognition of the clinic-pathologic



pattern and its associations with underlying autoimmune disease can prevent misdiagnosis. This case report not only represents the first reported association with autoimmune hepatitis and the development of multiple recurrences of the lymphoma in the literature, but also it applies new successful treatment regimens as an alternative to current clinical practice.

Obiorah IE, Johnson L, Ozdemirli M. Primary mucosa-associated lymphoid tissue lymphoma of the liver: A report of two cases and review of the literature. *World J Hepatol* 2017; 9(3): 155-160 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i3/155.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i3.155>

## INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) lymphoma, is a distinct subgroup of non-Hodgkin's lymphoma (NHL) that accounts for 7%-8% of all B cell lymphomas<sup>[1]</sup>. MALT lymphomas are considered low-grade and they can occur in a variety of organs, including the stomach, orbit, conjunctiva, salivary gland, skin, thyroid, lung, stomach, intestine, dura and rarely liver. MALT lymphomas usually arise in areas that are devoid of lymphoid tissue, but are preceded by chronic inflammation, either infectious or autoimmune, which result in the accumulation of extranodal lymphoid proliferation<sup>[1]</sup>. Prolonged lymphoid proliferation can eventually result in the development of a malignant clone. The stomach is the most common site of MALT lymphoma and its association with *H. pylori* is well documented<sup>[2-4]</sup>. An increased occurrence of MALT lymphomas, especially in the salivary glands has been reported in patients with Sjögren's syndrome<sup>[5-7]</sup>. Patients with Hashimoto's thyroiditis have a 67- to 80-fold increased risk of developing primary thyroid lymphoma<sup>[8-10]</sup> and B-cell type NHL is the most common type and features of MALT lymphoma can be seen in over one-third of cases<sup>[10]</sup>. Case studies on MALT lymphoma of the liver have been rarely reported and very little is known about this disease entity. Here we present two cases with primary MALT lymphoma of the liver, one with no underlying chronic liver disorder and the other is associated with autoimmune hepatitis.

## CASE REPORT

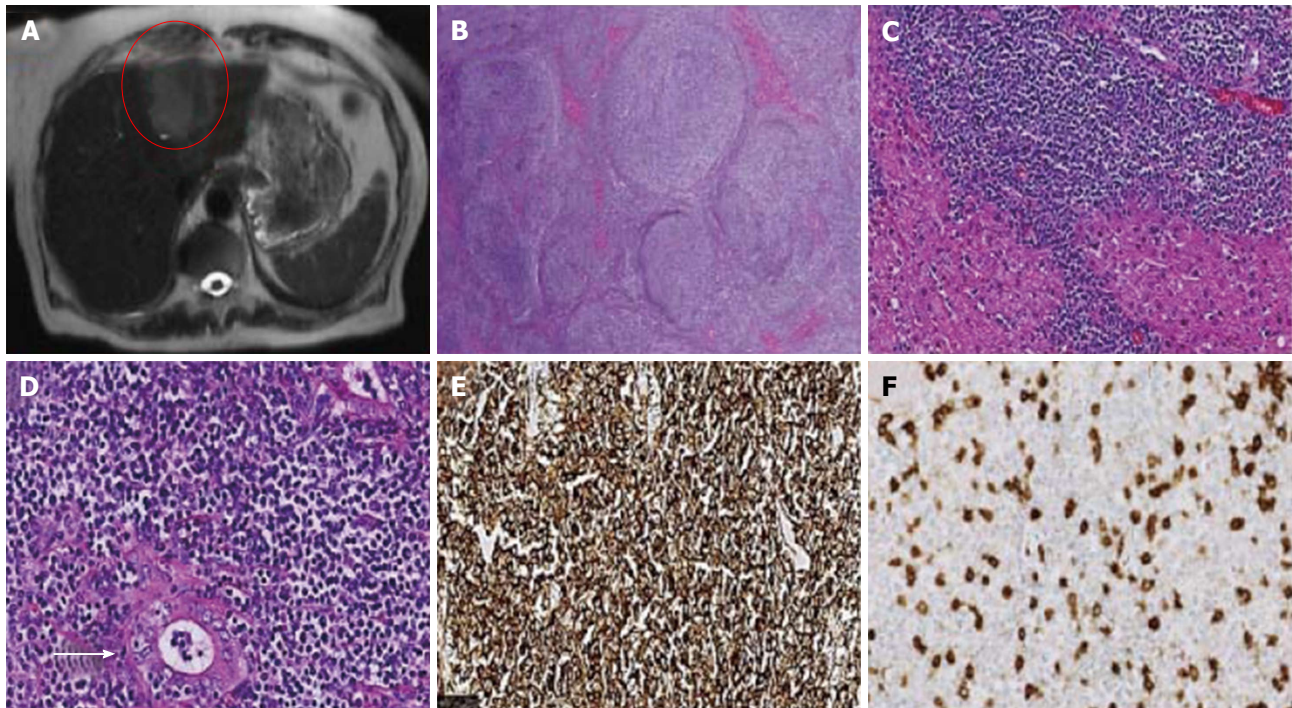
### Case 1

An 80-year-old Caucasian female, presents with a history of nausea, loss of appetite and a 20-pound weight loss. History was negative for any underlying infectious or autoimmune process. Physical examination was notable for weight loss. Abnormal laboratory results obtained was as follows: WBC 13.5 K/UL, aspartate aminotransferase (AST) 137 U/L and alanine aminotransferase (ALT) 166 U/L, alkaline phosphatase 53 U/L, albumin 2.5 g/dL, bilirubin total 1.1 mg/dL, Bilirubin direct 0.2 mg/dL. Abdominal computed tomography (CT) scan identified

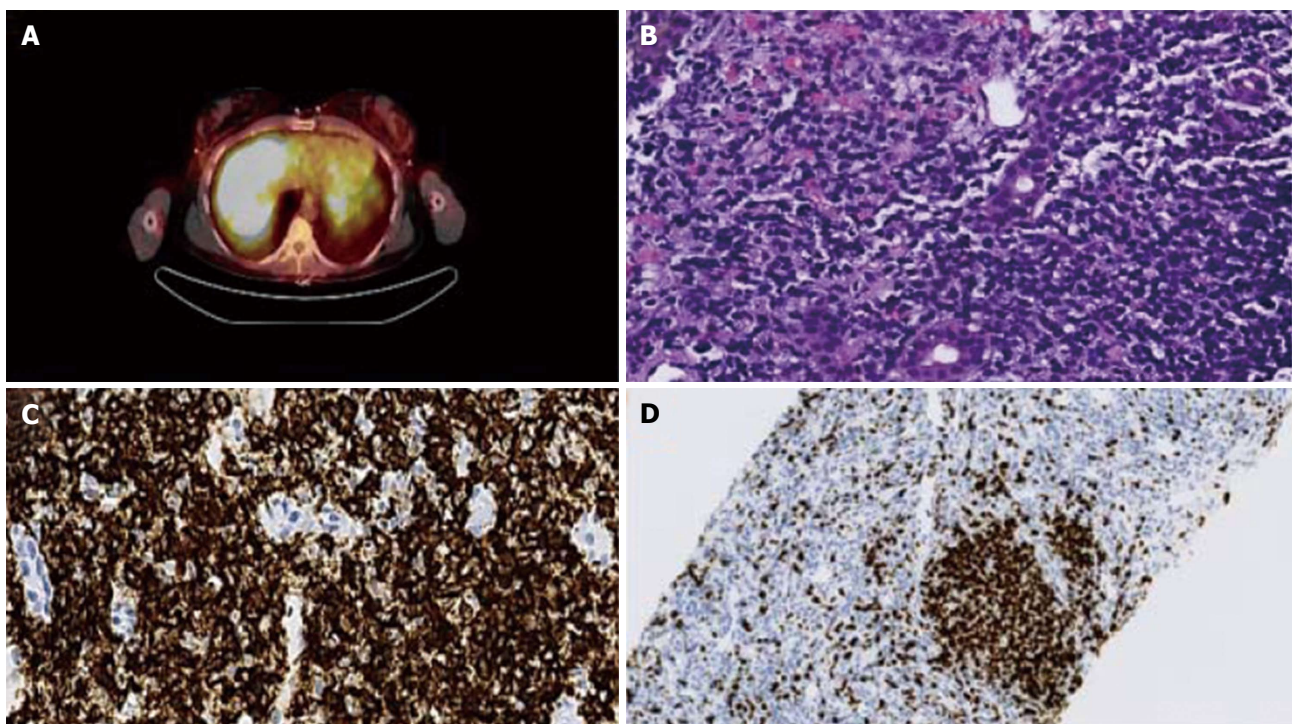
a bi-lobed mass in the left hepatic lobe (Figure 1A). No dilatation of the intra- or extra-hepatic bile ducts was seen. The spleen, pancreas, gallbladder kidneys and adrenal glands were unremarkable. Histological sections of the mass revealed a nodular infiltrate of atypical lymphocytes with small irregular nuclei and abundant clear cytoplasm surrounding occasional reactive germinal centers (Figure 1B and C). Focal fibrosis and plasmacytosis was identified at the periphery of the nodules. There were occasional lymphoepithelial lesions (Figure 1D). Immunohistochemical staining showed that the neoplastic cells were positive for CD20 (Figure 1E), CD79a and BCL-2 and negative for CD10, CD5 (Figure 1F), CD23, BCL-6, CD43, CD3, CD21, CD138 and IgD. Ki-67 was positive in approximately 30% of the cells. Polymerase chain reaction (PCR) analysis by capillary electrophoresis was clonal for immunoglobulin heavy chain (IgH) rearrangement. Bone marrow biopsy showed normocellular marrow with trilineage hematopoiesis and no evidence of lymphoma. These results supported the diagnosis of MALT lymphoma of the liver. On further follow-up, a year later, the patient developed pulmonary nodules which were proven to be MALT lymphoma on biopsy. She went into remission following treatment with rituximab for one year. Seven years later she presented with a right neck mass which was positive for MALT lymphoma of the parotid gland. The lung and parotid MALT lymphoma showed the same IgH rearrangement by PCR, which indicated that they were the same clones.

### Case 2

A 30-year-old lady presented to the clinic for management of a previously diagnosed autoimmune hepatitis. At 10 years of age, she was diagnosed with Hashimoto's thyroiditis and further work up revealed autoimmune hepatitis on a liver biopsy which was managed on azathioprine. In the previous year prior to presentation, she had a flare up of the autoimmune hepatitis when her liver enzymes were found to be in the 500's range and remained abnormal despite having been on medication. At the time of presentation, the patient was asymptomatic. The laboratory results obtained were as follows: WBC 1.5 K/UL, platelet 70 K/UL, HB 11.1 g/dL, AST 209 mg/dL, ALT 232U/L, alkaline phosphatase 239 U/L, bilirubin total 1.4 mg/dL, bilirubin direct 0.5 mg/dL. Biochemical investigation for chronic viral hepatitis was negative. Although previously positive, her current report for anti-smooth muscle antibodies was negative. Positron emission tomography/CT abdomen showed an enlarged liver (20 cm) with heterogeneity and diffuse FDG activity (Figure 2A), which was highly suspicious for malignancy. The spleen was slightly enlarged with mild portacaval and left paraaortic adenopathy. Liver biopsy identified atypical lymphoid infiltrate with focal interface activity, fibrosis and lymphoepithelial lesions on histopathological examination (Figure 2B). Immunohistochemistry analysis showed predominantly CD20 positive B-lymphocytes in the infiltrate (Figure 2C) that were negative for CD5, CD10, or CD43. Ki-67 proliferative index was low (30%)



**Figure 1 Abdominal computed tomography scan and histology of the mass.** A: Computed tomography of the abdomen reveals a large mass, highlighted in red, in the left lobe; B: Effacement of normal liver by nodules of atypical lymphocytes [hematoxylin-eosin (H and E), 2.5  $\times$ ]; C: Hepatocytes are admixed with neoplastic lymphocytes (H and E, 20  $\times$ ); D: Lymphoma cells infiltrate into the bile duct forming lymphoepithelial lesions (arrows) (H and E, 40  $\times$ ); E, F: By immunohistochemistry, the neoplastic cells are positive for CD20 (E) and negative for CD5 (F) (40  $\times$ , each).



**Figure 2 Positron emission tomography-computed tomography and histology of the liver mass.** A: Liver shows diffuse fluorodeoxyglucose activity throughout the parenchyma with the maximum standardized uptake value of 8.6; B: The atypical lymphocytes grow diffusely forming mass with occasional lymphoepithelial lesions (40  $\times$ ); C, D: By immunohistochemistry, the neoplastic lymphocytes are positive for CD20 (C, 40  $\times$ ) and have low MIB-1 proliferative index (D, 40  $\times$ ).

except in the reactive germinal center (Figure 2D). PCR analysis of the liver biopsy was positive for the *IgH* gene rearrangement, indicating monoclonal B cell proliferation.

Her bone marrow biopsy showed trilineage maturation with no evidence of lymphoma. Treatment with rituximab was commenced and the patient is still in remission three



years later.

## DISCUSSION

The etiology of MALT lymphoma of the liver is still unclear. In most extranodal MALT lymphomas, chronic inflammation due to either an infectious process or autoimmune process has been implicated. Several disease conditions have been associated with the development of hepatic MALT lymphoma which makes initial diagnosis very difficult. Nagata *et al*<sup>[11]</sup> reviewed 51 cases of MALT lymphoma of the liver. They reported that 25% was not associated with any disease condition. However, hepatic MALT lymphoma was associated with carcinomas (21%), viral and drug related-hepatitis (20%), biliary cirrhosis (10%), liver cirrhosis (10%), ascariasis (4%), gastric MALT lymphoma (4%), rheumatoid arthritis (2%), multiple biliary unilocular cysts (2%) and no information was reported in 2% of the cases. Majority of the cases presented as a solitary mass and were effectively treated with surgical resection without any adjuvant therapy. Our first case had no underlying history of hepatitis, infection, cancer or autoimmune condition. The only concerning clinical sign was drastic weight loss. The abnormal liver enzymes and liver mass were incidental findings. Clinically an initial diagnosis of hepatocellular carcinoma was made and the patient underwent surgery with complete resection of the lesion, which prevented recurrence in the liver but the lymphoma recurred in the lungs. The most frequent location of recurrence of hepatic MALT lymphoma following treatment appears to be the lungs<sup>[11,12]</sup> and this occurs at a mean average of 65 mo. Of the 3 reported cases in the literature, 2 patients were treated with resection and the remaining one, with radiation. Hepatic recurrence is rare after complete resection and only one case has been reported<sup>[11]</sup>. Our patient developed pulmonary recurrence only after one year, but after treatment with rituximab the patient remained in remission for 7 years. However, the lymphoma recurred in the right parotid gland which was treated with 7 mo of idelalisib. The patient is currently in remission a year later. MALT lymphoma generally has an indolent course but recurrences can occur over many years and it tends to involve other common extranodal sites<sup>[13]</sup>. In our experience, we report for the first time multiple recurrences of MALT lymphoma following complete resection of hepatic MALT lymphoma.

The mean age of patients with MALT lymphoma of the liver is about 60 years of age<sup>[11,14]</sup>, however our second case was 30 years old with an underlying confirmed diagnosis of autoimmune hepatitis which is a disorder frequently seen in young women. Wöhrer *et al*<sup>[15]</sup> analyzed 158 patients with MALT lymphoma and 39% had an autoimmune disease. The patients were predominantly women and significantly younger at lymphoma diagnosis. The most commonly reported autoimmune disorder associated with liver MALT lymphoma is biliary cirrhosis<sup>[14,16,17]</sup> and the usual presentation is as a solitary mass. To our knowledge, this is the first reported case associated with autoimmune

hepatitis. Autoimmune hepatitis is a chronic progressive liver disease, characterized by hepatocellular inflammation and liver damage and a tendency to progress to liver cirrhosis. Most patients also have other autoimmune diseases including type 1 diabetes, thyroiditis, vitiligo and Sjögren's syndrome. In support of this, in addition to autoimmune hepatitis, our patient had hashimoto thyroiditis. Similar to MALT lymphoma in other organs, chronic immune activation in autoimmune hepatitis may contribute to lymphomagenesis in the liver. Perhaps the prolonged history and severity of the disease in case 2 explains the diffuse liver involvement with MALT lymphoma. It is important to note that reactive lymphoid hyperplasia, also known as pseudolymphoma, is associated with autoimmune hepatitis<sup>[18]</sup>. Interestingly pseudolymphoma of the liver can cause a focal liver mass with atypical lymphoid proliferation on histology, which predominantly stain positive for CD20 and reactive germinal center formation<sup>[19]</sup>. The benign entity can be difficult to differentiate from hepatic MALT lymphoma without further molecular investigation and majority of these lesions are resected due to suspicion for a malignancy. Sato *et al*<sup>[20]</sup> reported transformation of a pseudolymphoma of the liver, in a background of biliary cirrhosis, into a diffuse B cell NHL. Accordingly, patients with pseudolymphoma will require close follow-up to prevent a misdiagnosis of MALT lymphoma. Indeed, further studies are needed to determine if there is an association between pseudolymphoma and subsequent transformation into low grade lymphomas such as MALT lymphoma.

Rituximab has shown promise as an effective treatment in extra-gastric MALT lymphoma<sup>[21,22]</sup> but very few cases involving treatment with rituximab have been reported in hepatic MALT lymphoma. Stable remission have been described in patients treated with rituximab following surgical resection<sup>[23]</sup> or in combination with chemotherapy<sup>[24]</sup> or radiofrequency ablation<sup>[25]</sup>. Both of our patients were treated with rituximab and our second case had no previous surgical treatment or chemotherapy. Although high remission rates are achieved by chemotherapy<sup>[26]</sup>, treatment with rituximab can minimize toxicity while maintaining efficacy. Idelalisib, a selective inhibitor of the delta isoform of phosphatidylinositol 3-kinase<sup>[27]</sup>, which plays an important role in B-cell development, proliferation, migration, adhesion and survival<sup>[28]</sup> has shown efficacy by inducing apoptosis in malignant B-cells in patients with relapsed follicular lymphoma or refractory chronic lymphocytic lymphoma<sup>[27,29]</sup>. To our knowledge, our study is the first reported case to use idelalisib in the treatment of recurrent MALT lymphoma of the liver and should be considered as a possible effective therapy following treatment failure with rituximab.

Primary hepatic MALT lymphoma is very rare. From our experience, we recommend that young patients with autoimmune hepatitis with sudden elevation of liver enzymes, should raise the suspicion of MALT lymphoma of the liver. Since the liver was diffusely involved, rituximab as the sole agent can be used as an effective treatment of choice. Patients with hepatic MALT lymphoma should

be closely followed up for recurrence especially in common extranodal sites. Rituximab or Idelalisib can be a suitable treatment in patients with relapse. In solitary cases of MALT lymphoma of the liver, complete resection of the lesion with adjuvant rituximab therapy should be considered to prevent recurrence.

## COMMENTS

### Case characteristics

Mucosa-associated lymphoid tissue (MALT) lymphoma of the liver is a very rare condition, which can be misdiagnosed.

### Clinical diagnosis

Hepatic MALT lymphoma is associated with various inflammatory and autoimmune diseases, and one of the authors' patients had autoimmune hepatitis. The disease may also occur in the absence of any underlying disorder.

### Differential diagnosis

Hepatocellular carcinoma (HCC), hepatic reactive lymphoid hyperplasia, cholangiocarcinoma, metastatic carcinoma to the liver.

### Laboratory diagnosis

The liver enzymes were elevated in both cases of MALT lymphoma of the liver.

### Imaging diagnosis

Lesions of hepatic MALT lymphoma may resemble HCC, cholangiocarcinoma or hepatic metastasis on computed tomography or position emission tomography scan, often leading to misdiagnosis.

### Pathological diagnosis

MALT lymphoma of the liver often presents as infiltration of the liver with atypical lymphocytes, forming lymphoepithelial lesions on histologic sections and monoclonal immunoglobulin heavy chain gene rearrangement on PCR analysis.

### Treatment

Complete surgical excision of lesion or rituximab in localized disease and the use of rituximab or idelalisib in recurrent or advanced disease.

### Related reports

Although MALT lymphoma of the liver is considered a low grade lymphoma, patients should be closely followed up for recurrence especially in common extranodal sites. Recurrences or advanced disease can be treated with biological targeted therapy to achieve remission while avoiding the toxic effects of chemotherapy.

### Term explanation

MALT lymphoma of the liver is associated with an inflammatory or autoimmune condition such as, primary biliary cirrhosis, hashimoto disease or like in our patient, autoimmune hepatitis. In some cases, there may be no underlying associations.

### Experiences and lessons

MALT lymphoma of the liver should be considered when imaging studies show a hepatic lesion with an underlying autoimmune condition or when a solitary liver mass is identified in a patient with no clear risk factors for HCC and cholangiocarcinoma.

### Peer-review

Well written and excellent case report on two patients with MALT lymphoma of the liver.

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## Miliary tuberculosis infection during hepatitis C treatment with sofosbuvir and ledipasvir plus ribavirin

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### Abstract

Chronic hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide. In the last 5 years, treatment for HCV infection has experienced a marked development. In 2014, the use of ledipasvir/sofosbuvir with or without concomitant weight-based ribavirin was approved with a very significant increase in the sustained virological response. However, new side effects have been associated. We report the first case of an HCV infected patient treated for 12 wk with the combination of sofosbuvir/ledipasvir plus ribavirin who developed a miliary tuberculosis (TB) infection while on therapy. The patient was a 65-year-old woman, who referred malaise, asthenia, hyporexia, 7 kg weight loss, productive cough, evening fever and night sweats, right after finishing the treatment. The chest computed tomography-scan revealed a superior mediastinal widening secondary to numerous lymphadenopathies with extensive necrosis and bilateral diffuse lung miliary pattern with little subsequent bilateral pleural effusion, highly suggestive of lymph node tuberculosis with lung miliary spread. A bronchoscopy was performed and bronchial suction showed more than 50 acid-alcohol resistant bacillus per line. A *Mycobacterium tuberculosis* DNA was detected in blood by polymerase chain reaction, which confirmed the diagnosis of miliary tuberculosis. Some cases of TB infection have been identified with  $\alpha$ -interferon-based therapy and with the triple therapy of pegylated interferon, ribavirin and boceprevir or telaprevir. However, significant infection has not been reported with sofosbuvir/ledipasvir plus ribavirin.

We believe that the case is relevant to increase awareness of opportunistic infections and particularly TB infection. Although the international guidelines offer no recommendation regarding TB screening, we wonder whether it would be advisable to screen for opportunistic infections prior to the introduction of HCV therapy.

**Key words:** Tuberculosis; Ledipasvir; Ribavirin; Sofosbuvir; Hepatitis C

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**Core tip:** Cases of tuberculosis (TB) infection have been identified with  $\alpha$ -interferon-based therapy and the triple therapy with pegylated-interferon, ribavirin and boceprevir or telaprevir. This is the first case of a TB infection during treatment with sofosbuvir/ledipasvir plus ribavirin. It is relevant to increase awareness of TB due to its variety of symptoms, which can be confused with those associated to the hepatitis C virus or the antiviral treatment. Considering the impaired immune system of cirrhotic patients and that these drugs arrived slightly more than one year ago it is important to be conscious of the potential events that can be related with the treatment.

Ballester-Ferré MP, Martínez F, García-Gimeno N, Mora F, Serra MA. Miliary tuberculosis infection during hepatitis C treatment with sofosbuvir and ledipasvir plus ribavirin. *World J Hepatol* 2017; 9(3): 161-166 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i3/161.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i3.161>

## INTRODUCTION

Affecting more than 160 million people and at an increasingly higher rate, hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide. In the last 5 years, treatment for HCV infection has experienced a marked development, especially in the case of treatment for genotype 1. Previously, treatment was based on pegylated interferon and ribavirin for 48 wk, which achieved a sustained virological response (SVR) rate of 45% to 50%. In 2011, the first generation of protease inhibitors-telaprevir and boceprevir-reached the market. The triple therapy with peg-interferon, ribavirin and a protease inhibitor was then adopted as the standard of care, followed in 2013 by the approval of the second generation of protease inhibitor, simeprevir, and the polymerase inhibitor, sofosbuvir. In 2014, the use of ledipasvir/sofosbuvir was approved, as well as the use of ombitasvir/paritaprevir/ritonavir plus dasabuvir for the treatment of HCV genotype 1 infections with or without concomitant weight-based ribavirin. These changes have brought a very significant increase in the SVR, with rates rising above 90%. However, new side effects, such as decompensation of cirrhosis, liver toxicity and some

infections, have been associated<sup>[1]</sup>.

We report the first case of an HCV-infected patient treated for 12 wk with the combination of sofosbuvir/ledipasvir plus ribavirin who developed a miliary tuberculosis infection while on therapy.

## CASE REPORT

The patient was a 65-year-old woman from Equatorial Guinea, who had been residing in Spain since 2000 (16 years), without any recent trip reported. Her medical history included a blood transfusion event in 1996, diabetes mellitus type 2 diagnosed in 1997 under treatment with insulin, no current nor previous smoking habits and no underlying pulmonary disease or symptoms. In 2000 the patient was admitted to the hospital with abdominal pain. On physical examination hepatomegaly was detected. The blood test revealed hypertransaminasemia and the serology test permitted the diagnosis of chronic HCV infection. HCV genotype was 1a and viral load was 720000 IU/mL. An abdominal ultrasonography revealed a discrete homogeneous hepatomegaly with no focal liver lesions and a transient elastography resulted in 7.9 kPa. A liver biopsy was performed showing chronic HCV infection with Batts and Ludwig<sup>[2]</sup> stage 1, grade 1. In January, 2008, the patient was treated with the standard at that time, based on the combination of peg-interferon- $\alpha$  2a (180  $\mu$ g/wk) with ribavirin (1200 mg/d) during 12 mo. Unfortunately, treatment had to be stopped three mo after starting due to the appearance of side effects: Gluteus abscess, leucopenia and anemia. The patient was followed up in outpatients' clinic without further therapy for hepatitis C until 2015 when she was evaluated for starting treatment with the new direct antiviral agents (DAAs). She was asymptomatic and there were no signs of ascites, edema, bleeding or encephalopathy nor lymphadenopathies on physical examination. Body weight was 54 kg. Hemoglobin level was 12.9 g/dL (reference range -RR-: 11.5-15.5), leucocyte count 5.56 cells/ $\mu$ L (RR: 3.9-11), absolute neutrophil count 1.14 cells/ $\mu$ L (RR: 2.5-7.5), absolute lymphocyte count 2.46 cells/ $\mu$ L (RR: 1.5-4.5) and platelet count 144.000 cells/ $\mu$ L (RR: 160.000-400.000). Albumin was 4 g/dL (RR: 3.5-5.2), total bilirubin 0.43 mg/dL (RR: 0.10-1.00), INR was 1.09 (RR: 0.85-1.35), alanine aminotransferase (ALT) 126 U/L (RR: 1-31), aspartate aminotransferase 120 U/L (RR: 1-31), gamma glutamyl transpeptidase 52 U/L (RR: 1-38) and alkaline phosphatase 90 U/L (RR: 30-120). Alpha-fetoprotein was 9.3 ng/mL (RR: 0.0-7.0) and glycosylated-hemoglobine was 5.2% (RR: 4.0-6.1). Human immunodeficiency virus (HIV) serology was negative. Viral load was 1300000 IU/mL and HCV genotype was 1a and the *IL28B* gene was TC. An abdominal ultrasound exam revealed signs of hypertrophy of the left and caudate liver segments with a focal benign lesion (hemangioma) of 9 mm in segment IV with no changes when compared to previous tests, normal portal vein caliber with hepatopetal flow and a splenomegaly of 13 cm. Transient elastography scored a

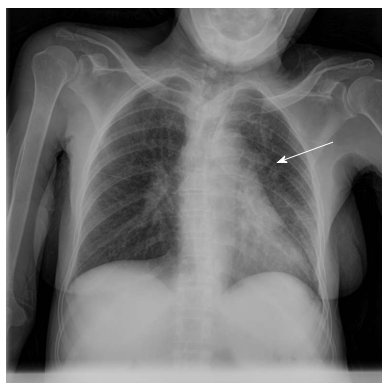


Figure 1 Chest X-ray: Left hilar widening (arrow).

result of 11.1 kPa. Treatment with ledipasvir/sofosbuvir plus ribavirin during 12 wk was initiated in June, 2015. The check-up at the clinic one month later showed no symptoms or pathological signs on physical examination. The treatment was completed but in September, 2015, the patient complained about malaise, asthenia, hyporexia, 7 kg weight loss and dry cough. She referred that these symptoms had appeared one month before she was attended at the emergency unit. However, due to the clinical and hemodynamic stability, the patient was discharged with further outpatient control. In the next 2 wk, in early October, a more productive cough with white sputum, evening fever and night sweats were added to the previous symptoms and upon examination at the emergency unit again, the patient's temperature was 39 °C, blood pressure was 131/94 mmHg, heart rate was 130 bpm with 98% oxygen saturation. She was conscious with no signs of neurologic impairment. Cardiac and pulmonary auscultation were normal, the abdomen was soft with a 3 cm hepatomegaly and a 2 cm splenomegaly without signs of ascites or abdominal pain, limbs showed no signs of edema and no cervical, axillary or inguinal adenopathies were found. The blood test highlighted a sodium level of 129 mmol/L (RR: 135-145), ALT 38 U/L, CRP 94 mg/L (RR: < 5), hemoglobin level 9.6 g/dL, leucocyte count 5.98 cells/ $\mu$ L, absolute neutrophil count 4.84 cells/ $\mu$ L, absolute lymphocyte count 0.78 cells/ $\mu$ L, platelet count 114000 cells/ $\mu$ L and INR 1.26 with the rest of parameters standing within the normal range. The arterial gasometry showed pH 7.51 (RR: 7.35-7.45), pO<sub>2</sub> 126 mmHg (RR: 83-108), pCO<sub>2</sub> 29 mmHg (RR: 35-45), lactate 1.6 mmol/L (RR: 0.6-1.17) and bicarbonate 25 mEq/L (RR: 20-29). The urine test, abdominal X-ray and cranial computed tomography (CT) scan that were carried out revealed no abnormalities. The chest X-ray showed a left hilar widening (Figure 1) and the patient was admitted to the hospital for further studies. The blood culture was negative, as well as the malaria and leishmania tests. The sputum direct vision showed mixed microbiota with predominance of gram-negative bacillus with no acid-alcohol resistant bacillus (AARB) seen. Nevertheless, *Mycobacterium tuberculosis* showed up in the culture of the sputum after 10 d. Chest and abdomen CT scan revealed a superior mediastinal

widening secondary to numerous lymphadenopathies with extensive necrosis, causing a displacement of the esophagus and trachea, and contiguous to these lymphadenopathies, a left hilar mass displaying an air bubble communicated with the left bronchial tree (Figure 2), as well as a bilateral diffuse lung miliary pattern with little subsequent bilateral pleural effusion (Figure 3). These findings were highly suggestive of lymph node tuberculosis with lung miliary spread, being less likely to be attributed to a malignant left hilar mass. A flexible bronchoscopy was performed showing a bossing in the upper part of the trachea and mucosa thickening in both main bronchi with partial stenosis of the left upper lobule bronchi. The retrotracheal mass was biopsied, displaying acute inflammation in the pathological study with negative Zielh Neelsen test. However, in the bronchial suction there were more than 50 AARB per line. A *Mycobacterium tuberculosis* DNA was detected in blood by RCP which confirmed the diagnosis of miliary tuberculosis. The first line treatment for tuberculosis with rifampin, isoniazid, pyrazinamide and ethambutol was initiated, presenting remission of the symptoms and a good tolerance with no signs of liver toxicity (Figure 4).

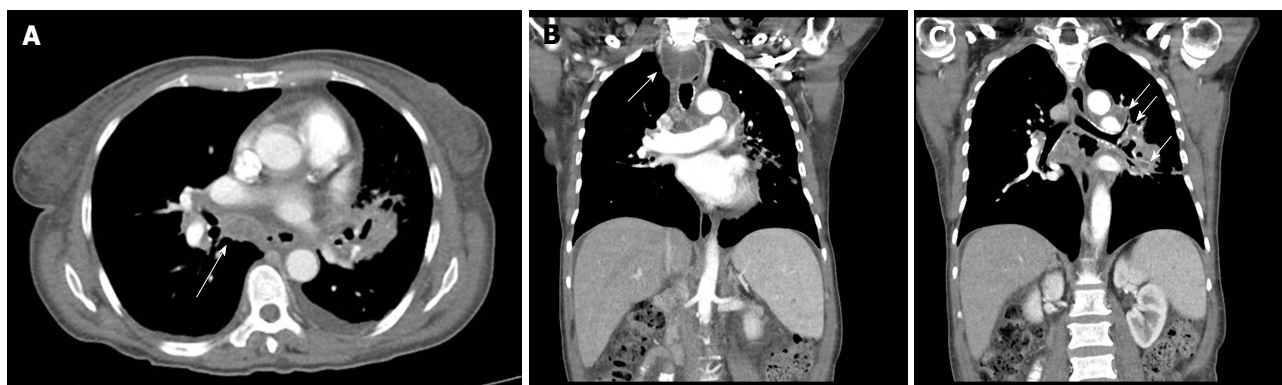
## DISCUSSION

Tuberculosis, caused by *Mycobacterium tuberculosis*, is a worldwide health problem, remaining as the leading cause of death from infectious diseases. It is estimated that 30% of the global population hosts TB in its latent form, which can be reactivated with the presence of several factors, such as aging, smoking, alcohol use, diabetes, chronic renal failure, cancer, a weakened immune system, glucocorticoids or tumor necrosis factor- $\alpha$  inhibitors use. Furthermore, miliary tuberculosis form, has only been described in immunocompromised hosts, especially in patients with underlying T-cell deficiencies such as HIV infection. The classic presenting symptoms of pulmonary TB include persistent fever, weight loss, drenching night sweats, persistent cough (often with sputum production), and hemoptysis; whilst extra-pulmonary TB can affect any organ with a wide variety of symptoms, and therefore requires a high index of clinical suspicion. Without treatment, TB has a mortality rate of 50% within 5 years. Various cell types and cytokines are crucial: T cells (CD4<sup>+</sup>, CD8<sup>+</sup>, and natural killer) and macrophages participate in protection against TB, and interferon- $\alpha$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are essential cytokines for the control of acute TB infection<sup>[3]</sup>.

Two conditions might have contributed to the TB infection. On one hand, cirrhosis by itself is associated with lymphocyte and macrophage dysfunction and decreased production of interferon- $\alpha$  and TNF- $\alpha$  and it may be linked to a higher TB risk. A Taiwan study, showed that active TB incidence rates were significantly higher among cirrhotic patients compared with non-cirrhotic patients, particularly those with alcoholism and HCV infection<sup>[4]</sup>.

On the other hand, TB infection has been reported

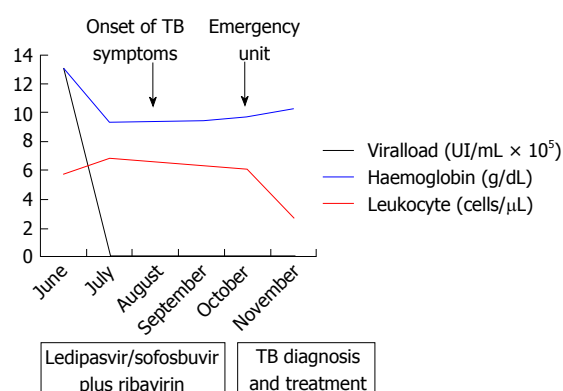




**Figure 2** Chest computed tomography scan (scale W500:L50): Soft tissue window. Axial (A) and coronal (B, C) views: Superior mediastinal widening secondary to numerous lymphadenopathies (arrows) and a left hilar mass.



**Figure 3** Chest computed tomography scan (scale W500:L50): Pulmonary windows. Axial view: Bilateral lung miliary pattern (arrows) with little bilateral pleural effusion.



**Figure 4** Course of haemoglobin, leucocytes and viral load during and after antiviral treatment. Attendance to the emergency unit, tuberculosis diagnosis and start of therapy are shown according to time. TB: Tuberculosis.

anecdotically in patients with HCV infection undergoing  $\alpha$ -interferon-based therapy, usually as a reactivation of latent cases. As an example, 18 cases of TB were observed in patients under HCV treatment in Brazil<sup>[5]</sup>. Many studies have already shown that  $\alpha$ -interferon inhibits type 1 immune response, which is characterized by IL-2, interferon- $\alpha$  and TNF- $\alpha$  production, cytokines that restrain *Mycobacterium tuberculosis*. On the contrary, ribavirin is a guanosine analogue that has demonstrated to have an immunomodulatory effect, shifting a type 2 response to a type 1 in plaque-forming cells *in vivo*. When human T-lymphocytes are activated, ribavirin enhances a type 1 cytokine response producing increased levels of IL-2, interferon- $\alpha$  and TNF- $\alpha$  while suppressing the type 2 response with IL-4, IL-5 and IL-10<sup>[6]</sup>. Therefore, ribavirin stimulates the host adaptive immune response responsible for protection against TB infection and it may act as a protective factor; however, no studies have been specifically performed to prove it. Some other cases of TB infection have also been reported with the triple therapy of pegylated interferon, ribavirin and boceprevir or telaprevir, the first generation of protease inhibitors<sup>[1]</sup>. Ledipasvir and sofosbuvir are both DAAs. Sofosbuvir works as an inhibitor of the HCV NS5B RNA-dependent RNA polymerase. Ledipasvir is

an NS5A inhibitor and its exact mechanism of action is unknown, but one suggested mechanism is its inhibition of hyperphosphorylation of NS5A, which seems to be required for viral production. Ledipasvir/Sofosbuvir phase III studies for HCV infection treatment showed that the most common adverse events reported by patients were fatigue, headache and nausea<sup>[7]</sup>. Addition of ribavirin to ledipasvir/sofosbuvir for HCV therapy for patients without cirrhosis or with compensated cirrhosis was associated with a greater incidence of common adverse effects, concomitant medication use and laboratory abnormalities such as anemia, increased levels of total bilirubin and lymphopenia; but rates of severe side effects and interruptions of treatment resulted similar, and no increased infection episodes were detected despite the reported decrease in lymphocytes<sup>[8]</sup>. In order to reduce the side effects of these drugs, advanced technologies are used during their development such as computer-aided leading drug optimization<sup>[9]</sup>.

Indeed, significant infection has not been reported with the new era of free-interferon regimen treatment. This case is, to the best of our knowledge, the first case report of a miliary TB infection during HCV infection treatment with sofosbuvir/ledipasvir plus ribavirin. Although the association with the DAAs has not been proven and

it may be a coincidence that the two infections have occurred in a close time frame, several data should make clinicians wonder: The patient had diabetes and chronic liver disease as a risk factors for TB, nevertheless both were well controlled; she was asymptomatic and with no signs on physical examination neither before starting the treatment nor after one month of the beginning, which goes against the hypothesis that she may have reactivated TB prior to therapy and it has just presented late; moreover, the mechanisms underlying these drugs effects are currently unknown and further immunological studies should be performed in order to find out how the innate and adaptive immune responses are altered by the different treatment regimens<sup>[10]</sup>.

We believe that the case we have reported is relevant to increase awareness of opportunistic infections, particularly TB infection due to its variety of symptoms which can be confused with those associated to the HCV infection or the antiviral treatment and the high mortality rate of TB infection without treatment. Considering the impaired immune system of cirrhotic patients and the fact that these DAAs arrived on the market slightly more than one year ago and no long-term side effects have been described, we consider that it is important to be conscious of the potential events that can be related with the HCV treatment. In addition, although the international guidelines for the management of HCV infection<sup>[11,12]</sup> offer no recommendation regarding TB screening, we wonder whether it would be advisable to screen for opportunistic infections, *via* tuberculin skin test and/or interferon gamma releasing assays, prior to the introduction of HCV therapy.

## COMMENTS

### Case characteristics

A 65-year-old woman with history of diabetes mellitus type 2, chronic hepatitis C virus (HCV) infection and no current nor previous smoking habits and no underlying pulmonary disease or symptoms.

### Clinical diagnosis

The patient presented with malaise, asthenia, hyporexia, 7 kg weight loss, productive cough with white sputum, evening fever and night sweats right after finishing the HCV treatment with sofosbuvir/ledipasvir plus ribavirin for 12 wk.

### Differential diagnosis

Respiratory infection, side effects of HCV treatment.

### Laboratory diagnosis

The blood test highlighted a sodium level of 129 mmol/L (RR: 135-145), ALT 38 U/L, CRP 94 mg/L (RR: < 5), hemoglobin level 9.6 g/dL, leucocyte count 5.98 cells/ $\mu$ L, absolute neutrophil count 4.84 cells/ $\mu$ L, absolute lymphocyte count 0.78 cells/ $\mu$ L, platelet count 114000 cells/ $\mu$ L and INR 1.26 with the rest of parameters standing within the normal range. The arterial gasometry showed pH 7.51 (RR: 7.35-7.45), pO<sub>2</sub> 126 mmHg (RR: 83-108), pCO<sub>2</sub> 29 mmHg (RR: 35-45), lactate 1.6 mmol/L (RR: 0.6-1.17) and bicarbonate 25 mEq/L (RR: 20-29).

### Imaging diagnosis

The chest X-ray showed a left hilar widening. The chest computed tomography scan revealed a superior mediastinal widening secondary to numerous

lymphadenopathies with extensive necrosis, causing a displacement of the esophagus and trachea, and contiguous to these lymphadenopathies, a left hilar mass displaying an air bubble communicated with the left bronchial tree, as well as a bilateral diffuse lung miliary pattern with little subsequent bilateral pleural effusion.

### Treatment

The first line treatment for tuberculosis with rifampin, isoniazid, pyrazinamide and ethambutol.

### Related reports

Cases of tuberculosis infection have been identified with  $\alpha$ -interferon-based therapy and the triple therapy with pegylated-interferon, ribavirin and boceprevir or telaprevir.

### Experiences and lessons

This is the first case of a TB infection during treatment with sofosbuvir/ledipasvir plus ribavirin.

### Peer-review

The manuscript is reasonably well written and is thought to have useful information for readers.

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*WJH* covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## Is laparoscopic hepatectomy superior to open hepatectomy for hepatocellular carcinoma?

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### Abstract

The low perioperative morbidity and shorter hospital stay associated with laparoscopic hepatectomy have made it an often-used option at many liver centers, despite the fact that many patients with hepatocellular carcinoma have cirrhosis, which makes the procedure more difficult and dangerous. Type of surgical procedure proves not to be a primary risk factor for poor outcomes after hepatic resection for hepatocellular carcinoma, the available evidence clearly shows that laparoscopic hepatectomy is an effective alternative to the open procedure for patients with early-stage hepatocellular carcinoma, even in the presence of cirrhosis. Whether the same is true for patients with intermediate or advanced disease is less clear, since laparoscopic major hepatectomy remains a technically demanding procedure.

**Key words:** Hepatocellular carcinoma; Laparoscopic hepatectomy; Open hepatectomy

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**Core tip:** Type of surgical procedure proves not to be a primary risk factor for poor outcomes after hepatic resection for hepatocellular carcinoma, the available evidence clearly shows that laparoscopic hepatectomy is an effective alternative to the open procedure for patients with early-stage hepatocellular carcinoma, even in the presence of cirrhosis.

Zhong JH, Peng NF, Gu JH, Zheng MH, Li LQ. Is laparoscopic hepatectomy superior to open hepatectomy for hepatocellular carcinoma? *World J Hepatol* 2017; 9(4): 167-170 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i4/167.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i4.167>



**Table 1** Propensity score studies comparing open and laparoscopic liver resection for hepatocellular carcinoma

Ref.	Country	Included period	Open/laparoscopic					P value	
			Sample size, n	Minor hepatectomy, %	Single tumor, %	Perioperative morbidity, %, P value	Perioperative mortality, %, P value	Overall survival	Disease free survival
Ahn <i>et al</i> <sup>[12]</sup>	South Korea	2005-2013	51/51	94/96	100/100	9.8/5.9, 0.470	0/0, 1.000	0.173	0.519
Cheung <i>et al</i> <sup>[11]</sup>	China	2002-2015	330/110	88/90	89/91	4.8/1.8, 0.266 <sup>1</sup>	1.8/0, 0.342	0.033	0.141
Han <i>et al</i> <sup>[13]</sup>	South Korea	2004-2013	88/88	68/65	80/76	20.4/12.5, 0.042	1.1/1.1, 1.000	0.944	0.944
Han <i>et al</i> <sup>[14]</sup>	South Korea	2002-2012	198/99	85/84	87/93	24.7/13.1, 0.020	-	0.086	0.701
Kim <i>et al</i> <sup>[15]</sup>	South Korea	2000-2012	29/29	100/100	83/97	13.8/37.9, 0.018	-	0.267	0.929
Meguro <i>et al</i> <sup>[17]</sup>	Japan	2003-2011	35/35	-	83/80	25.7/25.7, 1.000	-	0.672	0.954
Sposito <i>et al</i> <sup>[20]</sup>	Italy	2006-2013	43/43	100/100	81/86	48.8/18.6, 0.004	0/0, 1.000	0.802	0.990
Takahara <i>et al</i> <sup>[18]</sup>	Japan	2000-2010	387/387	79/77	-	13.0/6.7, 0.003	1.0/0.3, 0.178	0.358	0.422
Tanaka <i>et al</i> <sup>[19]</sup>	Japan	2007-2014	20/20	-	85/90	45.0/0, 0.001	0/0, 1.000	0.606	0.533
Yoon <i>et al</i> <sup>[16]</sup>	South Korea	2007-2011	174/58	88/93	100/100	22.4/6.9, 0.020	-	0.480	0.31

<sup>1</sup>With complication of Clavien-Dindo grade IIIA or above.

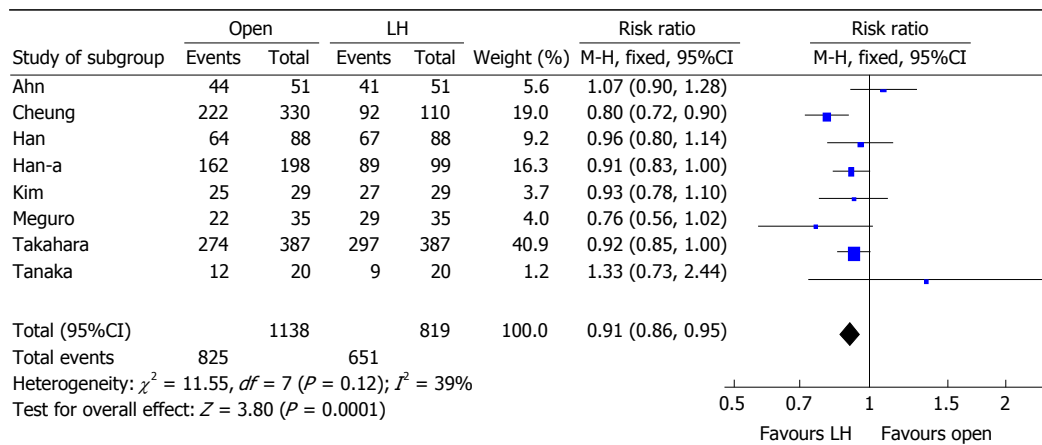
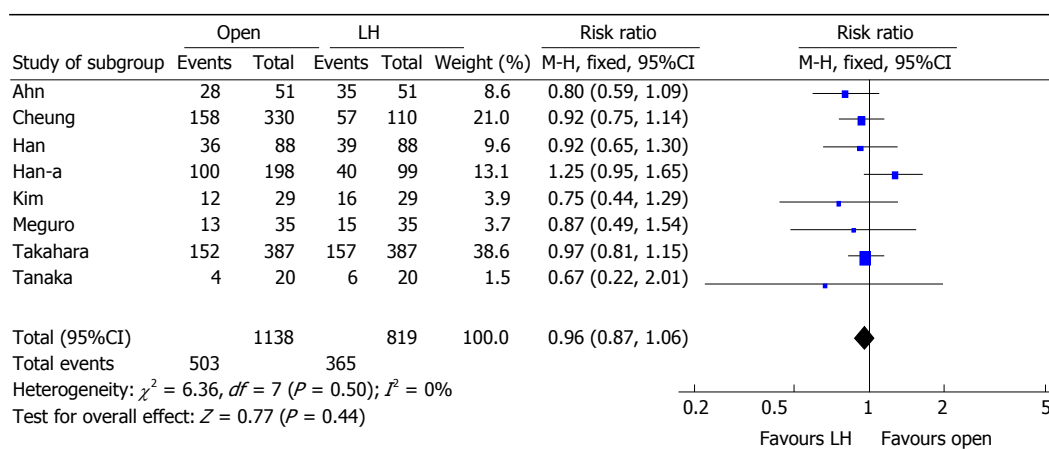
Recently, a large propensity score study comparing laparoscopic and open hepatectomy for treating hepatocellular carcinoma (HCC) was published in *Ann Surg*<sup>[1]</sup>. This parallel comparison comes at an important time, because technical and procedural improvements have led to increasing use of laparoscopic hepatectomy, including for more extensive hepatectomy and particularly in cases of left lateral sectionectomy<sup>[2]</sup>. In fact, the low perioperative morbidity and shorter hospital stay associated with laparoscopic hepatectomy have made it an often-used option at many liver centers<sup>[3-8]</sup>, despite the fact that many patients with HCC have cirrhosis, which makes the procedure more difficult and dangerous. The long-term benefits of laparoscopic hepatectomy remain controversial, and this study<sup>[1]</sup> provides the first evidence that it is associated with better long-term overall survival (OS) than open hepatectomy ( $P = 0.033$ ).

Our own clinical experience and evidence in the literature suggest that mortality risk following liver resection depends primarily not on the type of surgical procedure but on tumor-related factors<sup>[9-11]</sup>. In order to examine this possibility in more detail, we reviewed all randomized controlled trials and other studies involving propensity score analysis comparing laparoscopic and open hepatectomy published in 2014-2016. We identified 10 studies involving 2275 patients, comprising one from China<sup>[1]</sup>, five from South Korea<sup>[12-16]</sup>, three from Japan<sup>[17-19]</sup>, and one from Italy<sup>[20]</sup> (Table 1). Across these 10 studies, 90% of patients had single tumors and 84% underwent minor hepatectomy. This means that most patients had early-stage HCC and surgical procedures were relatively straight forward. In 7 of 10 studies (accounting for 73% of all patients), laparoscopic hepatectomy was associated with a significantly lower rate of perioperative morbidity. None of the studies found significant differences in perioperative mortality or disease-free survival (DFS) between the laparoscopic and open procedures. Eight of the 10 studies (accounting for 86% of all patients) reported 5-year OS and DFS<sup>[1,12-15,17-19]</sup>. Meta-analyses based on these eight studies revealed that patients in the laparoscopic group had significantly higher 5-year OS than those in the open group [risk ratio (RR) = 0.91,

95% confidence interval (95%CI): 0.86-0.95,  $P < 0.001$ ;  $I^2 = 39\%$ ; Figure 1A], but similar 5-year DFS (RR = 0.96, 95%CI: 0.87-1.06,  $P = 0.440$ ;  $I^2 = 0\%$ ; Figure 1B). Similar results were obtained when the study by Cheung *et al*<sup>[11]</sup> was excluded.

Thus, substantial evidence suggests that laparoscopic hepatectomy is associated with significantly better long-term OS than open hepatectomy. It is possible that this reflects less tissue manipulation - and therefore less hematogenous dissemination of malignant tumor cells - in "no-touch" anterior-approach laparoscopic hepatectomy<sup>[1]</sup>. However, the two techniques were associated with similar DFS, indicating similar rates of tumor recurrence, which is the main cause of death among HCC patients. In fact, patients in the two groups across all 10 studies showed similar tumor characteristics, including diameter, number, vascular invasion, and New Edmondson grade. Since these characteristics are the main risk factors of tumor recurrence, the available evidence appears to be consistent with the idea that mortality risk following liver resection depends on tumor-related factors and not on type of surgical procedure.

To examine this hypothesis rigorously, at least two questions must be answered. One is whether differences in blood loss and surgical complexity may help explain the difference in OS. Six of the 10 studies<sup>[1,13,16-19]</sup> reported significantly less blood loss in the laparoscopic group, yet the studies did not report whether tumors were close to the hepatic vein or portal hepatis, which would make the surgery more complex and increase risk of blood loss. Another question is whether economic differences may help explain the OS difference. Since laparoscopic hepatectomy costs substantially more than open hepatectomy, it stands to reason that patients opting for the laparoscopic procedure may be in a better financial position. This raises the possibility that such patients also receive better postoperative therapies, such as antiviral therapy, liver-protecting therapy, and/or psychological intervention. Such patients may also receive more extensive and/or more aggressive therapy after tumor recurrence. All these factors may explain the observed long-term OS advantage of laparoscopic hepatectomy

**A****B**

**Figure 1 Forest plots of meta-analysis comparing the efficacy of laparoscopic with open hepatectomy. A:** Rate of 5-year overall survival; **B:** Rate of 5-year disease-free survival. LH: Laparoscopic hepatectomy.

over open hepatectomy. Therefore, assessing the long-term impact of this procedure requires large randomized controlled trials that take surgical complexity and patient financial condition into account. At least, comparative studies with propensity score analysis should adjust surgical complexity and financial condition between groups.

Even if, as we suspect, type of surgical procedure proves not to be a primary risk factor for poor outcomes after resection, the available evidence clearly shows that laparoscopic hepatectomy is an effective alternative to the open procedure for patients with early-stage HCC, even in the presence of cirrhosis. Whether the same is true for patients with intermediate or advanced disease is less clear, since laparoscopic major hepatectomy remains a technically demanding procedure. Even so, we agree that laparoscopic hepatectomy is an alternative choice for treatment of HCC.

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## Hepatic Kaposi sarcoma: A case report and review of the literature

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### Abstract

Kaposi sarcoma (KS) is an aggressive cancer caused by human herpesvirus-8, primarily seen in immunocompromised patients. As opposed to the well-described cutaneous manifestations and pulmonary complications of KS, hepatic KS is rarely reported before death as most patients with hepatic KS do not manifest symptoms or evidence of liver injury. In patients with acquired immune deficiency syndrome, hepatic involvement of KS is present in 12%-24% of the population on incidental imaging and in approximately 35% of patients with cutaneous KS if an autopsy was completed after their death. Patients with clinically significant hepatic injury due to hepatic KS usually have an aggressive course of disease with hepatic failure often progressing to multi-organ failure and death. Here we report an unusual presentation of acute liver injury due to hepatic KS and briefly review the published literature on hepatic KS.

**Key words:** Herpesvirus 8; Acquired immune deficiency syndrome-related Kaposi sarcoma; Acquired immune deficiency syndrome hepatopathy; Human; Kaposi sarcoma

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**Core tip:** Hepatic Kaposi sarcoma (KS) is a clinical presentation that disproportionately affects the human immunodeficiency virus/acquired immune deficiency syndrome (AIDS) population. Up to 34% of patients with AIDS and KS have hepatic involvement. Usually hepatic KS is clinically indolent and diagnosed during autopsy. When clinically significant, hepatic KS presents with evidence of liver injury with elevation in bilirubin and liver enzymes, has characteristic findings on imaging and may progress to liver failure and death. Treatment is indicated in patients with progressive and symptomatic hepatic disease in the absence of other etiologies.



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## CASE PRESENTATION

A forty-eight years old African American male with HIV and CD4 count of 8/ $\mu$ L presented with conjunctival icterus. Physical exam showed cachexia, icterus, a violaceous 1 cm plaque on the soft palate and similar lesion on the chest wall, and a soft, non-distended, non-tender abdomen. He denied prior treatment with antiretroviral medications. Laboratory studies were significant for AST (SPGT) 172 U/L, ALT (SGOT) 201 U/L, total bilirubin of 20.0 mg/dL, direct bilirubin 14.9 mg/dL, alkaline phosphatase 947 U/L, INR 2.5, and platelet count 52000/ $\mu$ L. Acute and chronic serologies for hepatitis A, B and C, histoplasma, and cytomegalovirus as well as a toxicology screen were negative. Patient did report taking cotrimoxazole for five days which he completed a month prior to presentation. Magnetic resonance imaging (MRI) of the Abdomen showed innumerable 10 mm T2 intense hepatic nodules without enhancement (Figure 1). Liver biopsy was positive for Cytokeratin 7 and human herpes virus-8, consistent with infiltrative Kaposi sarcoma (KS) (Figures 2 and 3). There was no evidence of drug induced liver injury on histopathology. There was no lymphadenopathy indicative of hemophagocytic lymphohistiocytosis or Castleman's disease. The diagnosis was most consistent with acute liver injury (ALI) secondary to infiltrative hepatic KS, stage T1, I1, S1. The patient was not a candidate for cytotoxic therapy given progressive liver injury and was started on rituximab and ganciclovir. Liver injury progressed and was further complicated by acute kidney injury, hypoxic respiratory failure, consumptive coagulopathy and septic shock. The patient received broad-spectrum antibiotics, blood products, vasopressors and ventilator support but unfortunately expired.

## BACKGROUND

KS is an angioproliferative low-grade neoplasm that is associated with human herpesvirus-8 (HHV-8). KS can be codified into different clinical variants depending on the patient cohort and the presentation of the disease<sup>[1]</sup>. The "classical" form primarily affects men of Ashkenazic Jewish or Mediterranean background and follows an indolent cutaneous course. The "African endemic" form of the disease commonly affects Africans as the name implies, presents with lymphadenopathy and is usually fatal within 1-3 years. The "iatrogenic" form is due to HHV-8 activation caused by medical immunosuppression from treatment of autoimmune disorders or post-organ transplantation. The fourth and most common variant, acquired immune deficiency syndrome (AIDS)-related KS,

is rapidly progressive and holds the highest rate of hepatic involvement<sup>[2]</sup>.

The most common presentation of KS is a cutaneous papular disease with lesions on the legs, oral cavity, and genitalia. However, the most common site of visceral organ involvement is the gastrointestinal tract<sup>[3]</sup>. First described by Moritz Kaposi in 1872, hepatic KS was an autopsy diagnosis that rarely resulted in clinically significant disease or ALI<sup>[4]</sup>. To further understand hepatic KS, a systematic search of the literature was conducted on PubMed (1954 to 2015), EBSCO HOST (1956 to 2015), the Cochrane Database of Systematic Review, and the OVID interface (1946 to 2015) with comprehensive search terms as documented in Table 1.

## EPIDEMIOLOGY

While most herpesviruses are widespread in the adult population, the prevalence of HHV-8 varies with human immunodeficiency virus (HIV) status and exposure risk factors. In the United States, only 5% of HIV uninfected men are seropositive for HHV-8 compared to 25%-60% of HIV-positive men who have sex with men (MSM)<sup>[5]</sup>. These rates are reflective of HHV-8 and HIV co-infection in MSM in other countries as well<sup>[6]</sup>.

AIDS-patients have 20000 times greater risk of developing KS than the general population. Patients on HAART with a CD4 count of < 200 cells/ $\mu$ L are 18.9 times more likely to have KS than those with CD4  $\geq$  500 cells/ $\mu$ L<sup>[7]</sup>. In the era of ARV therapy, improved control of HIV viremia and preserved CD4 T-cell function has lead to an 80% decreased incidence of AIDS-associated KS<sup>[8]</sup>, AIDS-related KS currently affects < 1% of AIDS patients, compared to 15% in the pre-HAART era<sup>[9]</sup>.

## MODES OF TRANSMISSION

Behavioral risk factors for HHV-8 transmission are incompletely understood. Saliva exchange appears to be an important factor with HHV-8 DNA detected in the saliva of 61% of HHV-8-infected MSM<sup>[10,11]</sup>. With HHV-8 seropositivity higher in the MSM population, commercial sex workers and those with other sexually transmitted infections, a sexual route of transmission has also been proposed. HHV-8 DNA can be isolated from semen and vaginal secretions, but viral load is lower than that found in saliva, calling into question the clinical significance of sexual transmission<sup>[12]</sup>. HHV-8 seroprevalence in HIV-infected injection drug users is substantially lower than hepatitis B and C rates in HIV-infected MSM<sup>[13]</sup>. This finding is suggestive that blood exchange through contaminated needle sharing is a less significant route of HHV-8 transmission compared to salivary or sexual contact<sup>[14]</sup>.

## HEPATIC INVOLVEMENT

Hepatic KS is typically asymptomatic and rarely diagnosed in life. Therefore the true incidence of hepatic KS is not

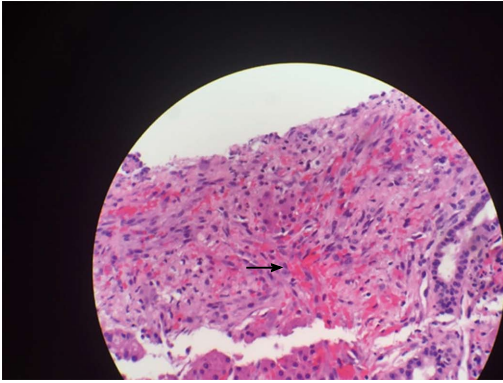


Figure 1 Spindle cells with cytokeratin 7 staining positive.

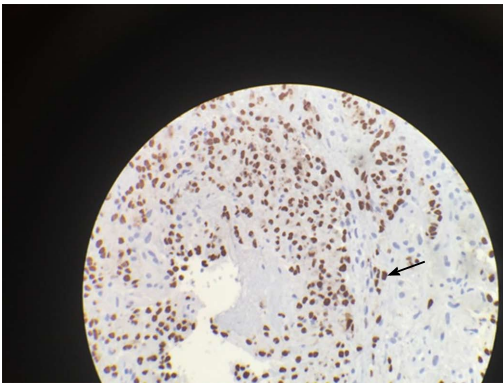


Figure 2 Positive human herpesvirus-8 immunohistochemical staining.

well-documented and is limited to small case series and reports. Prevalence of hepatic KS has primarily been determined from autopsy series with small sample sizes, which accounts for the wide variation in prevalence reported. In one autopsy series, approximately 34% of AIDS-related KS cases involved the liver while in another report, 8.3% had liver involvement<sup>[15,16]</sup>. In another retrospective review, hepatic involvement was present in 9 of 41 patients or 22% of cases of AIDS-related KS in post mortem dissection<sup>[17]</sup>. In this study ante-mortem hepatic KS was suspected in only one patient, in whom a computerized tomography (CT) scan demonstrated hepatosplenomegaly with a confluence of hypodense lesions in the left hepatic lobe. Autopsy confirmed disseminated KS. Schneiderman *et al.*<sup>[18]</sup> found KS on liver biopsy in 18.6% of AIDS patients making KS the most common hepatic pathological diagnosis caused by AIDS. All of these patients already had a diagnosis of extrahepatic KS at time of biopsy. In contrast, 66% of patients with extrahepatic KS did not manifest hepatic involvement.

As mentioned above, most cases of hepatic KS were not clinically significant. In the study by Schneiderman *et al.*<sup>[18]</sup>, there were no statistically significant differences in transaminases, lactic dehydrogenase, alkaline phosphatase or bilirubin based on liver involvement with KS. However, in the few reported patients with clinically significant disease, a rapid progression to liver and multi-organ failure has

Table 1 MeSH search terms

Liver
Hepatopathy
Hepatitis
Hepatology
Cholestatic injury
Hepatocellular injury
Kaposi sarcoma
Herpesvirus 8, human
AIDS-related Kaposi sarcoma
Non-AIDS-related Kaposi sarcoma
Liver neoplasms

AIDS: Acquired immune deficiency syndrome.

been reported, usually with fatal outcomes (Table 2).

In the non-HIV population, the incidence of KS is 0.2% in liver transplant patients from the United States, but the prevalence is higher in patients from Africa, the Middle East or the Mediterranean<sup>[19]</sup>. KS affected 4.7% of renal transplant patients in Saudi Arabia, 2.4% of recipients in Israel and 0.52% of recipients in France. While there is a well described clinical burden of post-transplant lymphoproliferative disorder including cutaneous and visceral manifestations of KS, there is no described literature of post-transplant hepatic KS.

## PATHOGENESIS OF HHV-8 ASSOCIATED TUMORS

HHV-8 consists of a large double stranded DNA genome that includes approximately 145 kilobases (kbases) long region encoding all the expressed viral genes, flanked by approximately 20-30 kbases that encode a number of mimicked human genes, several of which have immunologic or angiogenic properties<sup>[20,21]</sup>. HHV-8 has a tropism for both hematopoietic and non-hematopoietic cells including monocytes, B cells, endothelial cells and also hepatocytes<sup>[22]</sup>. Endothelial cells appear to be the most important host cells for oncogenic transformation as HHV-8 infection of these cells leads to their long-term proliferation and survival<sup>[23]</sup>. Similar to other herpesviruses, HHV-8 alternates between two metabolic cycles: Latent infection, where few genes are expressed, and the active lytic infection, where viral replication and multiple gene expression occurs. Lytic replication can be induced by oxidative stress, hypoxia, inflammatory cytokines, chemical exposure or concomitant infections, including HIV<sup>[24-27]</sup>.

In hepatocytes infected with HHV8 genome by DNA polymerase chain reaction amplification, immunohistochemistry demonstrates expression of the transcriptional regulator, latency-associated nuclear antigen-1 (LANA-1)<sup>[28]</sup>. It has also been directly implicated in oncogenesis because of its ability to bind to the tumour-suppressing protein p53<sup>[29]</sup>. Furthermore, hepatocyte growth factor/scatter factor, a kinase that mediates epithelial cell proliferation and angiogenesis<sup>[30]</sup> has been demonstrated to induce HHV-8 lytic replication, providing a means of KS pro-

**Table 2 Outcomes in patients with clinically symptomatic hepatic Kaposi sarcoma**

Age (yr)	Sex	HIV status	CD4 count (cells/mm <sup>3</sup> )	Liver chemistry profile	Pathology	Treatment	Hospital course + complications
45 <sup>[63]</sup>	M	(+)	192	T Bil 19.35 ALP 1309 AST 204 ALT 188 GGT 827	HHV-8 PCR VL (+) 24000 copies/mL. Liver biopsy revealed features of KS with spindle cells, extravasation of red blood cells and haemosiderin deposition. IHC staining HHV8 (+)	Paclitaxel, Montelukast	Continued on chemotherapy. Subsequently developed respiratory and renal failure, anemia and thrombocytopenia from aggressive metastatic KS
36 <sup>[64]</sup>	M	(+)	17	PTT 70 (s) ALT 185 T Bil 23	Necroscopy showed bile duct proliferation with diffuse fibrosis with lymphohistiocytic infiltration	Liposomal doxorubicin	Jaundice, renal failure, fulminant liver failure
28 <sup>[65]</sup>	M	(+)	NR	NR	Biopsy residues of spindle cells lining portal tracts. Immunoperoxidase staining factor VIII (+)	Palliative care	Liver function continued to decline and patient died from respiratory failure two weeks later
38 <sup>[66]</sup>	M	(+)	< 200 <sup>1</sup>	AST 147 ALT 180 ALP 573	Gross specimen with fibrous thickening of portal tracts and dark red nodules in periportal areas and diffusely infiltrating liver parenchyma	Chemotherapy, NOS	Partial cutaneous response, died several weeks later
40 <sup>[4]</sup>	M	(+)	NR	Reportedly, "normal"	KS present on biopsy of lymph nodes. US with three 7-12 mm hyperechoic nodules. Periportal groups of dilated blood filled cavernous spaces lined by flat endothelial cells and interspersed of spindle cells. Extravasated erythrocytes and minimal hemosiderin deposits	Combination Chemotherapy, NOS	Complete remission of cutaneous lesions and reduction in size of two of the lesions with the third not visible. Readmitted six months later for severe relapse of cutaneous KS. Reinitiated chemotherapy with rapid deterioration and death within one month
48 <sup>[67]</sup>	M	(+)	8	TBili 20.0 ALP 947 AST 186 ALT 155 INR 1.9	Liver biopsy was Cytokeratin-7 and HHV-8 staining positive	Ganciclovir and Rituximab	Presented with jaundice and acute liver injury with a cholestatic pattern, progressed to fulminant hepatic failure and ultimately death
44 <sup>[68]</sup>	M	(+)	CD4/CD8 ratio 0.08	AST 153 ALT 124 ALP 1228	Laproscopy demonstrated enlarged liver with multiple purple 2-3 mm nodules. Biopsy demonstrated spindle cells, vascular slits, extravasated red cells and lymphocytic infiltration	Platinum based chemotherapy, NOS	Primary hepatic manifestations without cutaneous lesions. Persistent abdominal pain after treatment. Progressed to cutaneous lesions six weeks after treatment. Lost to follow-up

<sup>1</sup>Less than 200, not otherwise reported. M: Male; F: Female; TBili: Total Bilirubin (units, mg/dL); ALP: Alkaline phosphatase (units, IU/L); AST: Aspartate transaminase (IU/L); ALT: Alanine transaminase (IU/L); GGT: Gamma glutamyl transpeptidase (units, IU/L); HHV-8: Human herpes virus-8; PCR: Polymerase chain reaction; VL: Viral load; IHC: Immunohistochemistry; NR: Not reported; NOS: Not otherwise specified; US: Ultrasound; KS: Kaposi sarcoma.

gression in the liver<sup>[31]</sup>.

## DIAGNOSIS OF KS

A full integumentary survey including oral and rectal examination quantifying extent of disease should be completed in patients with suspected KS. Cutaneous or visceral biopsy is required for diagnosis. On gross pathology, there are usually multiple, grossly irregular, variable sized red-brown spongiform nodules seen in the periportal connective tissue<sup>[32]</sup>. Histopathologic features of disease include thin walled vascular formations and inflammatory infiltration. Spindle cell formation is also characteristic of angioproliferative HHV-8-infected cells that have undergone reprogramming of vascular endothelium and tumorigenesis<sup>[33]</sup>. Immunohistochemical staining is characteristic for HHV-8 LANA expression within the spindle cell formation<sup>[34]</sup>. Immunohistochemistry staining for endothelial cell markers factor VIII, CD31, CD34 and lymphatic vessel endothelial receptor 1 further corroborate a diagnosis of KS<sup>[35]</sup>.

Further investigation of visceral KS is warranted in the presence of adenopathy or occult bleeding. Patients

with cutaneous KS and iron deficiency anemia, fecal occult blood or gastrointestinal symptoms warrant GI endoscopic evaluation. Patients with cutaneous KS and concurrent adenopathy should receive CT of the chest, abdomen, and pelvis to evaluate for visceral KS and HHV-8-related lymphoproliferative disorders including primary effusion lymphoma, Castleman disease, and plasmablastic lymphoma<sup>[36]</sup>.

## KS STAGING AND PROGNOSIS

As KS is a disseminated angioproliferative virally mediated malignancy, classic tumor, node, metastasis staging as used in other cancers does not accurately prognosticate disease or dictate treatment. AIDS Clinical Trials Group (ACTG) Oncology Committee has codified staging of AIDS-associated KS<sup>[37]</sup>. The ACTG staging system risk stratifies patients low risk (0) or high risk (1) based on three criteria: Tumor burden (T), immune status (I), and systemic illness (S). For tumor burden, poor risk (T1) is defined by presence of extensive cutaneous, oral disease or visceral disease. For immune status, poor risk (I1) is defined by CD4 count of less than 150 cells/ $\mu$ L. For

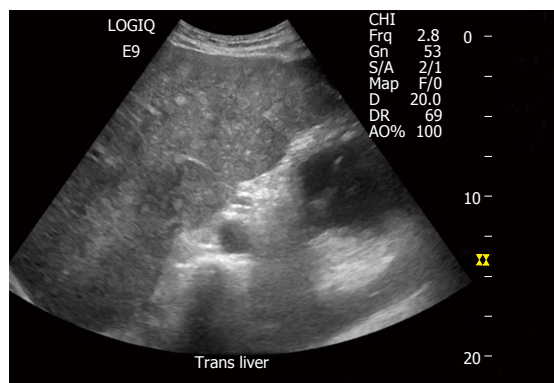


Figure 3 Ultrasound image with multiple small round hyperechoic nodules.

systemic illness, poor risk (S1) is defined by the presence of constitutional symptoms, poor performance status, or other opportunistic infections. While these criteria were validated in the pre-ART era, post-ART therapy, a CD4 cutoff of 100 cells/mm<sup>3</sup> has an unclear role in predicting mortality<sup>[38,39]</sup>.

## IMAGING IN HEPATIC KS

Hepatic KS has characteristic findings on individual imaging modalities that can help delineate clinically significant disease. Abdominal ultrasound imaging of the liver can demonstrate inhomogeneous cystic lesions with hyperechoic bands and nodules along the peripheral branches of portal veins. Likewise, computed tomography of the abdomen is characteristic for inhomogeneous hepatomegaly with multiple small hypodense nodules, often in the periportal area<sup>[40]</sup> (Figure 4). Mild hepatomegaly is a non-specific finding in 19% of patients with AIDS-related KS<sup>[41]</sup>. MRI shows hyperintense nodules on T1-weighted in-phase imaging and hypointense nodules on T1-weighted out-of-phase imaging (Figure 5). Neither T2-weighted imaging nor late hepatobiliary volumetric interpolated breath hold examination have any specific findings in hepatic KS<sup>[42]</sup>.

Image guided biopsy of hepatic nodules in patients suspected to have liver involvement demonstrate hyaline globules, hemosiderin accumulation, macrovacuolar steatosis, large fibrotic portal spaces, bile duct ectasia, neoductogenesis and spindle cells with large, irregular nuclei. Staining of the perinodular tissues is positive for CD31, CD34 and factor VIII as can be seen in extrahepatic KS as well.

## TREATMENT

As shown in Table 2, hepatic KS is predominantly manifested in patients with HIV/AIDS. While overall HIV mortality is improving in the era of ARV therapy, patients with AIDS-associated KS have an increased risk of death, compared to HIV controls, irrespective of CD4 count<sup>[43]</sup>. HIV-infected patients initiating ARV commonly have progression of their KS lesions<sup>[44]</sup>. However, long term



Figure 4 Computerized tomography scan enlarged inhomogeneous liver with multiple hypodense lesions.

ARV therapy is associated with a reduced incidence of KS. Guidelines currently recommend correcting underlying immunodeficiency by treating AIDS with ARV therapy. Studies indicate that control of KS progression is related to the degree of control of HIV, rather than the specific cART regimen utilized<sup>[45]</sup>. Beyond ARVs, a variety of systemic therapies may be used in KS. Usually systemic therapy is indicated in progressive disease, with symptomatic visceral involvement, in the presence of immune reconstitution inflammatory syndrome (IRIS) or with extensive cutaneous involvement. These strategies are not specific to hepatic dysfunction in the setting of KS.

Radiotherapy is a well-established treatment and has a robust clinical response for classic nodular KS but tends to be a palliative approach. While it may be a good modality for superficial lesions, electron beam radiation therapy (EBRT) has limited penetration below the dermis; deeper or unresponsive KS may be treated with standard non-EBRT approaches<sup>[46]</sup>.

Retinoid products appear to inhibit IL-6, a cytokine implicated in KS pathogenesis, and have an antiproliferative effect on KS lesions<sup>[47]</sup>. Application of alitretinoin can reduce cutaneous lesions of both classic and HIV-KS but has no role in systemic disease<sup>[48]</sup>.

The role of chemotherapy in addition to standard antiretroviral therapy has been explored. A meta-analysis of studies demonstrated that although chemotherapy in addition to ARVs did not have a mortality benefit, it did reduce disease progression<sup>[49]</sup>. Current first-line therapy for advanced AIDS-KS is liposomal anthracyclines, including pegylated liposomal doxorubicin (PLD). In a randomized control trial (RCT), PLD demonstrated superiority to previous conventional chemotherapy, bleomycin and vincristine with 58.7% vs 23.3% ( $P < 0.001$ ) response rate and a decreased adverse event rate (10.7% vs 26.7%)<sup>[50]</sup>. Another RCT of liposomal daunorubicin versus doxorubicin, bleomycin and vincristine showed no statistical difference in response rate or disease progression (25% vs 28%)<sup>[51]</sup>. When the analysis was restricted to patients receiving prior zidovudine, however, survival was improved in the liposomal daunorubicin group. Another non-randomized study showed a trend



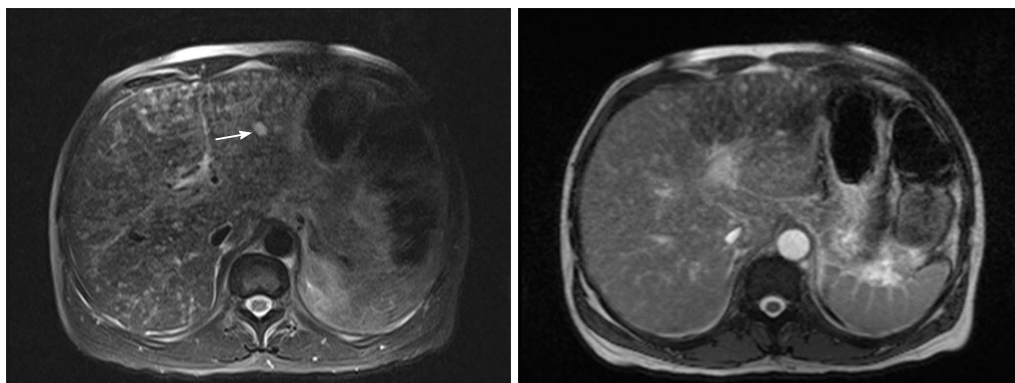


Figure 5 SPAIR and T2 images Kaposi sarcoma on magnetic resonance imaging.

toward mortality benefit with liposomal doxorubicin as compared to bleomycin plus vinblastine, vincristine or ARV monotherapy alone, although this did not reach statistical significance<sup>[52]</sup>.

Interferon-alpha, has an array of antiviral and antiangiogenic properties with efficacy in AIDS-KS, but its use is limited due to hepatotoxicity<sup>[48]</sup>.

Paclitaxel has systemic response rates from 59%-71% and is approved as second-line treatment for KS. In randomized controls, paclitaxel does not demonstrate benefit over PLD in complete or partial remission and no mortality data were available according to KS staging<sup>[53]</sup>. Less well tolerated than doxorubicin, adverse events include peripheral neuropathies, cytopenias, and gastrointestinal upset. Third line agents for AIDS related visceral KS include etoposide, bleomycin, vinblastine, and vincristine with overall response rates ranging from 23% to 36%. The median survival times are 11 (6 to 20) mo in the bleomycin only group and 13 (7 to 36) mo in the ABV group. With extensive side effect profiles, including secondary malignancies, these treatment modalities are maintained in resource-limited settings<sup>[54,55]</sup>.

Although HAART with or without chemotherapy is the current recommended treatment, novel targets are being explored including inhibitors of angiogenesis and matrix metalloproteinases. These drugs are currently in various phases of clinical trials<sup>[56]</sup>. Inhibition of HHV-8 replication with agents such as foscarnet and ganciclovir have also been explored<sup>[57]</sup>.

Finally, it bears mentioning that treatment for HIV/AIDS in patients co-infected with HHV-8 can cause a paradoxical worsening of disease. In the KS AIDS AntiRetroviral Therapy Trial, 23/112 (21%) of co-infected patients receiving ARV therapy developed KS-IRIS, which was defined as a rapid worsening of KS beyond its natural course within 12 wk of initiating ARV therapy. Of those 23 patients, 10 died, 9 of which had visceral KS. Eighteen patients in the study overall (16%) had worsening elevation in their liver enzymes and two patients (1.8%) died of liver failure. In this study, exclusion criteria included HIV-KS patients with direct serum bilirubin > 85  $\mu\text{mol/L}$  or aspartate aminotransferase or alanine aminotransferase > 2.5

times the normal range<sup>[39]</sup>.

Biologic and targeted molecular therapies may have a supplementary or alternative role in AIDS-KS, but are currently in early stages of clinical trials. In the AIDS Malignancy Consortium, a phase II trial of imatinib with a small sample size showed a partial response in approximately one third of patients<sup>[58]</sup>. In another study focusing on patients who did not respond to chemotherapy and chimeric antigen receptor T cell therapy, bevacizumab, an anti-vascular endothelial growth-factor monoclonal antibody, had a response rate in again approximately one third of patients<sup>[59]</sup>. The cytokine, interleukin-12 had a response rate of 71% in small phase I and phase II trials. However, patients were ineligible if they had transaminitis or a history of hepatic disease<sup>[60]</sup>. Ongoing studies include a phase II trial for the utility of combined PLD and bevacizumab in the treatment of advanced AIDS-KS<sup>[61]</sup> and a phase I study for dosing and side effect profile of combination therapy with ipilimumab, a cytotoxic T-lymphocyte antigen 4 antibody and nivolumab, an antibody against programmed cell death 1 for the treatment of advanced KS solid tumors<sup>[62]</sup>. These trials show that biologic and molecular therapies may have a role in the future as alternative treatment therapy for some patients with AIDS-KS.

Currently, HHV-8 infection cannot be eradicated but long-term remission is feasible. Treatment is indicated in patients with progressive hepatic disease in the absence of other etiologies.

## CONCLUSION

Hepatic KS is a clinical presentation that disproportionately affects the HIV/AIDS population. Up to 34% of patients with AIDS and KS have hepatic involvement. It is rarely clinically significant and often diagnosed during autopsy, but can cause liver injury or even fatal liver failure, as demonstrated in the case presentation above and case series in Table 2. In an immune compromised patient with ALI or failure, a thorough skin exam in addition to abdominal imaging and biochemical testing should be pursued and a diagnosis of hepatic KS should be considered. Treatment of hepatic KS does not differ

from systemic treatment of other KS manifestations with HAART and chemotherapy, and should be considered within the context of medical comorbidities and severity of disease. Due to wide population prevalence, a lack of clinically significant disease and variable presentations, there is little clinical data or dedicated clinical trials for liver specific disease, and further investigation is warranted.

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## Interferon-free regimens in patients with hepatitis C infection and renal dysfunction or kidney transplantation

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### Abstract

Treatment of patients with chronic kidney disease (CKD) and chronic hepatitis C (CHC) differs from that used in the general CHC population mostly when glomerular filtration rate (GFR) is below 30 mL/min, as sofosbuvir, the backbone of several current regimens, is officially contraindicated. Given that ribavirin free regimens are preferable in CKD, elbasvir/grazoprevir is offered in CHC patients with genotype 1 or 4 and ombitasvir/paritaprevir and dasabuvir in genotype 1b for 12 wk. Although regimens containing peginterferon with or without ribavirin are officially recommended for patients with CKD and genotype 2, 3, 5, 6, such regimens are rarely used because of their low efficacy and the poor safety and tolerance profile. In this setting, especially in the presence of advanced liver disease, sofosbuvir-based regimens are often used, despite sofosbuvir contraindication. It seems to have good overall safety with only 6% or 3.4% of CKD patients to discontinue therapy or develop serious adverse events without drug discontinuation. In addition, sustained virological response (SVR) rates with sofosbuvir based regimens in CKD patients appear to be comparable with SVR rates in patients with normal renal function. Treatment recommendations for kidney transplant recipients are the same with those for patients with CHC, taking into consideration potential drug-drug interactions and baseline GFR before treatment initiation. This review summarizes recent data on the current management

of CHC in CKD patients highlighting their strengths and weaknesses and determining their usefulness in clinical practice.

**Key words:** Chronic hepatitis C virus infection; Kidney; Renal; Kidney transplantation; Direct acting antiviral agents; Glomerular filtration rate; Hepatitis C

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**Core tip:** Recent evidence showed very good safety and efficacy of both interferon and ribavirin-free direct acting antivirals (DAAs) regimens in patients with severe kidney disease (CKD) or kidney transplantation. Nevertheless, sofosbuvir, the backbone of most antiviral schemes is officially contraindicated in patients with CKD (creatinine clearance < 30 mL/min). Accordingly, CKD patients with genotype 1 or 4 can be currently treated with available ribavirin free DAAs regimens without sofosbuvir, while those with non-1, non-4 genotype can officially be treated with peginterferon with or without ribavirin, but they are actually treated with sofosbuvir-based regimens mostly if they have advanced liver disease.

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## INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection among hemodialysis (HD) patients has been reported to range from 10% to 25%<sup>[1]</sup>. Chronic hepatitis C (CHC) has been related with high morbidity and reduced survival in both patients with renal dysfunction and kidney transplant (KT) recipients<sup>[2]</sup>. HCV treatment in patients with renal dysfunction has been a complex and challenging issue in the pre-direct acting antiviral (DAAs) era. Interferon-alpha (IFN) or pegylated IFN (PEG-IFN) with or without low doses of ribavirin (RBV) (200-400 mg three times weekly) was associated with low rates of sustained virological response (SVR) and several potentially dangerous side effects<sup>[3]</sup> such as steroid resistant acute allograft rejection in KT recipients<sup>[4]</sup>.

In general, the introduction of first generation DAAs (*i.e.*, telaprevir and boceprevir) improved the SVR rates in CHC patients infected with genotype 1 but did not substantially improve the treatment of such patients with renal dysfunction or KT<sup>[5]</sup>. Initially, both telaprevir and boceprevir had to be used in combination with PEG-IFN and RBV resulting in the potential appearance of limitations, worse tolerability and safety profile of both PEG-IFN and RBV. These could account for severe anemia with both drugs, rash and pruritus with telaprevir

and dysgeusia with boceprevir<sup>[5]</sup>. Moreover, glomerular filtration rate (GFR) deterioration was reported to develop in about 5% of CHC patients who received telaprevir- or boceprevir-based therapy, particularly if they had additional risk factors for renal impairment (*e.g.*, arterial hypertension)<sup>[6,7]</sup>.

After 2014, newer DAAs have been licensed for the treatment of CHC by EMA and FDA. They include a nucleotide analogue NS5B polymerase inhibitor, sofosbuvir (tablet of 400 mg, Sovaldi<sup>®</sup>, Gilead)<sup>[8]</sup>, the NS3/4 protease inhibitor, simeprevir (tablet of 150 mg, Olysio<sup>®</sup>, Janssen)<sup>[9]</sup>, the NS5A inhibitor, daclatasvir (tablet of 60 mg, Dankliza<sup>®</sup>, Bristol-Myers Squibb)<sup>[10]</sup>, the co-formulation of a NS5A inhibitor, ledipasvir, with sofosbuvir (tablet of 90/400 mg, Harvoni<sup>®</sup>, Gilead)<sup>[11]</sup>, the co-formulation of a NS5A inhibitor, ombitasvir, with a NS3/4 protease inhibitor, paritaprevir, boosted by ritonavir (r) (tablet of 12.5/75 per 50 mg, Viekirax<sup>®</sup>, Abbvie), with a non-nucleos(t)ide analogue NS5B polymerase inhibitor, dasabuvir (tablet of 250 mg, Exviera<sup>®</sup>, Abbvie)<sup>[12]</sup>, the co-formulation of a NS5A inhibitor, elbasvir, with a NS3/4 protease inhibitor, grazoprevir (tablet of 50/100 mg, Zepatier<sup>®</sup>, Merck)<sup>[13]</sup> and the co-formulation of a NS5A inhibitor, velpatasvir, with sofosbuvir (tablet of 100/400 mg, Epclusa<sup>®</sup>, Gilead)<sup>[14]</sup> (Table 1). IFN-free and often RBV-free combinations of the newer DAAs given for 8-24 wk have been associated with very high (> 95%) SVR rates in most subgroups of CHC patients. Such combinations seem to represent the optimal choice against HCV infection in patients with chronic kidney diseases (CKD) or KT recipients, although its potential effects on renal function in all HCV patients and in HCV patients with renal impairment have just started to be evaluated. All newer DAAs are mainly eliminated through the liver, except for sofosbuvir which is eliminated through the kidney<sup>[15]</sup>. According to licensed summaries of product characteristics, daclatasvir, dasabuvir, ombitasvir/paritaprevir/r and elbasvir/grazoprevir could be administered to patients with any severity of renal impairment. However, sofosbuvir and consequently its co-formulations, ledipasvir/sofosbuvir and velpatasvir/sofosbuvir, should not be used in patients with severe renal impairment [estimated GFR (eGFR) < 30 mL/min per 1.73 m<sup>2</sup>] and/or patients requiring HD. Furthermore, caution is required when simeprevir is offered in patients with severe renal impairment and/or on HD because the knowledge of how it affects kidney function is limited<sup>[15]</sup>.

The purpose of this review is to summarize the most recent data on the impact of the recent IFN-free anti-HCV regimes on kidney function in CHC patients as well as the safety and efficacy of these regimens in CHC patients with CKD and KT recipients.

## IMPACT OF NEW DAAS ON RENAL FUNCTION

### Non transplant setting

Given that sofosbuvir represents the back-bone of many current IFN-free regimens and at the same time it is the

**Table 1** Main characteristics of the approved direct acting antivirals that are currently used for the treatment of hepatitis C

DAA (commercial name), dose	Category	Dose adjustment in renal impairment	Antiviral activity	CNIs co-administration
Sofosbuvir (Sovaldi®), tablet 400 mg, once daily	Nucleotide analogue NS5B polymerase inhibitor	Contraindicated in patients with GFR < 30 mL/min	Genotypes 1-6 High genetic barrier	No change
Simeprevir (Olysio®), tablet 150 mg, once daily with food	NS3/4A protease inhibitor	No change in renal impairment	Genotypes 1,4 Low genetic barrier	Contraindicated with cyclosporine
Daclatasvir (Daklinza®), tablet 60 mg, once daily	NS5A inhibitor	No change in renal impairment	Genotypes 1, 2, 3, 4 Low genetic barrier	No change
Ledipasvir/sofosbuvir (Harvoni®), tablet 90/400 mg, once daily	NS5A inhibitor + nucleotide analogue NS5B polymerase inhibitor	Contraindicated in patients with GFR < 30 mL/min	Genotypes 1, 4, 5, 6 High genetic barrier	No change
Ombitasvir/paritaprevir/ritonavir (Viekirax®), tablet 12.5/75/50 mg, two once daily with food	NS5A inhibitor + NS3/4A protease inhibitor boosted by ritonavir boosted	No change in renal dysfunction	Genotypes 1, 4 Genetic barrier depending on HCV genotype	Cyclosporine: 20% of pretreatment total daily dose; tacrolimus: 0.2 mg/72 h or 0.5 mg once weekly
Dasabuvir (Exviera®), tablet 250 mg, every 12 h	Non-nucleos(t)ide analogue NS5B polymerase inhibitor	No change in renal dysfunction	Genotype 1 Low genetic barrier	
Elbasvir/Grazoprevir (Zepatier®), tablet 100/50 mg, once daily	NS5A inhibitor + NS3/4A inhibitor	No change in renal dysfunction	Genotypes 1,4	Co-administration increases tacrolimus concentrations
Velpatasvir/sofosbuvir (Epclusa®), tablet 100/400 mg, once daily	NS5A inhibitor + nucleotide analogue NS5B polymerase inhibitor	Contraindicated in patients with GFR < 30 mL/min	Genotypes 1-6 High genetic barrier	No change

CNI: Calcineurin inhibitor; DAA: Direct acting antiviral; GFR: Glomerular filtration rate.

only agent with renal elimination, only sofosbuvir based regimens have been evaluated for potential effects on renal function. One study<sup>[16]</sup> assessed the rate of renal impairment in patients treated with sofosbuvir-based regimens comparing it to that of telaprevir or boceprevir based regimens, which have been previously shown to cause renal impairment in 5%-7% of treated CHC patients<sup>[7]</sup>. In total, 442 patients (50% with cirrhosis, > 95% with baseline GFR  $\geq$  60 mL/min)<sup>[16]</sup>. Renal impairment (defined as increase in serum creatinine  $\geq$  50% from baseline) was observed at similar rates in all groups: 7% of 228 patients under boceprevir/telaprevir-based regimens, 5% of 76 patients under sofosbuvir plus PEG-IFN/RBV and 4% of 152 patients under IFN-free sofosbuvir-based regimens ( $P = 0.40$ ), but the on-treatment median creatinine peak was lower in the boceprevir/telaprevir group compared to sofosbuvir containing groups (1.4 mg/dL vs 2.0 mg/dL,  $P = 0.04$ ). In multivariable analysis, only ascites [odds ratio (OR) = 3.16] and preexisting proteinuria (OR = 5.74) were significantly associated with development of renal impairment and SVR did not differ between patients who did or did not develop renal impairment (88% vs 86%,  $P = 0.90$ ). According to the authors, monitoring of renal function and standard nephroprotective measures may be useful when sofosbuvir-based regimens are applied, particularly in patients with ascites or pre-existing kidney disease. This finding was confirmed in a recent study<sup>[17]</sup>, in which 90 patients with HCV infection were treated with sofosbuvir plus ledipasvir: 17 patients had abnormal baseline renal function (GFR < 60 mL/min), while 42% had worsening GFR while on treatment. In multivariate analysis, baseline GFR < 60 mL/min was independently associated with worsening renal function on treatment ( $P$

= 0.04).

On the other hand, HCV infection may have a negative impact on renal function, and thus, HCV eradication could be associated with improvement of GFR. This was shown in a recent study<sup>[18]</sup> including 124 patients treated with DAAs (mean age 53.8 years, 67.7% treatment experienced, 83% had genotype 1 and 41% had cirrhosis). The achievement of SVR was associated with GFR improvement (baseline:  $78.55 \pm 8.96$  vs SVR at week 12:  $81.85 \pm 12.87$  mL/min,  $P = 0.037$ ). Thus, renal function may be improved after effective treatment of HCV infection with DAAs-based regimens. However, caution is still advised if sofosbuvir is administered in patients with renal impairment, as renal function may get worse in addition to more adverse events particularly if RBV is also used in combination.

Another study assessed the potential effect of sofosbuvir-based regimens on renal function in patients with HCV decompensated cirrhosis, who represent a group at high risk for renal dysfunction<sup>[19]</sup>. The on-treatment changes of serum cystatin C, as a marker of glomerular function, and of neutrophil gelatinase-associated lipocalin (NGAL), as a marker of tubular function, were evaluated in 52 patients with Child-Pugh score  $\geq 7$  treated with sofosbuvir and a NS5A inhibitor (ledipasvir or daclatasvir) and RBV for 12 wk. Half of the patients had at least one renal risk factor (e.g., hypertension, diabetes, therapy with diuretics), while 14% of the patients had eGFR < 60 mL/min. The eGFR did not change significantly during antiviral therapy, but cystatin C and NGAL levels increased from baseline to week 4 of therapy (cystatin C: 1.46 mg/L vs 1.55 mg/L,  $P < 0.01$ ; NGAL: 28.1 ng/mL vs 32.8 ng/mL,  $P < 0.01$ ) indicating transient renal dysfunction. Unfortunately, the evolution of these renal markers at

longer follow-up was not provided.

### Transplant setting

The impact of sofosbuvir-based regimens on renal function was assessed in liver transplant (LT) recipients who are at high risk for renal dysfunction for several reasons including the long-term use of calcineurin inhibitors. A recent multicenter study<sup>[20]</sup> evaluated 193 LT recipients with HCV recurrence treated with sofosbuvir-based regimens (mean age  $58.7 \pm 9.0$  years, 30.6% cirrhotics). Renal dysfunction developed in 38% of patients. The presence of a preexisting renal disease (OR = 3.49), the baseline GFR (OR = 1.02) and tacrolimus-based immunosuppressive therapy (OR = 0.43) were all three predictive factors of renal dysfunction development. The same study group<sup>[21]</sup> focused on 20 patients with combined liver-kidney transplantation (cirrhosis 25%, genotype 1 in 70%) who received sofosbuvir-based therapy for HCV recurrence. The authors reported that GFR decreased significantly from baseline value 50.9 mL/min to 41.8 mL/min at week 12 and to 42.7 mL/min at 12 wk after the end of antiviral therapy ( $P$  values always  $\leq 0.0001$ ).

Finally, 165 LT patients with HCV recurrence<sup>[22]</sup> received sofosbuvir-based regimens. A decline in renal function was observed in 22% of patients, particularly in those with baseline eGFR < 30 mL/min ( $P = 0.01$ ), cirrhosis ( $P = 0.01$ ) and prior treatment failure ( $P = 0.03$ ). Similarly to the non-LT setting<sup>[18]</sup>, renal function improvement after treatment was observed in 58% of patients and more commonly in those who achieved SVR, compared to those who did not (81% vs 19%,  $P < 0.05$ ).

## INTERFERON-FREE REGIMENS IN PATIENTS WITH CHC AND CKD

### Interferon-free antiviral schemes approved for CHC and CKD

**Ombitasvir/paritaprevir/dasabuvir based regimens:** The combination of ombitasvir/paritaprevir/r and dasabuvir, which has been abbreviated as 3D regimen, is used with or without the addition of RBV for the treatment of genotype 1a or 1b CHC patients. Moreover, the combination of ombitasvir/paritaprevir/r (2D) with RBV is administered for the treatment of genotype 4 CHC patients. The potential effect of renal impairment on the pharmacokinetics of 3D combination was evaluated in more than 2000 patients from seven phase 2/3 studies<sup>[23]</sup>. The severity of renal dysfunction was not found to affect the area under the plasma concentration curve (AUC) of 3D in 22 patients with GFR between 30 and 59 mL/min and therefore no dose-adjustments are required. However, no patients with end stage renal disease (GFR < 30 mL/min) were included in that initial evaluation. In a smaller study<sup>[24]</sup>, HCV patients with normal or mild renal impairment ( $n = 38$ ), were compared to those with stage 4 or 5 CKD patients (with or without HD) ( $n = 19$ ). During a 12-wk course with the

3D regimen, renal dysfunction did not affect significantly the pharmacokinetics of the 3D regimen. Ombitasvir and paritaprevir exposures were comparable (< 20% difference) in both groups and ritonavir and dasabuvir exposures were 33% and 37% lower, respectively. Thus, the authors concluded that no dose adjustment for the 3D regimen is required in HCV patients with severe renal impairment.

In the RUBY-I study<sup>[25]</sup>, the safety and efficacy of 3D given for 12 wk was evaluated in 20 genotype 1 treatment-naïve non-cirrhotics patients with CHC and CKD stage 4 or 5 (RBV was given at 200 mg/d in genotype 1a patients). Thirteen patients were under HD. The efficacy was high since SVR was achieved in 18 (90%) of 20 patients in the intention to treat analysis: One F3 genotype 1a patient relapsed 4 wk post-treatment, while a second patient died 14 d after the end of therapy due to left ventricular systolic dysfunction. Regarding safety profile, most adverse events were of mild to moderate severity. There were nine serious adverse events in 4 patients (including the patient who died), but none of them was considered to be related with antiviral therapy (including RBV). Four patients received erythropoietin for anemia but none required blood transfusion. No deterioration of liver or kidney function was observed during the study period.

More recently, real life data have been reported from two studies<sup>[26,27]</sup> which evaluated the safety and effectiveness of 3D with or without RBV in 69 CHC patients with stage 4 or 5 CKD (*i.e.*, GFR < 30 mL/min) or under HD. Sixty-five (94.2%) patients had genotype 1 including 29 (44.6%) cases with genotype 1a. Twenty five (75.7%) of 33 patients were treatment naïve<sup>[26]</sup> and 31 (45%) of 69 patients had cirrhosis<sup>[26,27]</sup>. 3D was given for 12 wk in all 69 patients, combined with RBV in 32 (46.3%) of them<sup>[26,27]</sup>. SVR rates at week 12 (SVR12) were 97% (65/67) [94.4% (17/18) for 3D and 94.4% (17/18) for 3D plus RBV, as provided by the study data]. In regards to safety profile, no patient discontinued 3D, two patients stopped RBV and five out of 69 patients (7.2%) developed serious adverse events requiring hospitalization (1 urinary tract infection, 2 heart failure, 1 arthritis and 1 atrial fibrillation) (Table 2).

**Elbasvir/grazoprevir:** Elbasvir/grazoprevir co-formulated in one tablet, with or without the addition of RBV, has been recently licensed by FDA and EMA for the treatment of HCV genotype 1 and 4<sup>[13]</sup>. Given that these agents are cleared by the liver, they can be a good option for patients with CKD stages 4 and 5. In the C-SURFER phase III study<sup>[28]</sup>, 224 patients with eGFR < 30 mL/min were randomized to receive elbasvir/grazoprevir ( $n = 111$ ) or placebo ( $n = 113$ ) for 12 wk. At week 16, unmasking occurred and all patients in the placebo arm received elbasvir/grazoprevir as well. Almost half (52%) of the patients had genotype 1a, 83% were HCV treatment-naïve, 6% had cirrhosis, 19% had CKD stage 4 and 81% CKD stage 5 (76% of them under HD). In the intention to treat analysis, SVR was achieved in 94%



**Table 2** Studies of interferon free regimens for treatment of hepatitis C virus patients with severe renal disease or under hemodialysis

Ref.	Patients, <i>n</i>	Patient characteristics	Regimen: Patients number (dose of sofosbuvir)	Sustained virological response at 12 wk, <i>n/N</i>	Adverse events, <i>n</i>
Pockros <i>et al</i> <sup>[25]</sup>	20	GT1: 20 patients (1a: 13)	3D ± RBV: 20	18/20 (EOT-VR: 20/20)	Death from drug unrelated cause (cardiac arrest at 14 d after the end of therapy): 1
Gomez <i>et al</i> <sup>[26]</sup>	33	GT1: 29 (1a: 6) Age: 57 yr	3D ± RBV: 33	31/31	Serious adverse events: 5 (all unrelated to study drugs)
Basu <i>et al</i> <sup>[27]</sup>	36	GT1: 36 (1a: 23)	3D ± RBV: 36	34/36	No serious adverse event
Roth <i>et al</i> <sup>[28]</sup>	122	GT1: 122 patients	Elbasvir/grazoprevir: 122	115/122	Serious adverse events: 16
Czul <i>et al</i> <sup>[29]</sup>	28	GT1: 26 (1a: 16) Age: 58 yr	SOF + SMV: 26 SOF + RBV: 2 (200 mg/eod-400 mg/d)	21/25	Encephalopathy: 1 Uncontrolled diarrhea: 1
Beinhardt <i>et al</i> <sup>[30]</sup>	15	GT1: 11 patients Age: 52 yr	SOF + DCV: 9 SOF + SMV: 5 SMV + DCV: 1 (400 mg/d)	1/1 (EOT-VR: 5/5)	Pancytopenia at week 7: 1 (change SOF from every 24 h to every 48 h)
Dumortier <i>et al</i> <sup>[31]</sup>	50	GT1: 28 patients Age: 60 yr	SOF + RBV: 7 SOF + RBV + PEG-IFN: 2 SOF + DCV ± RBV: 30 SOF + SMV ± RBV: 11	24/26 (EOT-VR: 50/50)	No serious adverse event
Gane <i>et al</i> <sup>[32]</sup>	10	GT1: 9 (1a: 7) Age: 62 yr	SOF + RBV: 10 (200 mg/d)	4/10	Serious adverse events: 2 (diabetic acidosis, angina)
Nazario <i>et al</i> <sup>[33]</sup>	40	GT1: 26 (1a: 26) Age: 57 yr	SOF + LDV: 9 SOF + DCV: 2 SOF + SMV: 29 (400 mg/d)	29/29	Drug discontinuation: 1 (unknown reason)
Baliellias <i>et al</i> <sup>[34]</sup>	21 (10 on hemodialysis)	GT1: 20 patients (1a: 2) Age: 57 yr	SMV + DCV: 12 SMV + DCV + RBV: 9	17/19	No serious adverse event
Moreno <i>et al</i> <sup>[35]</sup>	42	GT1: 25 (1a: 8) Age: 54 yr	SOF + RBV: 5 LDV/SOF: 8 SOF + DCV: 14 SOF + SMV: 3 SMV + DCV: 12	32/42	Drug discontinuation: 11
Saxena <i>et al</i> <sup>[36]</sup>	19	GT1: 16 (1a: 8)	SOF + SMV + RBV: 2 SOF + SMV: 11 SOF + RBV: 5 SOF + RBV + PEG-IFN: 1 (400 mg/d)	SOF + SMV + RBV: 2/2 SOF + SMV: 8/10 SOF + RBV: 4/4 SOF + RBV + PEG: 1/1	Therapy discontinuation: 1 Serious adverse events: 3
Martin <i>et al</i> <sup>[37]</sup>	10	GT1: 8 patients Age: 58 yr	SOF + RBV: 10 (400 mg/d)	6/10	Acute respiratory failure - drug discontinuation: 1, hematemesis: 1

DCV: Daclatasvir; EOT-VR: End of treatment virological response; GT: Genotype; RBV: Ribavirin; LDV: Ledipasvir; PEG-IFN: Pegylated interferon-alfa; SMV: Simeprevir; SOF: Sofosbuvir; 3D: Ombitasvir/paritaprevir/ritonavir plus dasabuvir; eod: Every other day; HCV: Hepatitis C virus.

(115/122) of patients in the active arm: 1 noncirrhotic patient relapsed during the first 12 wk after the end of treatment, while 6 patients discontinued treatment for reasons unrelated to antiviral therapy. Serious adverse events occurred in 16 (14%) and 17 (15%) patients in the elbasvir/grazoprevir and placebo arms, respectively. None and 4% of the patients in the active and placebo groups, respectively, discontinued therapy due to an adverse event. The most common adverse events in the active arm were headache, nausea and fatigue (Table 2).

### Interferon-free antiviral schemes not approved for CHC and CKD

In total, nine studies<sup>[29-37]</sup> evaluated the safety and efficacy of various antiviral schemes in 235 patients with CHC and CKD. All patients had stage 4 or 5 CKD (*i.e.*, GFR < 30 mL/min) or were under HD. The mean age was provided in 7 studies and ranged between 52.4 and 62 years<sup>[29-35]</sup>. Based on the available data,

169 (71.9%) of 235 patients had genotype 1 [67/122 (54.9%) genotype 1a]<sup>[29,32-36]</sup>. One hundred (47.6%) of 210 patients were treatment naïve<sup>[29,31,33-36]</sup> and 121 (51.4%) of 235 patients had cirrhosis<sup>[29-37]</sup>.

Sofosbuvir was given for 12-24 wk in combination with RBV in 42 (and PEG-IFN in 3)<sup>[29,31,32,35-37]</sup>, simeprevir in 87<sup>[29-31,33,35,36]</sup> (and RBV in 2, unclarified in 11)<sup>[31,36]</sup>, daclatasvir in 55 patients<sup>[30,31,33,35]</sup> and ledipasvir in 17<sup>[33,35]</sup>. The dosage of sofosbuvir was 400 mg per day in 84<sup>[29,30,33,36,37]</sup>, 200 mg per day in 33<sup>[29,32,36]</sup>, 200 mg every other day in 2<sup>[29]</sup> and unclarified in 82 patients. The dosage of PEG-IFN was not provided in the few studies including PEG-IFN containing regimens, while the dosage of RBV was 200 mg per day in 20<sup>[32,37]</sup>, variable (200 mg three times per week to 600 mg per day) in 35<sup>[31]</sup> and unknown in the remaining patients receiving RBV. The daily dosage of simeprevir was 150 mg and of daclatasvir 60 mg in all patients. The dose of ledipasvir was dependent on the dose of sofosbuvir.

The efficacy of sofosbuvir-based antiviral therapy was provided in all studies. Based on the available data, the rates of end of treatment virological response and SVR at week 12 were 100% (91/91) and 87.1% (129/148), respectively [SVR: 55.2% (16/29) for sofosbuvir plus RBV, 92.1% (35/38) for sofosbuvir plus simeprevir (with or without RBV), 100% (14/14) for ledipasvir/sofosbuvir and 85.7% (12/14) for sofosbuvir plus daclatasvir]. The SVR rates were 80.6% (25/31) for simeprevir plus daclatasvir with or without RBV.

Regarding safety profile, only 14 (5.9%) of the 235 patients discontinued therapy due to adverse events (one under combination of sofosbuvir plus RBV due to acute respiratory failure and one under sofosbuvir plus simeprevir for unclarified cause, while no details were provided for 12 patients)<sup>[33,35-37]</sup>. In addition, one patient developed pancytopenia at week 7 under therapy (no further data were given regarding antiviral therapy, but sofosbuvir was reduced from 400 mg/d to 400 mg every other day)<sup>[30]</sup>. Finally, 8 (3.4%) of 235 patients developed serious adverse events requiring hospitalization without treatment discontinuation: Hematemesis<sup>[37]</sup>, new onset encephalopathy<sup>[29]</sup>, uncontrolled diarrhea<sup>[29]</sup>, diabetic ketoacidosis or angina<sup>[32]</sup> (unclarified causes in 3 patients)<sup>[36]</sup>. Renal safety was evaluated in two studies<sup>[31,36]</sup> which reported no significant change of GFR from baseline to the end of treatment in non-haemodialysis patients under sofosbuvir-based regimens (Table 2).

Recently, the co-formulation of velpatasvir/sofosbuvir was approved for the treatment of all HCV genotypes. Its short-term safety and pharmacokinetics were evaluated in 10 subjects with eGFR < 30 mL/min<sup>[38]</sup>. A single dose of 100 mg velpatasvir was followed by a 120-h intensive blood monitoring. Records were compared to control subjects with normal renal function (eGFR ≥ 90 mL/min) matched for age, sex and body mass index. Velpatasvir was well tolerated and all adverse events were of mild severity. Only an approximately 50% increase in the velpatasvir AUC was observed in the group of patients with renal dysfunction, while the maximum velpatasvir concentrations (C<sub>max</sub>) were similar between the two groups. The authors concluded that velpatasvir could be administered without dose adjustment in patients with any GFR. However, since velpatasvir is available only in co-formulation with sofosbuvir, its use is driven by the limitations of sofosbuvir in patients with renal impairment.

## INTERFERON-FREE REGIMENS IN KT RECIPIENTS WITH CHC

In total, 10 studies<sup>[39-48]</sup> evaluated the safety and efficacy of current DAAs based regimens in 330 KT recipients with CHC for 12-24 wk. The mean age ranged from 53 to 65 years. Based on the available data, 247 out of 281 patients (87.9%) had genotype 1 CHC [54/143 (37.8%) genotype 1a]<sup>[39-46]</sup>. One hundred and fifty one out of 238 patients (63.4%)<sup>[40,42-44,46,47]</sup> were treatment naïve and 64 out of 252 patients (25.4%) had cirrhosis<sup>[39,40,43,44,46,47]</sup>.

Sofosbuvir was given in combination with RBV in 30 patients, simeprevir (± RBV) in 31, daclatasvir (± RBV) in 20 and ledipasvir (± RBV) in 230 for 12-24 wk. The 3D (or 2D) combination (± RBV) was given in 12<sup>[46,48]</sup> and the combination of simeprevir and daclatasvir (± RBV) in 7 patients<sup>[46]</sup>. The daily dosage of RBV was provided in only 2 studies<sup>[42,43]</sup> ranging from 200 mg to 1200 mg per day.

Based on the available data, the week-12 SVR rates of sofosbuvir based regimens were 94.2% (193/205): 66.7% (10/15) for sofosbuvir plus RBV [100% (4/4) for genotype 2], 88% (22/25) for sofosbuvir plus simeprevir (with or without RBV), 75% (3/4) for sofosbuvir plus daclatasvir, 98% (158/161) for sofosbuvir plus ledipasvir (with or without RBV). In addition, in one study the week-12 SVR rates were 97.8% (45/46) for various antiviral schemes<sup>[46]</sup>. No data have been available for the efficacy of 3D or simeprevir plus daclatasvir regimens<sup>[46,48]</sup>.

Regarding safety profile, 7 (2.1%) of 330 KT recipients discontinued therapy (4 under combination sofosbuvir and RBV due to pruritus, myalgia, anemia and unclarified reason; 1 under sofosbuvir plus daclatasvir due to virological failure; 2 under ledipasvir/sofosbuvir plus RBV for unclarified reasons)<sup>[39,41,44,47]</sup>, while one patient died 4 wk after the end of antiviral therapy due to bleeding from donor aorta graft<sup>[40]</sup>. In addition, 15 KT recipients developed anemia requiring RBV dose reduction and/or erythropoietin injection or blood transfusion, one patient had an episode of bradycardia requiring pacemaker placement despite on regular amiodarone treatment, 2 patients presented worsening proteinuria (> 3 g/d), 4 patients developed rejection of kidney graft, and 12 patients developed unclarified serious adverse events<sup>[47]</sup>. No dose adjustment of calcineurin inhibitors was required. Renal and liver function tests remained stable during antiviral treatment (Table 3).

## DISCUSSION

Current DAAs against HCV have very good safety profiles. However, baseline GFR and potential drug-drug interactions should be always considered before treatment initiation. Since sofosbuvir is the only DAA with renal elimination, concerns for potential nephrotoxicity have been raised mainly for this agent. There have been reports suggesting that sofosbuvir might have a negative impact on renal function in patients at high renal risk (e.g., decompensated cirrhosis, LT, proteinuria), particularly if more sensitive renal function markers are used (e.g., cystatin C or serum or urine NGAL). However, renal function decline in such high renal risk patients does not necessarily reflect drug related toxicity, as shown in uncontrolled reports. In addition, improvement in renal function after treatment has also been reported in patients who achieved SVR despite the scarcity of long follow-up data after the end of therapy. Only nephrotoxicity related to sofosbuvir has been observed but seems to be minimal given the short duration of therapy. Therefore, no definite conclusion can be drawn,

**Table 3** Studies of interferon-free regimens for treatment of hepatitis C virus positive kidney transplant recipients

Ref.	Patients, <i>n</i>	Patient characteristics	Regimen: Patients number	Sustained virological response at 12 wk, <i>n/N</i>	Adverse events, <i>n</i>
Huard <i>et al</i> <sup>[39]</sup>	17	GT1: 16 patients (1a: 5) Age: 65 yr	SOF + RBV: 17 (400 mg/d)	1/6	Therapy discontinuation: 4 (3 due to pruritus, myalgia, anemia, 1 unclarified) Anemia: 8
Lin <i>et al</i> <sup>[40]</sup>	15	GT1: 14 (1a: 10) Age: 55.8 yr	SOF + SMV ± RBV: 12 (SOF + SMV: 9)	13/15	No serious adverse events under therapy (1 died by massive hemorrhage 4 wk after therapy) Proteinuria: 2 Bradycardia under amiodarone (pacemaker placement): 1
Bhamidimarri <i>et al</i> <sup>[41]</sup>	14	GT1: 14 (1a: 12) Age: 54 yr	SOF + RBV: 2 SOF + LDV: 1 SOF + LDV: 13 (in 9 plus RBV) SOF + SMV: 1	13/14	No serious adverse events Therapy discontinuation: 1 Anemia: 7
Hussein <i>et al</i> <sup>[42]</sup>	3	GT4: 3	SOF + RBV (400 mg/d)	3/3	No serious adverse events
Sawinski <i>et al</i> <sup>[43]</sup>	20	GT1: 17 (1a: 7) Age: 57 yr	SOF + SMV: 9 SOF/LDV: 7 SOF + RBV: 3 SOF + DCV: 1 (400 mg/d)	20/20	No serious adverse events
Moreno <i>et al</i> <sup>[44]</sup>	12	GT1: 11 (1a: 4) Age: 53 yr	SOF + SMV: 1 SOF/LDV: 8 SOF + DCV: 3 (400 mg/d)	11/12	Therapy discontinuation: 1
El-Halawany <i>et al</i> <sup>[45]</sup>	11	GT1: 10 (1a: 10) Age: 57.6 yr	SOF + SMV: 2 SOF/LDV: 8 SOF + RBV: 1	10/11	No serious adverse events
Londono <i>et al</i> <sup>[46]</sup>	74	GT1: 61 (1a: 6) Age: 54 yr	SOF/LDV ± RBV: 37 SOF + DCV ± RBV: 15 SOF + SMV ± RBV: 6 SMV + DCV ± RBV: 7 SOF + RBV: 4 3 "D" or 2 "D": 5	45/46	Rejection episodes: 3
Colombo <i>et al</i> <sup>[47]</sup>	114	GT1: 104	SOF/LDV	112/114	Therapy discontinuation: 1 Serious adverse events: 12 Rejection episode: 1
Reddy <i>et al</i> <sup>[48]</sup>	50		SOF/LDV ± RBV: 42 SOF + DCV ± RBV: 1 3 "D": 7	10/10	

DCV: Daclatasvir; GT: Genotype; RBV: Ribavirin; LDV: Ledipasvir; PEG-IFN: Pegylated interferon-alfa; SMV: Simeprevir; SOF: Sofosbuvir; 3D: Ombitasvir/paritaprevir/ritonavir plus dasabuvir; 2 "D": Ombitasvir/paritaprevir/ritonavir.

while it seems reasonable to apply nephroprotective measures and careful renal monitoring during treatment with sofosbuvir-based regimens in patients at high renal risk. Anyway, eGFR monitoring is currently recommended at 4 wk of therapy and as clinically indicated for all patients receiving any regimen with DAAs<sup>[49]</sup>.

All current DAAs can be given in CHC patients with mild to moderate renal impairment (*i.e.*, eGFR ≥ 30 mL/min) without dose modification. Similarly, they could all be administered in severe renal impairment (*i.e.*, eGFR < 30 mL/min) or end-stage renal disease without dose modification as well, except for sofosbuvir. Of note, the currently recommended regimens for CHC patients with severe renal impairment or end-stage renal disease according to the AASLD and EASL are presented in Table 4<sup>[49,50]</sup>. To date, HCV therapy is only recommended for patients with high urgency for treatment of the liver disease and without KT as an immediate option. Furthermore, antiviral therapy can be given after KT or even simultaneous liver and kidney transplantation,

when patients usually have eGFR > 30 mL/min and can receive any regimen. HCV therapy with an IFN free regimen is mandatory for CHC patients with cirrhosis and severe renal impairment usually due to hepatorenal syndrome, since HCV eradication may lead to liver function stabilization and such an improvement resulting in LT elimination. But more data are required in this subgroup before the optimal regimen can be decided. Regrettably, lack of adequate supporting evidence halts a widely disseminated recommendation.

The indication of elbasvir/grazoprevir as first line treatment for CHC patients with genotype 1 or 4 and severe renal impairment, always given without RBV for 12 wk, has been based on the results of the C-SURFER trial. In contrast to genotype 1a patients with eGFR > 30 mL/min who should be tested for NS5A resistance associated variants (RAVs) before therapy and require 16 instead of 12 wk treatment period - of elbasvir/grazoprevir combined with RBV in case of NS5A RAVs presence-, there is no recommendation for such pre-

**Table 4** Recommended regimens from the American Association for the Study of Liver Diseases and European Association for the Study of the Liver for patients with chronic hepatitis C and severe renal impairment (glomerular filtration rate < 30 mL/min) who need urgent hepatitis C virus therapy and renal transplantation is not an immediate option

HCV genotype	AASLD recommended regimen	EASL recommended regimen <sup>3</sup>
1	Elbasvir/grazoprevir for 12 wk (for 1a or 1b) or ombitasvir/paritaprevir/ritonavir plus dasabuvir <sup>1</sup> (for 1b) for 12 wk	Elbasvir/grazoprevir or ombitasvir/paritaprevir plus dasabuvir (for 1a or 1b), for 12 wk (plus RBV 200 mg/d for 1a if the haemoglobin level is > 10 g/dL at baseline)
2, 3, 5 or 6	Pegylated interferon-alfa plus dose-adjusted ribavirin (200 mg daily) <sup>2</sup>	Sofosbuvir/velpatasvir or sofosbuvir plus daclatasvir (plus ribavirin if the haemoglobin level is > 10 g/dL at baseline for genotype 3) for 12 wk (or for 24 wk without ribavirin for genotype 3) <sup>4</sup>
4	Elbasvir/grazoprevir for 12 wk	Elbasvir/grazoprevir for 12 wk or ombitasvir/paritaprevir plus dasabuvir plus ribavirin (if the haemoglobin level is > 10 g/dL at baseline) for 12 wk

<sup>1</sup>For HCV genotype 1a: Ombitasvir/paritaprevir/ritonavir plus Dasabuvir plus ribavirin at reduced doses (200 mg thrice weekly to daily) may be also used; <sup>2</sup>Ribavirin should be discontinued when hemoglobin decreases by > 2 g/dL despite use of erythropoietin (or in case of severe anaemia (haemoglobin < 8.5 g/dL according to EASL guidelines); <sup>3</sup>According to EASL guidelines: (1) antiviral therapy is indicated in those without an indication for kidney transplantation otherwise after kidney transplantation may be preferred; and (2) sofosbuvir should be used with caution (no dose recommendation can currently be given for these patients) and with careful monitoring of renal function; <sup>4</sup>If treatment is urgently needed. HCV: Hepatitis C virus; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver.

treatment testing in patients with genotype 1a and eGFR < 30 mL/min. The higher exposure to antiviral agents, the lower baseline HCV RNA levels in CHC patients and the severe renal impairment attribute for the previous difference. The 3D combination is considered an acceptable alternative for genotype 1 patients based on the results of the smaller RUBY I study and few real life data. The 3D combination is more attractive for patients with at least severe renal impairment and genotype 1b given for 12 wk without RBV. In contrast, the need for the addition of RBV makes it less attractive for such patients with genotype 1a. The safety and efficacy of the 2D regimen in patients with genotype 4 and CKD is currently under evaluation in the RUBY II trial.

The progress in HCV therapy seems to have been minimal for CHC patients with non-1, non-4 genotype and CKD, since current guidelines still recommend the PEG-IFN and RBV combination, which is associated with low efficacy, poor tolerance and potentially several adverse events. Therefore, several efforts have been focused on sofosbuvir based regimens despite its official contraindication in patients with stage 4 or 5 renal impairment (*i.e.*, with GFR < 30 mL/min or under HD)<sup>[51]</sup>. The package labels record that up to 20-fold accumulation of the sofosbuvir metabolite GS-331007 is expected in patients with severe renal dysfunction, but the clinical significance of GS-331007 accumulation remains unknown. Moreover, a recent prospective observational study<sup>[52]</sup> evaluated the pharmacokinetics of sofosbuvir in 2 dosing (400 mg per day or 3 times per week after HD), in HCV-infected patients under HD. No accumulation of sofosbuvir or GS-331007 was observed, while HD removed 53% of GS-331007.

Since sofosbuvir was chronologically the first licensed current DAA in most countries and is still required for the IFN-free treatment of patients with non-1, non-4 genotype, the safety and efficacy of sofosbuvir based regimens in patients with end stage renal disease (CKD stage 4 or 5) on or off HD have been reported in several

“real life” studies (Table 2). Its overall safety profile has been very good even in this setting with only 6% of patients (14/235) discontinuing therapy and 3.4% of patients (8/235) developing serious adverse events but without drug discontinuation. The SVR rates seem to be comparable with SVR rates in patients with normal renal function, although no definite conclusion can be drawn due to the suboptimal design of the studies, the suboptimal regimens used in some studies according to chronological availability, the small patient numbers and the variable sofosbuvir dosage. Provided that reduced sofosbuvir dosage reduces not only the plasma concentrations of GS-331007, but also the liver concentrations of the active sofosbuvir metabolite, GS-461203<sup>[53]</sup> and no major safety issues have been raised with the use of any sofosbuvir dosage in patients with at least severe renal impairment, the standard dose of sofosbuvir (400 mg daily) seems to be optimal even for this setting but should be linked with close clinical, biological, cardiovascular, and therapeutic drug monitoring. Nevertheless, further studies including more patients are required to provide stronger answers to all unresolved issues with sofosbuvir use in patients with CKD. In addition, further studies are needed in children and adolescents with CHC. It is estimated that the prevalence of chronic HCV infection is low (*e.g.*, < 0.5 among European children)<sup>[54]</sup> and currently no data on the efficacy and adverse effects of DAA are available in children with CHC.

For KT recipients, IFN-free, sofosbuvir based regimens are highly recommended providing that there is no severe underlying renal dysfunction because they are very effective with good tolerance, safety and minimal drug-drug interactions. Alternatively, the 3D or 2D regimens and the fixed elbasvir/grazoprevir combination could be the additional treatment options for patients with genotypes 1 and 4, but their safety and efficacy in the KT setting has not been evaluated yet. In general, the concurrent use of immunosuppressive agents has not been shown to affect the efficacy of any DAA



regimen and the main concern in transplant patients has been the potential drug interactions. Of the currently licensed DAAs, sofosbuvir, daclatasvir and ledipasvir have no interaction with the usual immunosuppressive agents and require no dosage modifications in transplant patients. On the other hand, simeprevir should not be given in patients receiving cyclosporine and initiation of 3D or 2D regimens should be given with reduced daily dose of cyclosporine (start with 20% of previous dose) or tacrolimus (start with 0.2 mg every 72 h or 0.5 mg once per week) in parallel with close level monitoring and dosage adjustment as required. Similarly, close monitoring of tacrolimus levels should be performed in patients undertaken elbasvir/grazoprevir because their co-administration results in increased tacrolimus plasma concentrations (Table 1).

In conclusion, IFN-free recent DAAs regimens offer for the first time the opportunity to treat effectively and safely most CHC special populations including those with severe renal dysfunction or KT. In particular, excellent IFN and RBV free options are already available for patients with genotypes 1 and 4 and severe renal impairment (eGFR < 30 mL/min) on or off HD such as elbasvir/grazoprevir for genotypes 1 and 4 and 3D for genotype 1b. To date, the patients with severe renal impairment and genotype 2, 3, 5 or 6 can be treated officially with PEG-IFN with or without RBV. Nevertheless, sofosbuvir-based regimens are actually applied if urgent treatment for the liver disease is required. Otherwise, such patients can wait for HCV treatment after KT or for future options with safer kidney profile, anticipated within the next few years.

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Basic Study

## Regulation of hepatic microRNA expression by hepatocyte nuclear factor 4 alpha

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**Author contributions:** Lu H wrote the paper; Lu H, Lei X and Liu J performed the experiments and analyzed the data; Lu H and Klaassen C conceived and designed the experiments.

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**Institutional animal care and use committee statement:** All animal procedures in the study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. The animal protocol was designed to minimize the pain or distress to the mice. Age-matched young-adult HNF4 $\alpha$  Liv-KO mice and their wild-type control littermates were fed rodent chow (#8064, Teklad; Harlan, Indianapolis, IN). Mice were housed at an ambient temperature of 22 °C with alternating 12-h light/dark cycles and allowed water and feed ad libitum.

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### Abstract

#### AIM

To uncover the role of hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) in regulating hepatic expression of microRNAs.

#### METHODS

Microarray and real-time PCR were used to determine hepatic expression of microRNAs in young-adult mice lacking Hnf4 $\alpha$  expression in liver (Hnf4 $\alpha$ -LivKO). Integrative genomics viewer software was used to analyze the public chromatin immunoprecipitation-sequencing datasets for DNA-binding of HNF4 $\alpha$ , RNA polymerase- II, and histone modifications to loci of microRNAs in mouse liver and human hepatoma cells. Dual-luciferase reporter assay was conducted to determine effects of HNF4 $\alpha$  on the promoters of mouse and human microRNAs as well as effects of microRNAs on the untranslated regions (3' UTR) of two genes in human hepatoma cells.

#### RESULTS

Microarray data indicated that most microRNAs remained unaltered by Hnf4 $\alpha$  deficiency in Hnf4 $\alpha$ -LivKO mice. However, certain liver-predominant microRNAs were down-regulated similarly in young-adult male and female Hnf4 $\alpha$ -LivKO mice. The down-regulation of miR-101, miR-192, miR-193a, miR-194, miR-215, miR-802, and miR-122 as well as induction of miR-34 and miR-29 in male Hnf4 $\alpha$ -LivKO mice were confirmed by real-time



PCR. Analysis of public chromatin immunoprecipitation-sequencing data indicates that HNF4 $\alpha$  directly binds to the promoters of miR-101, miR-122, miR-194-2/miR-192 and miR-193, which is associated with histone marks of active transcription. Luciferase reporter assay showed that HNF4 $\alpha$  markedly activated the promoters of mouse and human miR-101b/miR-101-2 and the miR-194/miR-192 cluster. Additionally, miR-192 and miR-194 significantly decreased activities of luciferase reporters for the 3'UTR of histone H3F3 and chromodomain helicase DNA binding protein 1 (CHD1), respectively, suggesting that miR-192 and miR-194 might be important in chromosome remodeling through directly targeting H3F3 and CHD1.

## CONCLUSION

HNF4 $\alpha$  is essential for hepatic basal expression of a group of liver-enriched microRNAs, including miR-101, miR-192, miR-193a, miR-194 and miR-802, through which HNF4 $\alpha$  may play a major role in the post-transcriptional regulation of gene expression and maintenance of the epigenome in liver.

**Key words:** Liver; Hepatocyte nuclear factor 4 alpha; Knockout; Mice; Human; miR-122; miR-192; miR-194; miR-101; miR-802

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**Core tip:** Hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) is a liver-enriched master regulator of liver development and function. HNF4 $\alpha$  plays a key role in regulating hepatic transcriptome and epigenome. However, little was known about the role of HNF4 $\alpha$  in regulating hepatic expression of microRNAs, essential modulators of the transcriptome and epigenome. Results from this study uncover species differences and similarities between humans and mice in the role of HNF4 $\alpha$  in regulating hepatic expression of certain important microRNAs. Such novel knowledge will help understand the role of HNF4 $\alpha$  in post-transcriptional regulation of gene expression and maintenance of the normal epigenome and physiology in mouse and human liver.

Lu H, Lei X, Liu J, Klaassen C. Regulation of hepatic microRNA expression by hepatocyte nuclear factor 4 alpha. *World J Hepatol* 2017; 9(4): 191-208 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i4/191.htm> DOI: <http://dx.doi.org/10.4254/wjgh.v9.i4.191>

## INTRODUCTION

Hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) is a master regulator of liver development and function<sup>[1]</sup>. HNF4 $\alpha$  is essential for hepatocyte differentiation in fetal liver<sup>[2-4]</sup>, maintenance of liver function in adult<sup>[5,6]</sup>, and protection against liver cirrhosis and liver cancer<sup>[7,8]</sup>. HNF4 $\alpha$  is

critical in regulating hepatic metabolism of fatty acids, bile acids, and ureagenesis<sup>[5,9-11]</sup>. Moreover, HNF4 $\alpha$  is essential in regulating hepatic expression of drug processing genes, namely cytochrome P450s, phase-II conjugation enzymes, and transporters<sup>[1,12,13]</sup>.

There are very large individual variations in hepatic basal expression of HNF4 $\alpha$  in humans<sup>[14]</sup>, and mutation of HNF4 $\alpha$  causes maturity onset diabetes of young humans<sup>[15]</sup>. The expression and/or transcriptional activity of HNF4 $\alpha$  is decreased markedly in severe cirrhotic livers, alcoholic liver disease, tumor necrosis factor- $\alpha$ -induced hepatotoxicity, and hepatoma progression<sup>[16-19]</sup>. Thus, it is important to understand how HNF4 $\alpha$  deficiency affects hepatic gene expression and its underlying mechanism.

Interestingly, overexpression of HNF4 $\alpha$  in hepatocellular carcinoma (HCC) markedly decreases the stemness of gene expression and the percentage of cancer stem cells in HCC<sup>[7]</sup>; however, the underlying mechanism is unknown. Epigenetic modifications play key roles in regulating gene expression and stem cell differentiation. Our recent study demonstrates that *Hnf4 $\alpha$*  deficiency in young-adult mouse livers causes marked alteration in histone methylation and acetylation, which is associated with induction of certain key epigenetic enzymes, including enhancer of zeste homolog 2 (EZH2), G9a and DNA methyltransferase (cytosine-5) 1 (Dnmt1)<sup>[20]</sup>. EZH2 plays a key role in maintaining the stemness of stem cells<sup>[21]</sup>. Therefore, establishment and maintenance of the epigenome of differentiated hepatocytes may be a key mechanism in the regulation of gene expression and cell differentiation by HNF4 $\alpha$ .

The importance of HNF4 $\alpha$  in regulating hepatic expression of mRNAs has been well established, however, the underlying mechanism remains less clear. HNF4 $\alpha$  directly binds to a large number of gene promoters in human and mouse liver<sup>[22-24]</sup>. *Hnf4 $\alpha$*  deficiency in young-adult mouse liver caused induction of certain key epigenetic modifiers<sup>[20]</sup>. However, our analysis of published data of chromatin immunoprecipitation-sequencing (ChIP-seq) of *Hnf4 $\alpha$*  in adult mouse liver<sup>[25]</sup> revealed no binding of *Hnf4 $\alpha$*  to these epigenetic modifiers, suggesting indirect regulation of these epigenetic modifiers by *Hnf4 $\alpha$*  in liver. microRNAs are important post-transcriptional regulators of gene expression, and deregulation of microRNAs is common in human hepatocarcinogenesis<sup>[26]</sup>. Through binding to the untranslated regions (UTRs, usually the 3'UTR) of mRNAs, microRNAs affect the stability/translation of mRNAs and thus the mRNA and/or protein levels of their target genes. We hypothesized that HNF4 $\alpha$  can indirectly regulate hepatic gene expression through directly regulating hepatic expression of certain microRNAs. Thus, the purpose of this study was to uncover the role of HNF4 $\alpha$  in regulating hepatic expression of microRNAs. We used microarray and real-time PCR to determine hepatic expression of microRNAs in young-adult mice lacking *Hnf4 $\alpha$*  expression in liver (*Hnf4 $\alpha$* -LivKO). We used integrative genomics viewer (IGV) software to analyze the public ChIP-seq datasets

for DNA-binding of HNF4 $\alpha$ , RNA polymerase- II, and histone modifications to loci of microRNAs in mouse liver and human hepatoma cells. Additionally, we conducted dual-luciferase reporter assay to determine effects of HNF4 $\alpha$  on the promoters of mouse and human microRNAs as well as effects of microRNAs on the 3'UTR of two putative target genes in human hepatoma cells.

## MATERIALS AND METHODS

### Preparation of liver samples

The livers of male and female young-adult mice with liver-specific knockout of *Hnf4 $\alpha$*  (*Hnf4 $\alpha$ -LivKO*) (*Hnf4 $\alpha$*  flox/flox, Alb-cre/+) and age-matched wild-type (*Hnf4 $\alpha$*  flox/flox, Alb-cre/-) littermates at the age of 45 d were collected in the previous study<sup>[27]</sup> and stored at -80 °C until use. All animal procedures in the study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center<sup>[27]</sup>.

### Microarray profiling of microRNA expression in *Hnf4 $\alpha$ -LivKO* mice

Pooled total RNAs from livers of young-adult (42-45 d old) male and female *Hnf4 $\alpha$ -LivKO* and their age-matched wild-type littermates ( $n = 5-6$ ) were used for microarray analysis of microRNAs, utilizing miRCURY™ LNA array version 11.0 (Exiqon, Denmark), which contains probes targeting all mouse microRNAs registered in the miRBASE version 13.0. Background correction was conducted utilizing normexp plus offset method with offset value 10<sup>[28]</sup>. The non-linear regression method was used for data normalization to remove certain systematic biases from microarray data, such as dye effects or intensity dependence.

### Heat map and unsupervised hierarchical clustering of microRNAs

The heat map diagram shows the result of the 2-way hierarchical clustering of microRNAs and samples<sup>[29]</sup>. Each row represents a microRNA and each column represents a pooled liver sample. The microRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the bottom illustrates the relative expression level of a microRNA across all samples: Red color represents an expression level above mean, blue color represents expression lower than the mean. The clustering is performed on log<sub>2</sub>(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; LogMedianDRatios differences > 0.58, corresponding to 50% differential expression.

### Quantification of microRNAs using real-time PCR

miRCURY LNA™ Universal RT microRNA PCR (Exiqon) was used to quantify microRNAs in individual RNA samples from livers of male *Hnf4 $\alpha$ -LivKO* mice. All PCR reagents and specific LNA-modified PCR primer sets were purchased from Exiqon. The PCR primer sets for mmu-miR-19b, 26a, 29b, 34a, 122, 192, 193a-3p, 194 and

195 target both human and mouse microRNA homologs, whereas PCR primer sets for mmu-miR-101b, 215, and 802 were specific for mouse microRNAs. The relative expression of each microRNA was normalized by 5s rRNA and U6 rRNA with values of wild-type mice set at 100.

### Use of public database to analyze DNA-binding of HNF4 $\alpha$ and the chromatin status of microRNAs in mouse liver, intestine, and human hepatoma HepG2 cells

Actively transcribed genes typically remain in loosely-packed euchromatin, where DNA is more accessible to the transcriptional machinery. DNase- I hypersensitive sites (DHSs), determined by DNase-sequencing (DNase-seq), is a key determining factor of the chromatin accessibility of transcription factors. DNA-binding of RNA polymerase 2 (Pol2) is widely used as a marker of active transcription. Histone H3 trimethylation at lysine-4 (H3K4me3) is enriched around the transcription start sites (TSS) and correlates tightly with active gene transcription<sup>[30,31]</sup>, whereas H3 trimethylation at lysine-36 (H3K36me3) along the gene coding regions after TSSs correlated highly with transcription elongation<sup>[32]</sup>. Our previous study shows that alterations of H3K4me3 correlate bi-directionally with mRNA expression in HNF4 $\alpha$ -null livers<sup>[20]</sup>. Conversely, Histone H3 trimethylation at lysine-27 (H3K27me3) and at lysine-9 (H3K9me3) are well-established epigenetic signatures of gene silencing<sup>[31,33]</sup>. The public genome-wide datasets of DNase-seq (GSM1003818) as well as ChIP-seq of H3K4me3 (GSM769014), H3K36me3 (GSM1000151), H3K9me3 (GSM1087075), H3K27me3 (GSM1087069), Pol2 (GSM722763) and HNF4 $\alpha$  (GSM1390711) in wild-type mouse liver were retrieved from GEO DataSets and uploaded into the IGV software<sup>[34]</sup> to visualize the DNA-binding of HNF4 $\alpha$ , Pol2 and these epigenetic signatures in each microRNA locus in mouse liver. Similarly, the public genome-wide datasets of DNase-seq (GSM816662) as well as ChIP-seq of H3K4me3 (GSM945182), H3K36me3 (GSM945211), H3K9me3 (GSM1003519), H3K27me3 (GSM945231), Pol2 (GSM935543), and HNF4 $\alpha$  (GSM935619) in HepG2 cells were retrieved from GEO DataSets for their visualization in the IGV software. Additionally, to determine the role of tissue-specific binding of HNF4 $\alpha$  in the tissue-specific regulation of miRs, ChIP-seq data for DNA-binding of HNF4 $\alpha$  in the mouse liver (GSM1390711) and small intestinal villus cells (GSM851120) were compared using the IGV software.

### Generation of expression vectors for wildtype and mutant mouse *Hnf4 $\alpha$ 1*

The mouse *Hnf4 $\alpha$ 1* cDNA was synthesized by Integrated DNA Technologies, Inc (IDT, Coralville, IA) and cloned into the pcDNA3 backbone to generate the expression vector for wildtype *Hnf4 $\alpha$ 1*, which was named as pcDNA3-*Hnf4 $\alpha$ 1*. The expression vector for the 304 serine to aspartic acid (S304D) mutant of *Hnf4 $\alpha$ 1* was generated using pcDNA3-*Hnf4 $\alpha$ 1* and the Q5® Site-Directed Muta-

genesis Kit (New England Biolabs), and verified by sequencing.

#### **Generation of reporter constructs for the promoters of human miR-101-2 and mouse miR-101b**

miR-101 is mainly transcribed from the human miR-101-2 and mouse miR-101b loci<sup>[35]</sup> which are located in the intron8-9 of RNA terminal phosphate cyclase-like 1 (*RCL1*) gene. The first base of the pre-miR-101-2 was assigned as chr9:4840297<sup>[35]</sup>, located within intron5-6 of *RCL1*, around where prominent peaks of H3K4me3 and DNA-binding of Pol2 and HNF4 $\alpha$  were identified. Thus, we PCR cloned a 739-bp fragment of miR-101-2 proximal promoter (-926 to -190 bp), located within the intron5-6 of *RCL1*, into the KpnI/MluI sites of pGL3-Basic reporter vector, which was named as pGL3-miR-101-2. In mice, miR-101b is predominantly expressed in the liver<sup>[35]</sup>. Similar to its human ortholog miR-101-2, we found prominent peaks of HNF4 $\alpha$ , H3K4me3 and Pol2 that start at the intron5-6 of *Rcl1* and extend to intron7-8 and intron8-9 of *Rcl1*. Thus, we PCR cloned a 933-bp fragment of the miR-101b promoter, located within intron5-6 of *Rcl1* that contains the peaks of HNF4 $\alpha$  and Pol2, into the KpnI/MluI sites of pGL3-Basic reporter vector, which was named as pGL3-miR-101b.

#### **Generation of reporter constructs for the proximal and/or distal promoters of human and mouse miR-194-2/miR-192 cluster**

Mouse miR-194-1/miR-215 and miR-194-2/miR-192 forms gene clusters in chromosome 1 and 19, respectively. The miR-194-1/miR-215 loci is expressed lowly in mouse liver<sup>[36]</sup>. In mouse liver, we found prominent peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 located approximately 1.6 kb upstream of the miR-194-2. Thus, we PCR cloned a 1973 bp fragment (-1694 to + 279 bp) of the promoter of the mouse miR-194-2/miR-192 cluster into the MluI/XhoI site of pGL3-Basic reporter vector, which was named as pGL3-mmIR-194-2. The sequences of all the primers used for PCR cloning of miR promoters are listed in Supplemental Materials.

A previous study indicates that a single approximately 2.4 kb transcript contains the human pri-miR-194-2 transcript and a 5' AK092802 cDNA. In the human colon cancer Caco-2 cells, HNF1 $\alpha$  binds to a HNF1 site located between -70 and -52 bp upstream of the transcription start site (TSS) of AK092802 to activate the promoter of pri-miR-194-2<sup>[37]</sup>. The upstream genomic region close to the TSS of pri-miR-194-2 contains some highly conserved regions between humans and mice<sup>[37]</sup>. We found prominent peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 within a 350 bp fragment from -329 to +21 bp upstream of the TSS of AK092802, which was PCR cloned into the KpnI/MluI sites of pGL3-Basic reporter vector and named as pGL3-hmiR-194-2-Dist. Genomic DNA prepared from C57BL/6 mouse liver and human embryonic kidney 293 cells were used as the PCR templates. In addition to the prominent peaks of HNF4 $\alpha$  and Pol2 identified in

approximately 2 kb upstream of the human miR-194-2 loci, smaller peaks of HNF4 $\alpha$  and Pol2 were also found in the proximal promoter of human miR-194-2. A DNA fragment of 417 bp that contains 5' KpnI and 3' Hind III restriction sites as well as a wild-type and mutant 405 bp human miR-194-2 promoter (from -405 to +1) were synthesized and verified by sequencing (GenScript United States Inc., Piscataway, NJ), and ligated into the KpnI/HindIII site in the pGL3-basic vector, which was named as pGL3-hmiR-194-2-Pro and pGL3-hmiR194-2 TriM. The mutant 405-bp human miR-194-2 promoter had mutations of 3 putative HNF4-binding sites predicted by software of NHR-scan<sup>[38]</sup> and HNF4 Binding Site Scanner<sup>[39]</sup> (for DNA sequences see Supplemental Materials).

#### **Generation of reporter construct for the mouse miR-802 promoter**

We PCR cloned a 2 kb fragment of the mouse miR-802 promoter (-2004 to -1 bp) into the MluI/XhoI sites of pGL3-Basic to generate the reporter vector for mouse miR-802 promoter, which was named as pGL3-mmIR-802 Pro.

#### **Determination of effect of HNF4 $\alpha$ on the promoter activities of human and mouse miRs**

Human hepatocellular adenoma HepG2 cells were maintained in D-MEM with 5% FBS. Cells were added to 96-well plates and grown to approximately 80% confluence. Plasmid DNA including pGL3 reporter vectors, the pRL-CMV luciferase (as control for transfection efficiency), pCDNA3-HNF4 $\alpha$ 2 (Addgene), pCMV-CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) (gift from Dr. Magnus Nord, Karolinska Institute), or pCDNA3 were complexed with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) and applied to individual wells, according to the manufacturer's protocol. Transfected cells were lysed with passive lysis buffer (Promega) 24 h after transfection. Promoter activities of cell lysates were quantified by Dual-Glo<sup>TM</sup> luciferase assay (Promega) with the control values of pGL3-Basic vs pRL-CMV set at 1.0. To study the role of SP1 in mediating the transactivation of human miR-194-2 proximal promoter by HNF4 $\alpha$ , the SP1 inhibitor mithramycin was added 1 h after transfection and cells were lysed 24 h after transfection for dual-luciferase assay.

#### **Generation of reporter construct for the 3'UTR of mouse chromodomain helicase DNA binding protein 1 (*Chd1*) and *H3f3* mRNAs**

The chromatin remodeling factor Chd1 is required to maintain the open chromatin and pluripotency of mouse embryonic stem cells<sup>[40]</sup>. DNA sequence containing 48 bp of the 3'UTR of mouse Chd1 mRNA (NM\_007690.3, 6708-6756, in bold), namely CTAGTGATTGGCTTT AATATAAAACTGTTACAGTACACACTGATTGTATATA CGCGTA, and its antisense sequence AGCTTACGCG TATATACAATCAGTGTGTACTGTAACAGTTTTTATATTTAA



GCCAATCA were synthesized by IDT. DNA sequence containing 48 bp of the 3'UTR of mouse H3f3b mRNA (NM\_008211.3, 1593-1639, in bold), namely CTAGTAA GTATCCTATTGAAGTTTTAGGTCAATTATGTATGTTGA CTAATACGCGTA, and its antisense sequence AGCTT ACGCGTATTTAGTCAACATACATAATTGACCTAAAAA CTTCAATAGGATACTTA were synthesized by IDT. The two sense and antisense oligos were annealed and ligated into the Spe I /HindIII site between the luciferase cDNA and SV40 polyA in pMIR-REPORT™ microRNA Expression Reporter Vector (Applied Biosystems/Ambion, Austin, TX), which was named pMIR-Chd1 and pMIR-H3f3, respectively. The correctness of pMIR-Chd1 and pMIR-H3f3 was verified by the unique restriction site (ACGCGT) for MluI that was introduced into the synthetic oligo.

#### **Determination of effect of miR-194 and miR-192 on the stability of mouse Chd1 and H3f3 3'-UTR using dual-luciferase assay**

HepG2 human hepatocellular adenoma cells were maintained in D-MEM with 5% FBS. Cells were added to 96-well plates and grown to approximately 80% confluence. Plasmid DNA including pmiR-Chd1 (or pmiR-H3f3), the pRL-CMV luciferase, and a synthetic mimic of miR-194/miR-192 (miScript miR-194/miR-192, QIAGEN Inc, Valencia, CA), or AllStars Negative Control siRNA (QIAGEN, as negative control for microRNAs) were co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol of DNA-RNAi co-transfection. Transfected cells were lysed with passive lysis buffer (Promega) 24 h after transfection. Promoter activities of cell lysates were quantified by Dual-Glo™ luciferase assay (Promega) with the control values of pmiR-Chd1/pmiR-H3f3 vs pRL-CMV set at 1.0.

**Animal care and use statement:** The animal protocol was designed to minimize the pain or distress to the mice. Age-matched young-adult HNF4 $\alpha$  Liv-KO mice and their wild-type control littermates were fed rodent chow (#8064, Teklad; Harlan, Indianapolis, IN). Mice were housed at an ambient temperature of 22 °C with alternating 12-h light/dark cycles and allowed water and feed *ad libitum*.

#### **Statistical analysis**

Data are presented as mean  $\pm$  SE. Differences between two groups were determined using Student's *t*-test. For multiple comparisons, analysis of variance was performed, followed by the Student-Newman-Keuls Method in SigmaPlot 12.5, with significance set at  $P < 0.05$ .

## **RESULTS**

#### **Results of microarray analysis of microRNAs in pooled young-adult male and female Hnf4 $\alpha$ -LivKO mouse livers**

Generally, there were few gender differences in hepatic expression of microRNAs in mice (Figure 1), which is similar to that in rats<sup>[41]</sup>. Hepatic expression of most microRNAs

remained unchanged ( $< 50\%$  differential expression among the 4 pooled samples) in *Hnf4 $\alpha$ -LivKO* mice (data not shown). However, *Hnf4 $\alpha$ -LivKO* mouse livers had up- or down-regulation of a small portion of microRNAs that are important in regulating cell proliferation, differentiation, and apoptosis (Figure 1). Thirty microRNAs were found to have  $\geq 50\%$  differential expression among the 4 pooled samples, namely male WT and *Hnf4 $\alpha$ -LivKO* as well as female WT and *Hnf4 $\alpha$ -LivKO* mice. Fourteen microRNAs had  $> 50\%$  lower expression in *Hnf4 $\alpha$ -LivKO* mice than in WT mice (Figure 1A). Among them, the 4 liver-predominant microRNAs miR-194, miR-192, miR-215 and miR-193 were 71%, 72%, 70% and 70% lower, respectively, in *Hnf4 $\alpha$ -LivKO* male mouse livers than WT males (WTM). miR-101a and 101b, which are expressed moderately in liver, also decreased  $> 50\%$  in male *Hnf4 $\alpha$ -LivKO* mice. Female *Hnf4 $\alpha$ -LivKO* mouse livers had very similar lower expression of these microRNAs than WT females (Figure 1A). In contrast, two microRNAs that are expressed highly in liver, namely miR-122 and miR-26a<sup>[42,43]</sup>, had less than 50% differential expression in all the groups (Supplemental Table 1).

In contrast to the down-regulation of certain liver-predominant microRNAs, hepatic expression of 16 microRNAs were  $> 50\%$  higher in *Hnf4 $\alpha$ -LivKO* mice than in WT mice (Figure 1B). The tumor-suppressor miR-34a<sup>[44]</sup> was expressed at relatively low levels in wild-type mouse liver, but was induced 2.6 fold in male *Hnf4 $\alpha$ -LivKO* mouse livers. Tumor-suppressor miR-29b and miR-195<sup>[45]</sup> were highly and modestly expressed in WT mouse livers, respectively, and were 90% and 70% higher, respectively, in male *Hnf4 $\alpha$ -LivKO* mouse livers than WTM (Figure 1B).

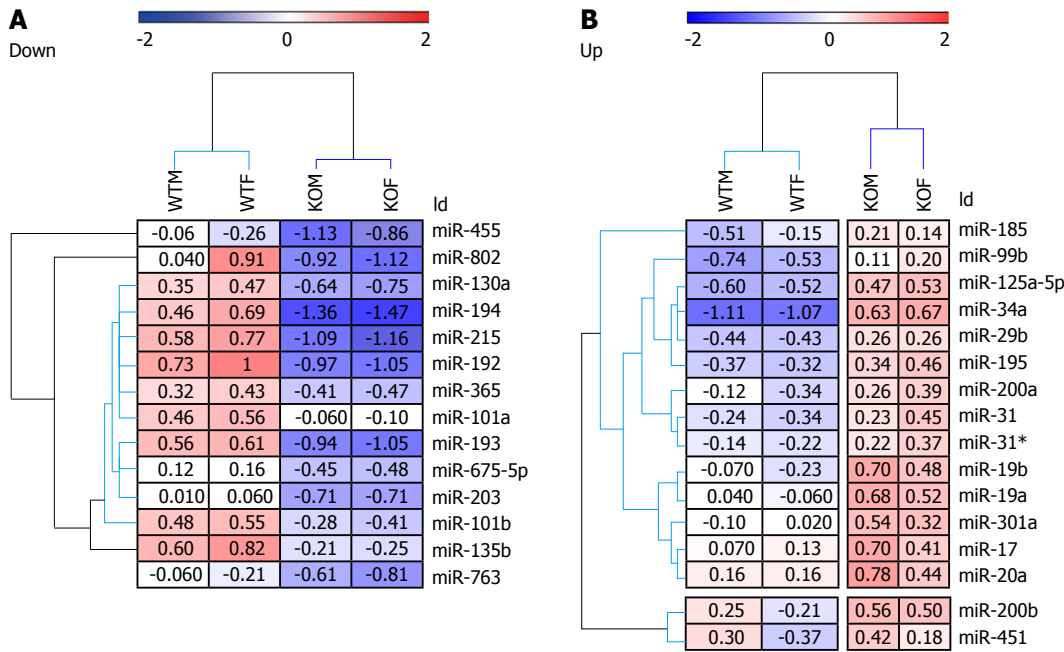
The oncogenic miR-17-92 locus encodes a cluster of 7 microRNAs transcribed as a single primary transcript<sup>[46]</sup>. Four miR-17-92 members, namely miR-17, 19a, 19b and 20 tended to be higher in *Hnf4 $\alpha$ -LivKO* mouse liver (Figure 1B).

#### **Verification of changes in hepatic microRNAs in male Hnf4 $\alpha$ -LivKO mice by real-time PCR**

To verify the changes in microRNAs detected by microarray in the pooled liver samples, real-time PCR was used to quantify 12 microRNAs in individual samples from *Hnf4 $\alpha$ -LivKO* mice (Figure 2). Because similar alterations of these microRNAs were found in male and female *Hnf4 $\alpha$ -LivKO* mice (Figure 1), only individual male *Hnf4 $\alpha$ -LivKO* liver samples were used in this study. The selection of these 12 microRNAs for verification was based on their relative expression levels (Supplemental Table 1) and their reported importance in cellular pathophysiology.

Compared to male WT mice, male *Hnf4 $\alpha$ -LivKO* mice had markedly lower levels of miR-101b (7% of WT values), miR-192 (24%), miR-193a (24%), miR-194 (16%), miR-215 (59%) and miR-802 (33%) (Figure 2A-B), but higher levels of miR-29b (190%) and miR-34a (244%) (Figure 2C). In contrast, hepatic levels of miR-26a and miR-195 were similar between male WT and *Hnf4 $\alpha$ -LivKO* mice (Figure 2C-D). Hepatic miR-122 was





**Figure 1** Heat map and unsupervised hierarchical clustering of hepatic microRNAs in male and female *Hnf4a*-LivKO mice. The heat map diagram shows the results of the 2-way hierarchical clustering of microRNAs and samples. Each row represents a microRNA and each column represents a pooled liver sample. The microRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the top illustrates the relative expression level of a microRNA across all samples: Red color represents an expression level above mean, blue color represents expression lower than the mean. The clustering is performed on log<sub>2</sub>(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; LogMedianDRatios differences > 0.58, corresponding to 50% differential expression. WTM: Wild-type male; WTF: Wild-type female; KOM: Knockout male; KOF: Knockout female.

modestly (30%) lower in male *Hnf4a*-LivKO mice than male WT mice (Figure 2D).

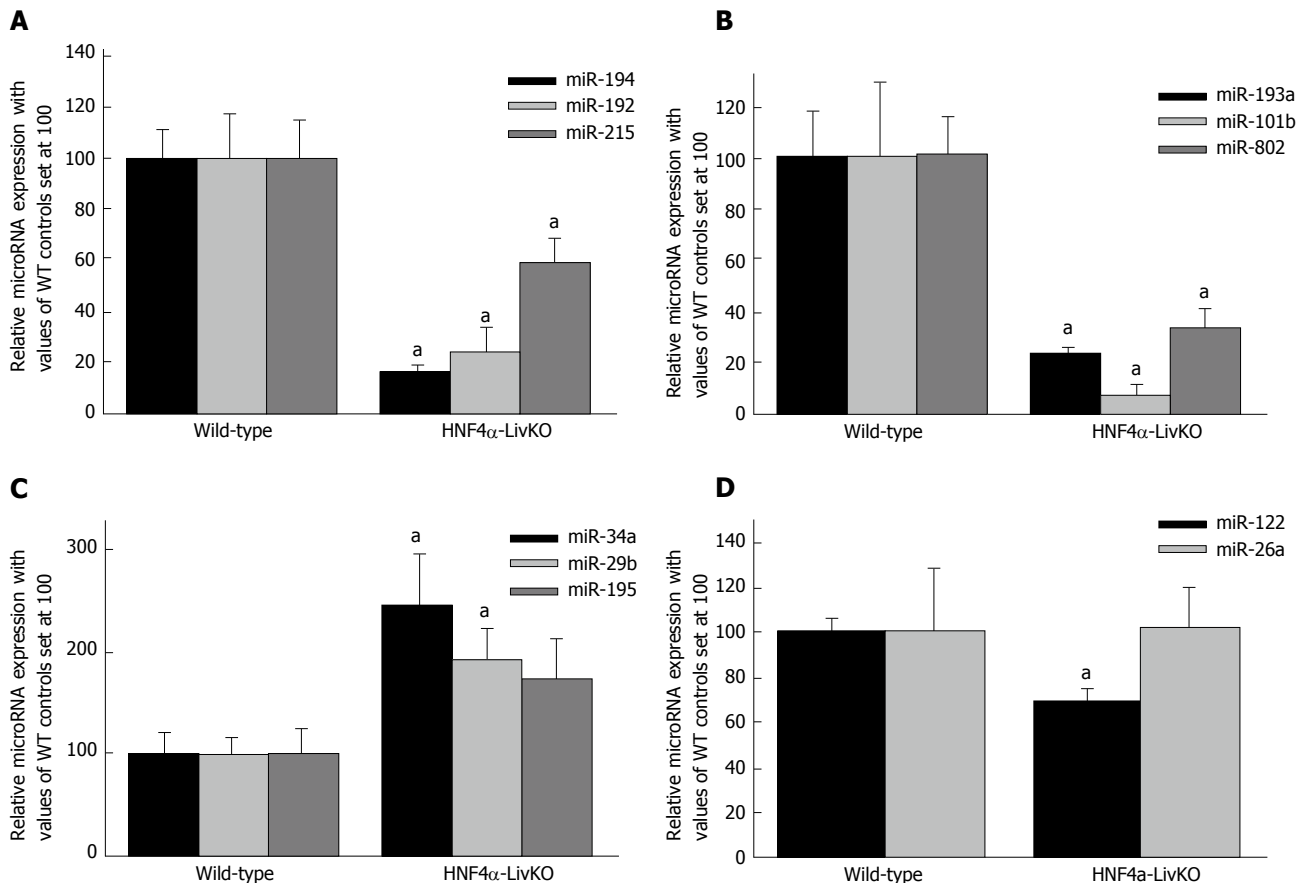
#### DNA-binding of HNF4 $\alpha$ in mouse liver and small intestine as well as the chromatin status of microRNAs in mouse liver

To understand the mechanism of regulation of microRNA expression by HNF4 $\alpha$  in mouse liver, we used IGV software to analyze the published genome-wide DNase-seq and ChIP-seq data on DNA-binding of HNF4 $\alpha$  as well as the presence of DHSs, Pol2 and active (H3K4me3 and H3K36me3) and suppressing (H3K9me3 and H3K27me3) epigenetic signatures, in the loci of several microRNAs in mouse liver and/or small intestine. Consistent with their high expression in mouse liver, miR-122a, miR-194-2/miR-192 and miR-101b had large peaks of DHSs in their gene loci, which were associated with sequential prominent peaks of HNF4 $\alpha$ , Pol2, H3K4me3 and H3K36me3 downstream (Figure 3A-3C). This strongly suggests that the binding of HNF4 $\alpha$  to the promoter of these miR genes causes the recruitment of Pol2 and the introduction of H3K4me3 and H3K36me3, the active marks of transcription initiation and elongation. Consistent with the liver-specific and liver-predominant expression of miR-122a and miR-101b, respectively, no binding of HNF4 $\alpha$  to the promoters of miR-122a and miR-101b was found in mouse small intestine (Figure 3A and 3C). In contrast, large peaks of HNF4 $\alpha$  were identified in the distal and proximal promoters of the miR-194-2/miR-192 cluster, consistent with their high expression in the mouse intestine<sup>[37]</sup>.

Similarly, peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 were also found in the gene loci of miR-193 and miR-802 (Figure 3D and E); however, the peaks were smaller and less sequential compared to those in the gene loci of miR-122, miR-194-2/miR-192 and miR-101b. In contrast, no clear peaks of H3K36me3 were found in regions that encode the mature transcripts of miR-193 and miR-802 (Figure 3D and E). Interestingly, the silencing mark H3K27me3 was found to span the whole locus of miR-802, whereas a peak of H3K9me3 was found 3' downstream of the miR-802 (Figure 3E). In summary, the data suggest that these five microRNAs might be directly regulated by HNF4 $\alpha$  in mouse liver.

Much smaller peaks of HNF4 $\alpha$  were found in the gene loci of miR-194-1/miR-215, miR26a-1 and miR26a-2, and DNA-binding of HNF4 $\alpha$  was not associated with prominent peaks of Pol2 or H3K4me3 in mouse liver (Figure 4A-C). Conversely, although prominent peaks of DHSs, HNF4 $\alpha$ , Pol2 and H3K4me3 were found in the miR-26b locus, the direction of HNF4 $\alpha$ , Pol2 and H3K4me3 peaks was toward the upstream of miR-26b, rather than the transcription initiation of miR-26b (Figure 4D). These data suggest that HNF4 $\alpha$  may not have a direct and/or important role in regulating hepatic expression of miR-194-1/miR-215, miR-26a and miR-26b. In contrast, large peaks of HNF4 $\alpha$  were found in the distal and proximal promoter of the miR-194-1/miR-215 cluster in mouse small intestine (Figure 4A), suggesting that HNF4 $\alpha$  may be important in regulating the high expression of the miR-194-1/miR-215 cluster in mouse small intestine<sup>[36]</sup>.

It was reported that HNF4 $\alpha$  binds to the proximal



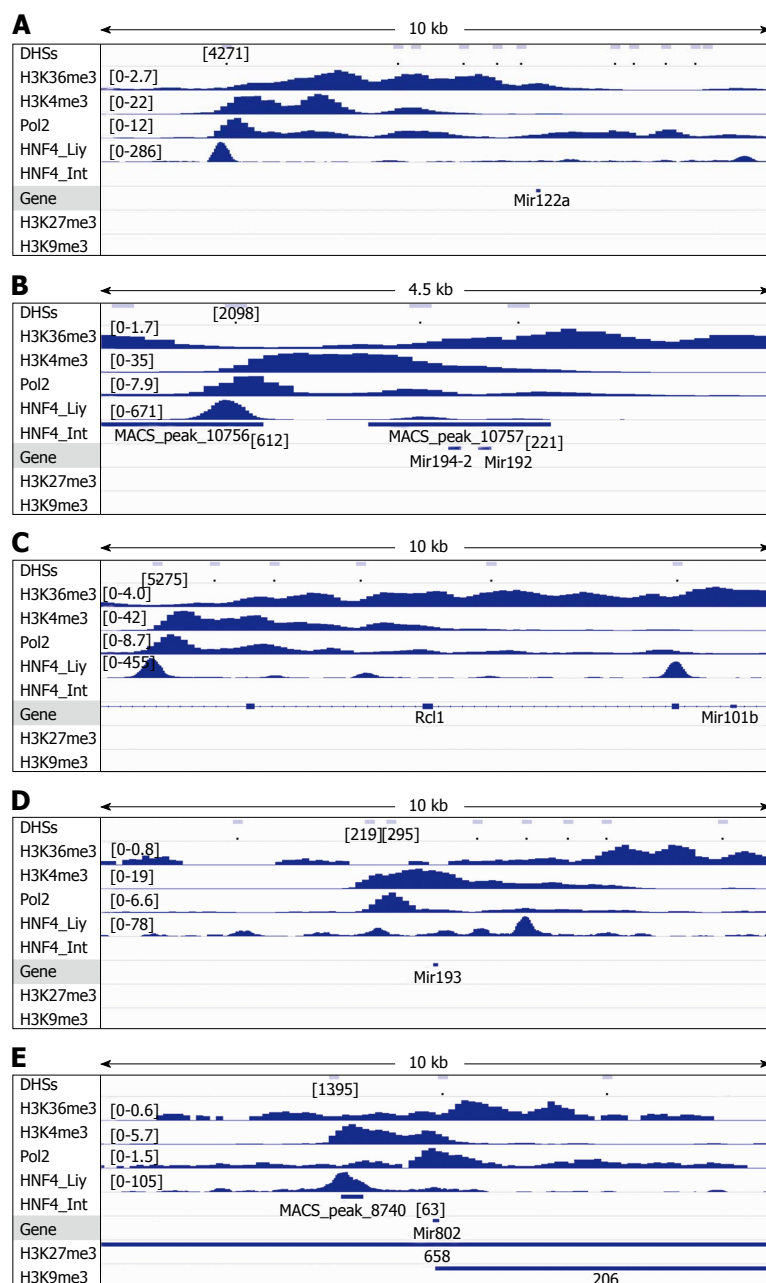
**Figure 2** Hepatic microRNA expression in young-adult male mice with liver-specific deletion of *Hnf4a* (*Hnf4a*-LivKO) (A-D). microRNAs in total RNA from livers of *Hnf4a*-LivKO and wild-type (WT) control mice ( $n = 5-6$ ) were determined by miRCURY LNA<sup>TM</sup> Universal RT microRNA PCR (Exiqon). Mean  $\pm$  SE.  $^*P < 0.05$  compared to WT control. HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha.

promoter of miR-29 a-b cluster in cultured mouse hepatocytes, and acute loss of HNF4 $\alpha$  decreased the levels of miR-29a and miR-29b in isolated hepatocytes and livers from mice on a mixed background of SvJ129/FVB<sup>[47]</sup>. However, only a small peak of HNF4 $\alpha$  was found within 10 kb of the mouse miR-29 a-b loci in adult liver from C57BL/6 mice, and the small HNF4 $\alpha$  peak was not associated with peaks of Pol2 or H3K4me3 (Figure 5A). In contrast, a larger peak of HNF4 $\alpha$  was found in the promoter of the miR-29 a-b loci in the small intestine (Figure 5A). Thus, the role of HNF4 $\alpha$  in regulating hepatic expression of miR-29 a-b cluster in mice may be strain and/or cell-context dependent.

Recent studies indicate that HNF4 $\alpha$  directly regulates miR-124 and miR-134 in human liver, and down-regulation of HNF4 $\alpha$  is associated with reduction of miR-124 and miR-134 in human HCC<sup>[48,49]</sup>. However, our microarray data showed that miR-124 and miR-134 were expressed very lowly in mouse liver, and *Hnf4a* deficiency had no effect on hepatic expression of miR-124 and miR-134 in mice (Supplemental Table 1). Consistently, there were no clear peaks of HNF4 $\alpha$ , Pol2, or the activating signatures H3K4me3, H3K36me3 in the loci of the 3 mouse miR-124 genes, namely miR-124a-1, 124a-2 and 124a-3 in livers of C57BL/6 mice (Figure 5B-D). In contrast, large peaks of the silencing mark H3K27me3 were found

in the whole loci of miR-124a-1, 124a-2 and 124a-3, and a large peak of H3K9me3 was found in the miR-124a-1 locus (Figure 5B-D). Similarly, there were no prominent peaks of DHSs, HNF4 $\alpha$ , Pol2, H3K4me3, or H3K36me3 detected in the locus of mouse *miR-134* gene, where the silencing mark H3K9me3 was found (Figure 5E). Taken together, the very low signal of miR-124s and miR-134 in the microarray data (Supplemental Table 1) and the lack of activating epigenetic signatures but enrichment of silencing epigenetic signatures in the loci of miR-124s and miR-134 strongly indicate that miR-124 and miR-134 are expressed very lowly in adult mouse liver, and they are not HNF4 $\alpha$ -target genes in mouse liver. Thus, there appear to be species differences between humans and mice in hepatic basal expression and regulation of miR-124 and miR-134 by HNF4 $\alpha$ .

Because our data of microRNA expression and analysis of public database for ChIP-seq strongly suggest that HNF4 $\alpha$  has a critical direct role in maintaining hepatic expression of miR-194/miR-192 and miR-101b in mice, we further examined DNA-binding of HNF4 $\alpha$  and chromatin status in the gene loci of miR-194-2/miR-192 and miR-101-2 in the human hepatoma HepG2 cells using the data from public database (Figure 6). Very similar to the mouse miR-194-2/miR-192 cluster (Figure 3B), starting from approximately 2 kb upstream of the



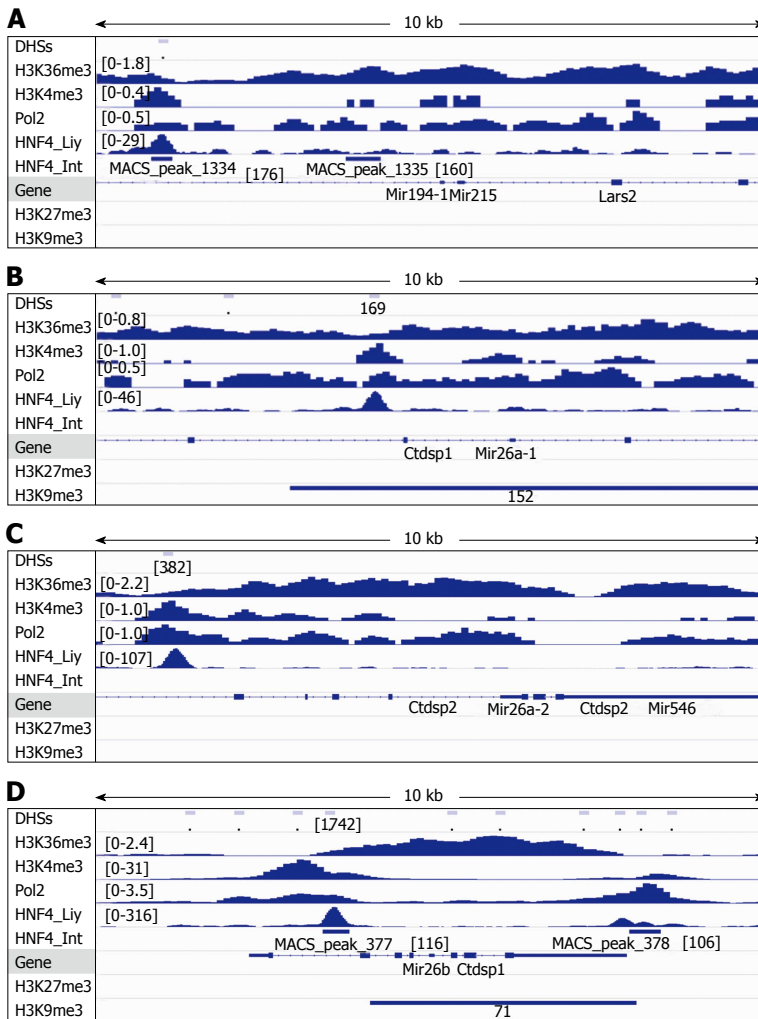
**Figure 3** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-122a (A), miR-194-2/miR-192 (B), miR-101b (C), miR-193 (D) and miR-802 (E) in wildtype mouse liver. DNA-binding of HNF4 $\alpha$  to these microRNA loci in the mouse small intestine (HNF4 $\alpha$ \_Int) was compared to those in the mouse liver (HNF4 $\alpha$ \_Liy). Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

human pri-miR-194-2, prominent sequential peaks of DHSs, HNF4 $\alpha$ , Pol2, H3K4me3 and H3K36me3 were identified in the human *miR-194-2/miR-192* gene cluster in HepG2 cells (Figure 6A). Very similar to the mouse miR-101b, the human *miR-101-2* gene body is located in the intron8-9 of the *RCL1* gene, and clear (but weaker than miR-194-2) sequential peaks of HNF4 $\alpha$ , Pol2, H3K4me3 and H3K36me3 were identified in the intron5-6 of *RCL1* (Figure 6B). These data strongly suggest that HNF4 $\alpha$  may also have a direct critical role in

regulating hepatic expression of miR-194-2/miR-192 and miR-101-2 in humans. In contrast, there were no clear peaks of HNF4 $\alpha$ , Pol2, or H3K4me3 (Figure 6C) in the miR-122 locus which is known to be silenced in HepG2 cells<sup>[42]</sup>.

#### Regulation of the mouse and human miR-194-2/miR-192 gene cluster by HNF4 $\alpha$

Hepatic expression of miR-194 is markedly down-regulated in mice null for *Hnf1 $\alpha$* <sup>[36]</sup>, a down-stream target

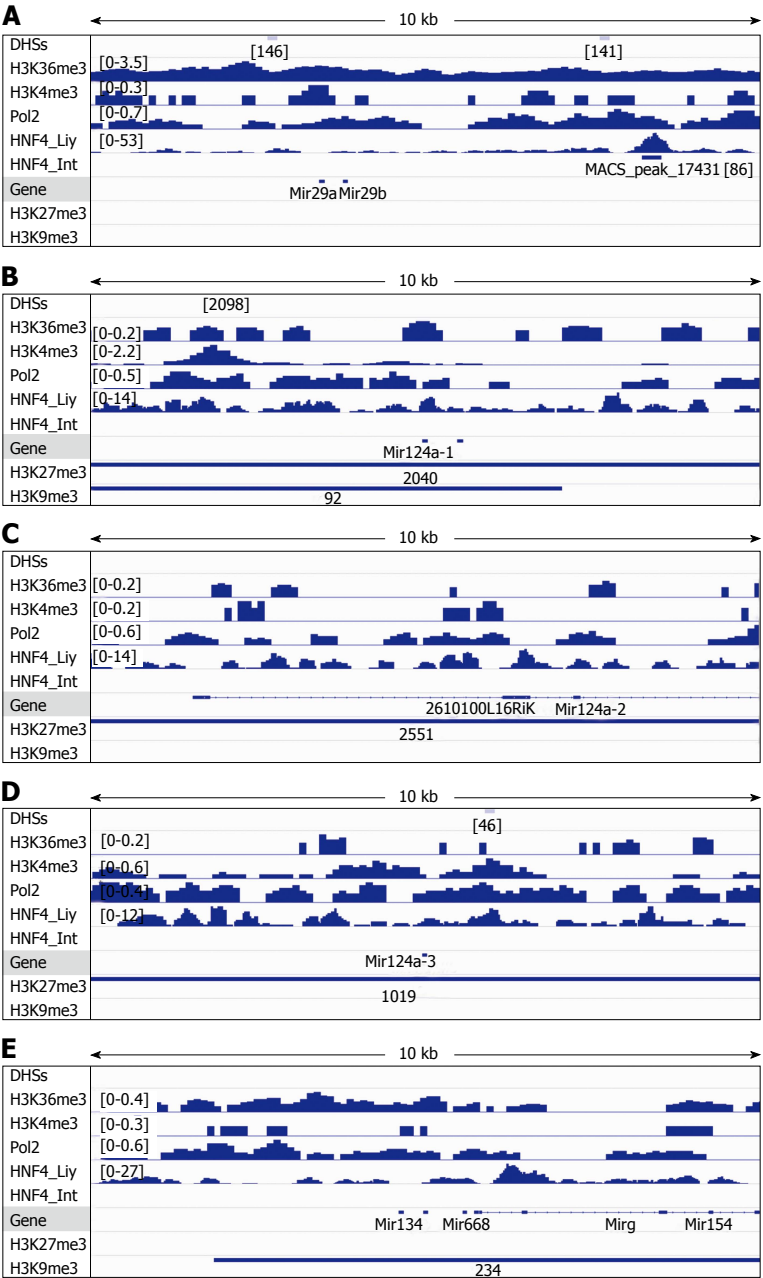


**Figure 4** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-194-1/miR-215 (A), miR-26a-1 (B), miR-26a-2 (C) and miR-26b (D) in wildtype mouse liver. DNA-binding of HNF4 $\alpha$  to these microRNA loci in the mouse small intestine (HNF4 $\alpha$ \_Int) was compared to those in the mouse liver (HNF4 $\alpha$ \_Liv). Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

of HNF4 $\alpha$ . In small intestine, miR-194 is transcriptionally up-regulated by Hnf1 $\alpha$ <sup>[37]</sup>. Hepatic mRNA expression of Hnf1 $\alpha$  decreased modestly in *Hnf4 $\alpha$ -LivKO* mice<sup>[1]</sup>. We found that HNF1 $\alpha$  and HNF4 $\alpha$  modestly activated the reporter for the mouse *miR-194-2/miR-192* gene cluster 1.5 and 2.8 fold, respectively, and they synergistically activated mouse miR-194-2/miR-192 promoter 7.5 fold (Figure 7A). ChIP-seq results showed that HNF4 $\alpha$  bound strongly to the distal promoter but weakly to the proximal promoter of human miR-194-2/miR-192 cluster (Figure 6A). To determine the role of HNF4 $\alpha$  in regulating the *miR-194-2/miR-192* gene cluster in humans, we generated reporter vectors for the distal and proximal promoters of human miR-194-2/miR-192 cluster. Surprisingly, HNF4 $\alpha$  only modestly activated the distal promoter 3 fold, but very strongly activated the proximal promoter of human miR-194-2/miR-192 cluster by 200 fold (Figure 7B). To identify the critical cis-elements responsible for the very strong transactivation

of this proximal promoter by HNF4 $\alpha$ , we engineered luciferase reporter constructs for the mutated 400-bp proximal promoter of human *miR-194-2* gene cluster. Surprisingly, mutations of the 3 putative HNF4-binding sites (HNF4-RE) within the 400-bp miR-194-2 promoter had little effects on the transactivation of this promoter by HNF4 $\alpha$  (Figure 7C). HNF4 $\alpha$  can transactivate the human p21 promoter *via* physically interacting with the general transcription factor SP1, independent of DNA-binding of HNF4 $\alpha$ , because the S304D mutant of HNF4 $\alpha$  which has markedly decreased DNA-binding activity<sup>[50]</sup>, is equally active as the WT HNF4 $\alpha$  in transactivating p21<sup>[51]</sup>. Thus, we tested the hypothesis that HNF4 $\alpha$  can DNA-binding-independently transactivate the proximal human miR-194-2 promoter *via* interacting with SP1. We found that mithramycin, a widely used SP1 inhibitor<sup>[52]</sup>, dramatically suppressed the HNF4 $\alpha$ -transactivation of both the WT and HNF4RE-mutant miR-194-2 promoter by 94% and 95%, respectively (Figure 7C). Moreover,





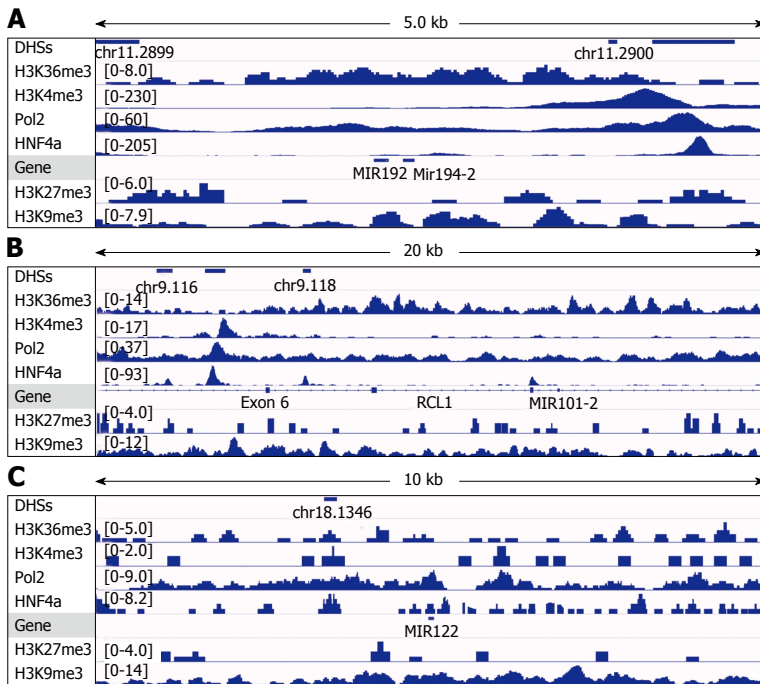
**Figure 5** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-29a/miR-29b (A), miR-124a-1 (B), miR-124a-2 (C), miR-124a-3 (D) and miR-134 (E) in wildtype mouse liver. DNA-binding of HNF4 $\alpha$  to these microRNA loci in the mouse small intestine (HNF4 $\alpha$ \_Int) was compared to those in the mouse liver (HNF4 $\alpha$ \_Liv). Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.

the S304D-mutant of HNF4 $\alpha$  was equally active as the WT HNF4 $\alpha$  in transactivating the proximal human miR-194-2 promoter (Figure 7D). Taken together, these data strongly indicate that HNF4 $\alpha$  can DNA-binding-independently transactivate the proximal human miR-194-2 promoter *via* interacting with SP1.

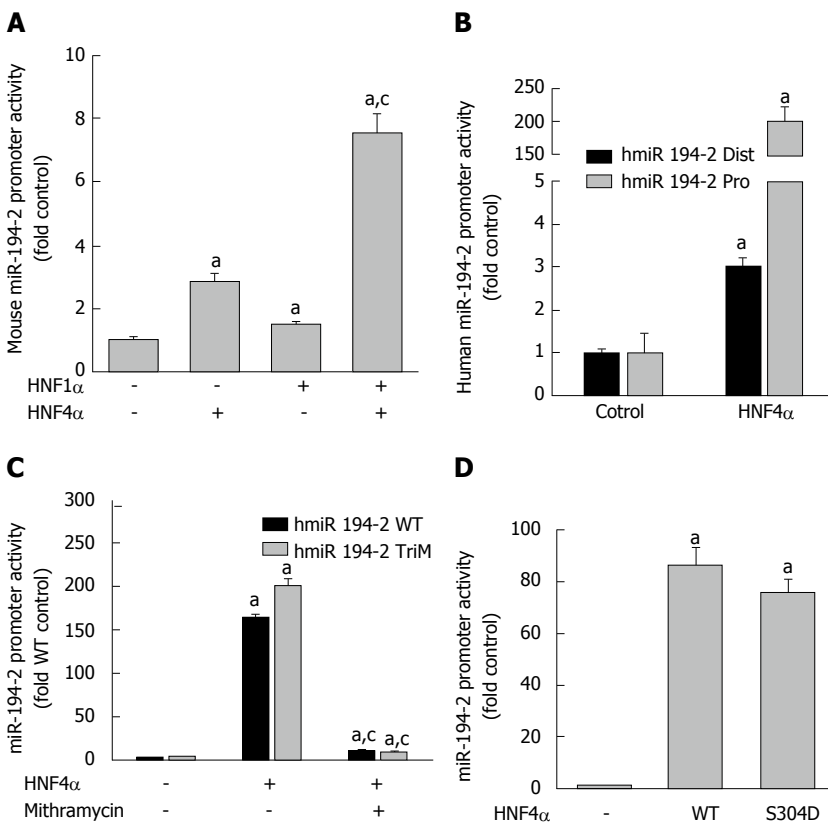
### Regulation of mouse miR-101b and human miR-101-2 promoters by HNF4 $\alpha$

The mouse miR-101b promoter was moderately active in

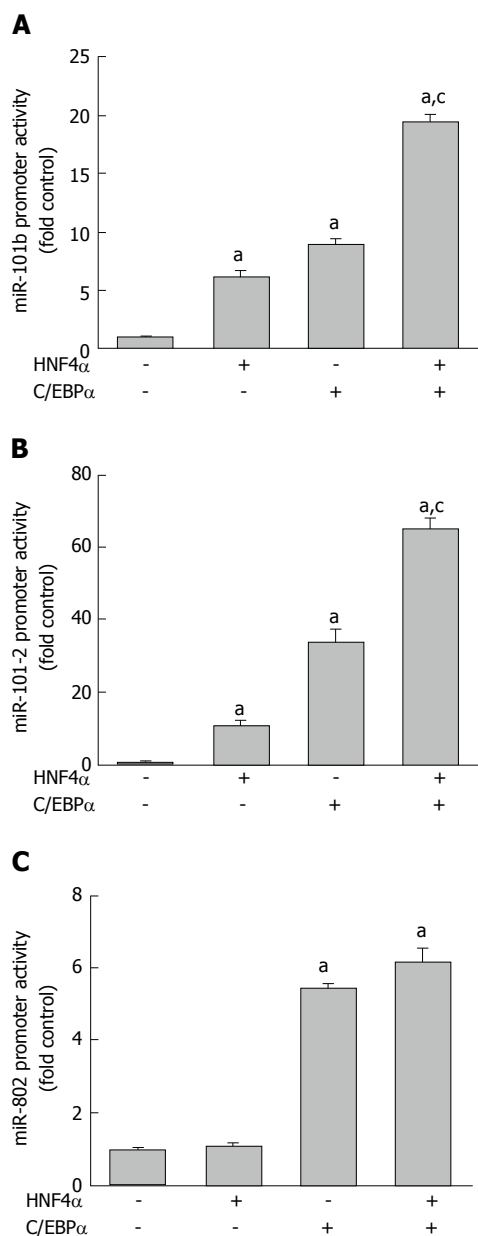
HepG2 cells (Figure 8A), whereas the human miR-101-2 promoter was largely inactive in HepG2 cells (Figure 8B). C/EBP $\alpha$ , a liver-enriched transcription factor, plays a key role in regulating liver-specific gene expression. The expression of C/EBP $\alpha$  is low in HepG2 cells, and re-expression of C/EBP $\alpha$  in HepG2 cells can reactivate certain liver-specific genes<sup>[53]</sup>. Our analysis of published ChIP-seq data for C/EBP $\alpha$  in mouse liver (GSM1037657) showed that C/EBP $\alpha$  bound to the miR-101b promoter, located in the Intron5-6 of Rcl1, in close proximity to



**Figure 6** Analysis of DNase-I hypersensitive sites as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II (Pol2), and methylated histones to loci of miR-194-2/miR-192 (A), miR-101-2 (B) and miR-122 (C) in human hepatoma HepG2 cells. Data of DHSs (determined by DNase-seq) and DNA-binding of proteins (determined by ChIP-seq) were retrieved from the public database of GEO DataSets and visualized in the IGV software. The peak values/ranges for each mark were shown in square brackets or under the line mark. DHSs: DNase-I hypersensitive sites; H3K36me3: H3 trimethylation at lysine-36; H3K4me3: H3 trimethylation at lysine-4; H3K27me3: H3 trimethylation at lysine-27; H3K9me3: H3 trimethylation at lysine-9; Pol2: Polymerase 2; HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; ChIP-seq: Chromatin immunoprecipitation-sequencing; IGV: Integrative genomics viewer.



**Figure 7** Activation of mouse (A) and human (B-D) miR-194-2/miR-192 promoter by HNF4 $\alpha$ . Human hepatoma HepG2 cells were transfected with firefly luciferase vectors containing wild-type and mutant miR-194-2 promoter, pRL-CMV, and an expression vector for HNF4 $\alpha$ /HNF1 $\alpha$ . Dual-luciferase reporter assay was conducted 24 h after transfection. The y-axis represents relative luciferase activity for microRNA promoter normalized by the renilla luciferase.  $n = 4$ , Mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  compared to vector control; <sup>b</sup> $P < 0.05$  compared to HNF4 $\alpha$  alone group. HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha.



**Figure 8** Activation of (A) mouse miR-101b, (B) human miR-101-2, and (C) mouse miR-802 promoter by HNF4 $\alpha$ . Human hepatoma HepG2 cells were transfected with firefly luciferase vectors containing microRNA promoter, pRL-CMV, and an expression vector for HNF4 $\alpha$  and/or C/EBP $\alpha$ . Dual-luciferase reporter assay was conducted 24 h after transfection. The Y-axis represents relative luciferase activity for microRNA promoter normalized by the renilla luciferase.  $n = 4$ , Mean  $\pm$  SE. <sup>a</sup> $P < 0.05$  compared to vector control; <sup>c</sup> $P < 0.05$  compared to HNF4 $\alpha$  alone group. HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; C/EBP $\alpha$ : CCAAT/enhancer-binding protein  $\alpha$ .

HNF4 $\alpha$ . Moreover, putative C/EBP binding sites are highly enriched in the human miR-101-2 promoter, predicted by the Alibaba2 software. We found that HNF4 $\alpha$  and C/EBP $\alpha$  activated the mouse miR-101b promoter 6.2 and 8.9 fold, respectively, and they synergistically activated the miR-101b promoter 19 fold in HepG2 cells (Figure 8A). Similarly, HNF4 $\alpha$  and C/EBP $\alpha$  activated the human miR-101-2 promoter 11 and 33 fold, respectively, and they synergistically activated the miR-101-2 promoter 65 fold in HepG2 cells (Figure 8B).

#### Regulation of mouse miR-802 promoter by HNF4 $\alpha$

Different from miR-101, HNF4 $\alpha$  had no effect on the 2 kb mouse miR-802 promoter, and HNF4 $\alpha$  did not enhance the transactivation of the miR-802 promoter by C/EBP $\alpha$  in HepG2 cells (Figure 8C).

#### Regulation of mouse Chd1 and H3f3 by miR-194 and miR-192

TargetScan was used to identify potential targets of liver-predominant microRNAs down-regulated in *Hnf4 $\alpha$* -LivKO livers. miR-192/215 and miR-194 have a perfect match (8 mer) and very high context score percentile of 96%-99% with human and mouse histone H3f3b (H3.3b) and Chd1, respectively, indicating a very high likelihood of inhibition (Table 1). Therefore, we generated luciferase reporters for the 3'UTR of H3.3 and Chd1. Results of dual luciferase assay showed that miR-194 and miR-192 significantly decreased the luciferase activity for the 3' UTR of Chd1 (Figure 9A) and H3.3 (Figure 9B) by 37% and 36%, respectively, in HepG2 cells.

## DISCUSSION

The present study demonstrates that *Hnf4 $\alpha$*  is essential for hepatic expression of certain liver-predominant microRNAs, namely miR-101, miR-192, miR-193 and miR-194. HNF4 $\alpha$  transactivates these miRs *via* direct DNA-binding to the promoters and/or interacting with the general transcription factor SP1. These miRs target essential epigenetic modifiers, such as EZH2 (by miR-101), histone H3.3 (by miR-192) and Chd1 (by miR-194) (Figure 10).

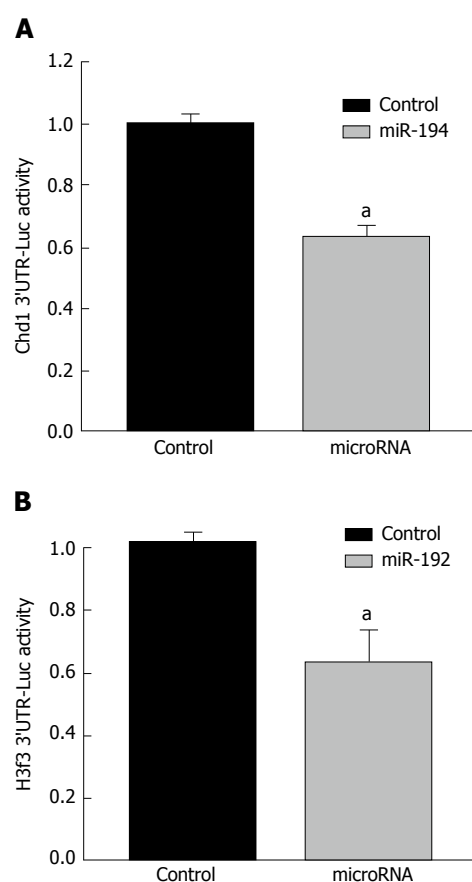
The present data provide the first evidence that HNF4 $\alpha$  is essential for hepatic expression of miR-194 in mice, and likely in humans. In both mice and humans, miR-194 is expressed highly in kidney and GI tract including liver and small intestine<sup>[37]</sup>. The tissue distribution of miR-194 parallels that of HNF4 $\alpha$ . In liver, miR-194 signals are detected in hepatocytes but not in non-parenchymal cells, and miR-194 is down-regulated during dedifferentiation of hepatocytes<sup>[54]</sup>. miR-194 inhibits the metastasis of mesenchymal-like liver cancer cells. Moreover, ChIP-seq results demonstrate direct binding of HNF4 $\alpha$  to the distal and proximal promoters of mouse and human miR-194-2 (Figure 3B and 6A). Furthermore, results of reporter assays indicate that HNF4 $\alpha$  potentially activates the promoter of mouse and human *miR-194-2/miR-192* gene cluster (Figure 7). Taken together, these data strongly indicate that HNF4 $\alpha$  plays a key role in maintaining hepatic expression of miR-194 in mice and humans.

Two recent studies of mice with inducible knockout of *Hnf4 $\alpha$*  demonstrate that acute loss of *Hnf4 $\alpha$*  in adult mouse liver triggers extensive hepatocyte proliferation, hepatomegaly, and increased HCC<sup>[55-57]</sup>. The increased intestinal cell proliferation in mice with specific loss of *Hnf4 $\alpha$*  in the adult intestinal epithelium is ascribed to the activation of the Wnt/beta-catenin system<sup>[58]</sup>. miR-194 negatively control expression of frizzled-6, which activates the beta-catenin pathway<sup>[36]</sup>. Therefore, *Hnf4 $\alpha$*  may

**Table 1 Targeting of human and mouse genes by liver-predominant microRNAs predicted by TargetScan**

	Predicted pairing of target region (top) and microRNA (bottom)	Seed match	Context score percentile
Position 1085-1091 of human H3F3B 3'UTR miR-192/215	5' ...AUUUACUGAAGUUUUUAGGUCAA...           3' CCGACAGUUAAGUAUCCAGUC	8 mer	96
Position 1064-1070 of mouse H3f3b 3'UTR miR-192/215	5' ...UCCUAUUGAAGUUUUUAGGUCAA...           3' CCGACAGUUAAGUAUCCAGUC	8 mer	99
Position 1109-1115 of human CHD1 3'UTR miR-194	5' ...GACUUUUAAUAUAAACUGUUACA...           3' AGGUGUACCUCAACGACAAUGU	8 mer	99
Position 1100-1106 of mouse Chd1 3'UTR miR-194	5' ...GCUUUAAUAUAAAAACUGUUACA...           3' AGGUGUACCUCAACGACAAUGU	8 mer	99

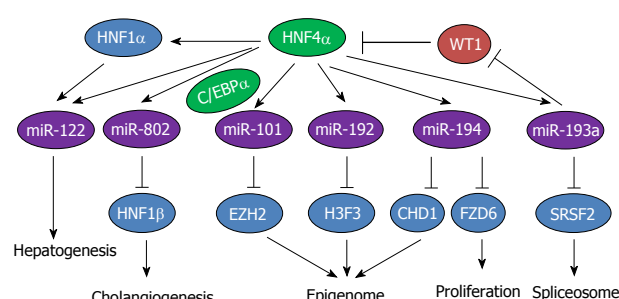
3'UTR: Untranslated regions.



**Figure 9 Effects of miR-194 and miR-192 on the activities of luciferase reporter vectors for the 3'UTR of mouse Chd1 and H3f3.** Human hepatoma HepG2 cells were transfected with plasmid DNA including pmir-Chd1 (or pmir-H3f3), the pRL-CMV luciferase, and a synthetic mimic of miR-194/miR-192, or AllStars Negative Control siRNA (as negative control for microRNAs) using Lipofectamine 2000. Dual-luciferase reporter assay was conducted 24 h after transfection. The Y-axis represents relative luciferase activity for the 3'UTR of Chd1 or H3f3 normalized by the renilla luciferase.  $n = 4$ , Mean  $\pm$  SE.  $^{\ast}P < 0.05$  compared to control (AllStars Negative Control siRNA). 3'UTR: Untranslated regions.

inhibit cell proliferation through the miR-194→frizzled-6→beta-catenin signaling pathway.

The chromatin remodeling factor CHD1 is required to maintain the open chromatin and pluripotency of mouse



**Figure 10** Diagram that illustrates the regulation of hepatic microRNA expression by Hnf4 $\alpha$  in mouse liver. HNF4 $\alpha$ : Hepatocyte nuclear factor 4 alpha; C/EBP $\alpha$ : CCAAT/enhancer-binding protein  $\alpha$ .

embryonic stem cells<sup>[40]</sup>. CHD1 is required for chromatin incorporation of the histone variant H3.3, which is generally associated with active genes<sup>[59]</sup>. However, CHD1 may also repress gene expression *via* association with HDACs<sup>[60]</sup>. Overexpression of HNF4 $\alpha$  in hepatoma cells dramatically decreased the “stemness” gene expression and the percentage of cancer stem cells in HCC<sup>[8]</sup>; however, the underlying mechanism is unknown. HNF4 $\alpha$ , *via* regulating miR-194, might inhibit stemness gene expression by targeting the chromatin remodeling factor CHD1, which deposits the unmodified or altered histone H3.3 into chromatin and increases the stemness of *Hnf4 $\alpha$* -LivKO hepatocytes.

The present data indicate that Hnf4 $\alpha$  is essential for hepatic expression of miR-192, and the histone variant H3.3 is a direct target of miR-192. Thus, down-regulation of miR-192 may be the underlying mechanism of hepatic induction of H3.3 in young-adult Hnf4 $\alpha$ -LivKO mice<sup>[21]</sup>. The replacement H3 variant H3.3 is encoded by two genes termed H3.3A and H3.3B, both code for the same amino acid sequence, but differ in nucleotide sequences and gene organization<sup>[61]</sup>. H3.3 is the exclusive substrate for replication-independent deposition, which provides a mechanism for the immediate activation of genes that are silenced by histone modification<sup>[62,63]</sup>, and H3.3 is important in epigenetic memory<sup>[64]</sup>. H3.3/H2A.Z double variant-containing nucleosomes mark “nucleosome-free regions” of active promoters and other regulatory



regions<sup>[65]</sup>. Deposition of H3.3 can rapidly derepress gene silencing<sup>[66]</sup>. Taken together, Hnf4 $\alpha$  directly regulates miR-192, and the down-regulation of miR-192 in *Hnf4 $\alpha$ -LivKO* livers may be the underlying mechanism of hepatic induction of H3.3, which contributes to the marked alteration of epigenome and transcriptome in *Hnf4 $\alpha$ -LivKO* livers<sup>[21]</sup>.

The present study indicates that Hnf4 $\alpha$  is required for hepatic expression of the tumor-suppressor miR-101. miR-101 is predominantly expressed in the liver<sup>[35]</sup>. miR-101 is down-regulated in HCC<sup>[67]</sup> and miR-101 directly represses EZH2<sup>[68,69]</sup>, a protooncogene that silences the expression of tumor-suppressors *via* H3K27me3. Down-regulation of miR-101 in *Hnf4 $\alpha$ -LivKO* mouse livers might be the underlying mechanism of induction of EZH2 and increased H3K27me3 observed previously<sup>[21]</sup>.

The present data indicate that Hnf4 $\alpha$  is important for hepatic basal expression of the tumor-suppressor miR-193a. miR-193a and miR-365 closely cluster in chromosome 11 in mice. The tumor-suppressor miR-193a is down-regulated in the majority of HCC in humans<sup>[70]</sup> and miR-193a prevents the resistance of HCC to 5-fluorouracil *via* repressing the expression of serine/arginine-rich splicing factor 2 (SRSF2)<sup>[71]</sup>. Through maintaining hepatic expression of miR-193a, HNF4 $\alpha$  might regulate expression of SRSF2 and the splicing of transcripts in liver. Interestingly, miR-193a also targets directly Wilms' tumor protein 1 (WT1)<sup>[72]</sup>. WT1 is overexpressed in cirrhotic liver and HCC<sup>[18,73]</sup>, and induction of WT1 down-regulates HNF4 $\alpha$  expression in liver<sup>[18]</sup>. The putative feedback regulatory loop of HNF4 $\alpha$ →miR-193a→WT1 and its significance in liver cirrhosis and carcinogenesis warrant further investigation.

The present data provide the first evidence that Hnf4 $\alpha$  is important for hepatic expression of miR-802 (Figure 2). Results of reporter assay (Figure 8C) suggest that HNF4 $\alpha$  may indirectly regulate hepatic miR-802 expression *via* C/EBP $\alpha$ , whose DNA-binding activity decreased in *Hnf4 $\alpha$ -LivKO* mice<sup>[27]</sup> and human hepatoma cells. Interestingly, the miR-802 locus is marked with both the activating signature H3K4me3 and the silencing signature H3K27me3, a feature of bivalent chromatin which allows a low basal expression but timely activation of developmentally-regulated genes<sup>[74]</sup>. Hnf1 $\beta$  is a direct target of miR-802<sup>[75]</sup>, and Hnf1 $\beta$  is overexpressed in adult *Hnf4 $\alpha$ -LivKO* mouse livers<sup>[4]</sup>. In mouse liver, miR-802 is expressed at 10-fold higher levels in hepatocytes than non-hepatocytes<sup>[75]</sup>. In contrast, Hnf1 $\beta$  is strongly expressed in cholangiocytes but weakly in hepatocytes, and Hnf1 $\beta$  plays a key role in bile-duct morphogenesis and glucose homeostasis<sup>[76]</sup>. Thus, the putative HNF4 $\alpha$ →C/EBP $\alpha$ →miR-802→HNF1 $\beta$  pathway might play a role in controlling cell-specific expression of HNF1 $\beta$  and liver morphogenesis during liver development.

The tumor-suppressor microRNAs miR-34a, miR-192, miR-215 and miR-194 are all p53-inducible microRNAs<sup>[77]</sup>. The induction of the p53-target gene p21 in *Hnf4 $\alpha$ -nul* mouse livers<sup>[13]</sup> suggests that p53 is activated by *Hnf4 $\alpha$*  deficiency, which may contribute to the induction of the p53-target miR-34a and miR-29b (Figure 2B). However,

hepatic expression of other p53-target microRNAs miR-192, miR-215 and miR-194 are markedly down-regulated in *Hnf4 $\alpha$ -LivKO* mice. It is interesting that HNF4 $\alpha$  can transactivate two p53-target genes, p21 and miR-194 (Figure 7), independent of DNA-binding of HNF4 $\alpha$  to the promoter. The AMP-activated protein kinase (AMPK) phosphorylates HNF4 $\alpha$  at S304, resulting in a marked decrease in the DNA-binding activity and decreased transactivation of apolipoprotein C3<sup>[50]</sup>. AMPK suppresses lipogenesis and carcinogenesis in liver<sup>[78,79]</sup>. The contribution of selective modulation of HNF4 $\alpha$ -target lipogenic genes and tumor-suppressors (p21 and miR-194) to the physiological and pharmacological roles of AMPK in liver diseases warrants further investigation.

miR-29 is broadly expressed at high levels in normal tissues. miR-29 sensitizes cholangiocarcinoma cells to TNF-induced cytotoxicity<sup>[80]</sup> and miR-29 activates p53<sup>[81]</sup>. miR-29 induces global DNA hypomethylation and tumor suppressor gene reexpression in lung cancer and acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1<sup>[82,83]</sup>. miR-29 also directly inhibits Dnmt3a and Dnmt3b in mice<sup>[84]</sup>. Thus, induction of miR-29b might contribute to the lack of global changes in hepatic DNA methylation, despite an induction of Dnmt1, in the young-adult *Hnf4 $\alpha$ -LivKO* mice<sup>[21]</sup>. Currently, the mechanism of induction of miR-29 in the young-adult *Hnf4 $\alpha$ -LivKO* mice remains unknown. miR-29 can be transactivated by p53<sup>[85]</sup>. Thus, activation of p53 might contribute to hepatic induction of miR-29b in *Hnf4 $\alpha$ -LivKO* mice.

The liver-specific miR-122 is important in regulating hepatic cholesterol and lipid metabolism<sup>[86,87]</sup>, and down-regulation of miR-122 contributes to HCC malignancy<sup>[88-90]</sup>. HNF4 $\alpha$  can directly activate the expression of miR-122 in mouse liver<sup>[91]</sup>. However, knockdown of HNF4 $\alpha$  does not affect the high expression of miR-122 in a HCC cell line, although miR-122 expression correlates strongly with HNF4 $\alpha$ <sup>[88]</sup>. In contrast, hepatic miR-122 expression is regulated by Hnf1 $\alpha$ <sup>[88]</sup>. The moderate down-regulation of miR-122 in *Hnf4 $\alpha$ -LivKO* mouse livers parallels the moderate decrease of Hnf1 $\alpha$  in these mice<sup>[27]</sup>. Taken together, these data suggest that HNF4 $\alpha$  has a positive but limited role in regulating hepatic expression of miR-122.

The present study demonstrates species differences between humans and mice in hepatic basal expression and regulation of miR-124 and miR-134 by HNF4 $\alpha$ . Interleukin-6 (IL6) plays a key role in inflammation and hepatocarcinogenesis<sup>[92]</sup>. Interestingly, HNF4 $\alpha$  exerts anti-inflammatory effects in human hepatocytes *via* the miR-124-IL6R-STAT3 pathway; knockdown of HNF4 $\alpha$  in human hepatocytes leads to down-regulation of miR-124, induction of IL6R and IL6, and activation of STAT3<sup>[49]</sup>. However, there is no induction of IL-6 or activation of STAT3 in adult mice with acute loss of HNF4 $\alpha$ <sup>[56]</sup>. Thus, there may be species difference between humans and mice regarding the interaction of HNF4 $\alpha$  with miR-regulated inflammatory and carcinogenic pathways in the liver.

Our previous study found that Hnf4 $\alpha$  deficiency in

young-adult mice causes marked alteration of histone modifications, which is associated with induction of epigenetic modifiers such as Ezh2 and histone H3.3<sup>[20]</sup>. However, ChIP-seq data reveal no direct binding of Hnf4 $\alpha$  to these epigenetic modifiers in adult mouse livers, suggesting that these epigenetic modifiers may not be directly regulated by Hnf4 $\alpha$ . The present study provides the first evidence of the essential role of Hnf4 $\alpha$  in maintaining hepatic expression of certain microRNAs, including miR-101, miR-192, miR-193a, miR-194 and miR-802. These microRNAs target certain key proteins in gene regulation and epigenetic modifications, such as WT1 (by miR-193a)<sup>[72]</sup>, HNF1 $\beta$  (by miR-802)<sup>[75]</sup>, CHD1 (by miR-194) (Figure 9), EZH2 (by miR-101)<sup>[69]</sup>, SRSF2 (by miR-193a)<sup>[71]</sup>, and histone H3.3 (by miR-192) (Figure 9). Establishment and maintenance of hepatic expression of these microRNAs by HNF4 $\alpha$  may play a key role in the indirect regulation of hepatic transcriptome and epigenome by HNF4 $\alpha$  (Figure 10).

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## COMMENTS

### Background

Hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) is a liver-enriched master regulator of liver development and function. HNF4 $\alpha$  plays a key role in regulating hepatic transcriptome and epigenome. However, little was known about the role of HNF4 $\alpha$  in regulating hepatic expression of microRNAs, essential modulators of the transcriptome and epigenome. Additionally, HNF4 $\alpha$  deficiency causes marked induction of a large number of genes in mouse liver; however, the mechanism of suppression of hepatic gene expression by HNF4 $\alpha$  remains poorly understood.

### Research frontiers

Previous studies demonstrate that HNF4 $\alpha$  regulates hepatic expression of miR-122, miR-124 and miR-29.

### Innovations and breakthroughs

This is the first study to use microarray and liver-specific knockout mice to determine the genome-wide role of HNF4 $\alpha$  in the regulation of hepatic expression of microRNAs in mice. The key changes in hepatic microRNA expression induced by HNF4 $\alpha$  deficiency were verified by real-time polymerase chain reaction. Moreover, hepatic microRNA expression were correlated with chromatin accessibility as well as DNA-binding of HNF4 $\alpha$ , RNA polymerase II, and activating/silencing epigenetic signatures to determine the role of HNF4 $\alpha$  in regulating hepatic expression of these microRNAs. The novel key role of HNF4 $\alpha$  in regulating liver-predominant expression of miR-101-2/miR-101b and the miR-194-2/miR-192 cluster was confirmed by luciferase reporter assay.

### Applications

Results from this study uncover species differences and similarities between humans and mice in the role of HNF4 $\alpha$  in regulating hepatic expression of certain important microRNAs. Such novel knowledge will help understand the role of HNF4 $\alpha$  in post-transcriptional regulation of gene expression and maintenance of the normal epigenome and physiology in mouse and human liver.

### Terminology

Epigenetic signatures/marks are modifications of the genome that do not change

the underlying DNA sequence but can switch genes on and off and thus affect how cells express genes. Typical epigenetic signatures/marks include DNA methylation and histone modifications.

## Peer-review

The study appears to be properly conducted and written. No major criticisms and/or weaknesses were noted.

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Basic Study

## Antioxidant effects of aqueous extract of Salep on Paraquat-induced rat liver injury

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### Abstract

#### AIM

To evaluate the effects of aqueous extract of Salep on Paraquat-mediated liver injury.

#### METHODS

In this experimental study, 56 adult male Wistar rats were divided randomly to 7 groups as control, sham, and 5 experimental groups. In control group, rats did not receive any substance during experiment. In Sham group, rats were given distilled water according to their body weight and in experimental groups, Paraquat alone and with different doses of Salep aqueous extract

(40, 80, 160 and 320 mg/kg) was given intraperitoneal daily for 14 d. After that, liver biochemical parameter and histologic changes were analyzed and compared in different groups.

## RESULTS

Paraquat compared to control and sham groups, significantly ( $P < 0.05$ ) increased serum level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, malondialdehyde (MDA) and total oxidant capacity (TOC); while level of total protein, albumin and total antioxidant capacity (TAC) were remarkably decreased by Paraquat. Salep at doses of 80, 160 and 320 mg/kg significantly decreased serum level of ALT, AST, ALP, bilirubin, MDA and TOC and significantly increased total protein, albumin and TAC level as compared to Paraquat exposed group in dose dependent manner. Aqueous extract of Salep at doses of 40 mg/kg made no significant changes in serum level of mentioned biochemical parameters. Liver microscopic observation revealed that Paraquat could cause hepatocyte necrosis, degenerative changes, proliferation and activation of Kupffer cells (sporadically) which were reduced by Salep treatment.

## CONCLUSION

Salep possesses remarkable hepatoprotection activity against Paraquat-induced hepatic injury by having antioxidant activity and reducing lipid peroxidation and oxidative stress.

**Key words:** Salep; Paraquat; Liver injury; Antioxidant; Oxidative stress

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**Core tip:** Oxidative stress has a key role in triggering Paraquat-mediated liver injury. Paraquat causes oxidative stress *via* modulation of redox cycling, generation of free radicals and reduction of endogenous antioxidant levels. Salep from orchid family (Orchidaceae) used in traditional medicine as a healing agent in the treatment of breast disorders, gastrointestinal disorders, tuberculosis, diarrhea, Parkinson, cancer, fever, and impotency. Salep is used in food engineering for preparation of ice cream and drinks. This study showed that Salep could have a protective effect against Paraquat-induced hepatic injury *via* reinforcing endogenous antioxidant systems, reduction of lipid peroxidation and free radical scavenging. The antioxidant and protective effect of Salep could be due to presence of flavonoids and polyphenols such as Quercetin, Ferulic Acid and Glucomannan.

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## INTRODUCTION

With the increasing population of human societies, providing nourishment without the use of advanced scientific farming is impossible. In this modern agriculture, using pesticides, herbicides and chemical fertilizers for more and higher quality crop is inevitable and may be toxic to man and animals. Paraquat (1, 1'-dimethyl-4, 4'-bipyridylium-dichloride) is a widely used herbicide for broadleaf weed control<sup>[1]</sup> and is extremely poisonous for humans and animals and many cases of acute poisoning and death have been reported over the past few decades<sup>[2]</sup>.

Paraquat is a bipyridyl compound with high toxicity for lungs, kidney, brain and liver<sup>[3]</sup>. When it is given in acute dose (50 mg/kg) in mice, liver necrosis and inflammation will develop<sup>[4]</sup>. Paraquat toxicity is due to oxidative damage to cells and generation of free radicals<sup>[5]</sup>. Herbicidal activity of paraquat can be explained by its interfering with photosynthesis and intracellular electron transfer system in plants and prevention of NADP reduction to NADPH. This could disrupt important NADPH-dependent biochemical processes<sup>[6,7]</sup>. In addition, Paraquat radical forms superoxide anion in presence of oxygen which leads to production of more toxic reactive oxygen species like hydrogen peroxides and hydroxyl radical and would cause oxidative stress<sup>[1,8]</sup>. Superoxide anion may also attack unsaturated lipids of membrane to form fatty acid hydroperoxide, resulting in lipid peroxidation, membrane injury, cell death and multi-system toxicity<sup>[9]</sup>.

Due to the role of oxidative stress mechanisms in Paraquat toxicity and the lack of an effective antidote, researchers are currently focused on the importance of antioxidant in Paraquat poisoning management<sup>[10]</sup>. Many herbal compounds have antioxidant properties and can protect the liver from damaging agents like Paraquat. One of these plants is Salep from orchid family (Orchidaceae) which has different species worldwide<sup>[11]</sup>. Salep contains Quercetin, Nitrogenic materials, Ferulic acid, starch, protein, Glucomannan, Glucose, Daucosterol, Cirsilineol and steroids<sup>[11-13]</sup>. This plant is used in traditional medicine as a healing agent in the treatment of breast disorders, gastrointestinal disorders, tuberculosis, diarrhea, Parkinson, cancer, fever, and impotency. Salep is used in food engineering for preparation of ice cream and drinks<sup>[13-15]</sup>.

Polyphenols, especially flavonoids such as quercetin, are important antioxidants found in Salep<sup>[12]</sup>. These compounds have hepatoprotective effects against liver damage caused by toxins and free radicals<sup>[16]</sup> and can also protect cells against depletion of glutathione by increasing the capacity of antioxidant enzymes such as glutathione reductase, glutathione peroxide and catalase<sup>[17]</sup>. Furthermore, glucomannan can inhibit oxidative stress and

effectively reduce alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels<sup>[18]</sup>. Therefore, the aim of this survey was to evaluate the effects of aqueous extract of Salep on Paraquat - induced hepatotoxicity.

## MATERIALS AND METHODS

### Chemicals

Paraquate was purchased from Ara Shimi-Iran Company. Paraquat was dissolved in distilled water and animals were given intraperitoneal injection in each case. Malondialdehyde (MDA), total antioxidant capacity (TAC) and total oxidant capacity (TOC) measurement kits were purchased from Diametra Company (Italy), ALT, AST, alkaline phosphatase (ALP), albumin and bilirubin and total protein (TP) measurement kits were purchased from Pars Azmoon Company (Iran).

### Collection and extraction of Salep

Salep plants were obtained from farmlands around Yasouj (a city in the southwest of Iran). Salep roots were washed and dried in laboratory and mixed with Ethanol 96° in 1 to 5 proportions, mixed for 24 h at room temperature and a homogeneous mixture was obtained. Then, the uniform solution was filtered and dried for 48 h to obtain solid extract without ethanol. The final dried extract was dissolved with distilled water<sup>[12]</sup>.

### Experimental animals

Fifty-six adult male Wistar rats (180-200 g) were obtained from the Animal House of Jahrom University of Medical Sciences. The animal house temperature was maintained at 22 °C ± 2 °C with a 12 h light/dark cycle. All animals were kept for two weeks prior to experiment and had free access to food and water. All ethical points regarding working with laboratory animals were considered in this research (Ethical Code: IR.JUMS.1394.722).

### Experimental design

The rats were divided randomly to 7 groups, 8 rats each, as followed: Control group: Rats did not receive any substance during experiment; Sham group: Rats were given distilled water according to their body weight during the experiment; Experimental group 1: Rats were given Paraquat 2 mg/kg per BW; Experimental groups 2, 3, 4 and 5: Rats were given Paraquat at a dose of 2 mg/kg per BW daily and Salep at doses of 40, 80, 160 and 320 mg/kg per BW, respectively. Salep doses were selected based on previous studies done on this herbal treatment<sup>[12]</sup>; Paraquat and Salep aqueous extract were administered intraperitoneally daily for 14 d in all 5 groups.

### Blood sampling and liver function evaluation

At the end of the study (day 15) after weighing the animals, blood sample were taken directly from their

hearts using 5 cc syringes (rats were anesthetized by barbiturate) and blood serum was collected after centrifugation (15 min, 3000 rpm) and stored at -20 °C until they were tested. Biochemical measurement kits (made in Iran and Italy) using the colorimetric method and an autoanalyzer machine (Selectera XL model made in Holland) were used for assessment of biochemical factors including ALT, AST, ALP, TP, albumin, bilirubin, MDA, TOC and TAC.

### Histological examination

After drawing the blood, for histological examination a small part of liver was separated, fixed by 10% formalin and embedded in paraffin wax. Paraffin sections with thickness of 5 µm were prepared, stained employing the haematoxylin and eosin and Masson Trichrome stain methods and histological and pathological changes were studied using a light microscope. Furthermore, The Degree of inflammation in the portal zone, liver necrosis and inflammatory cell infiltration were evaluated in the form of semiquantitative scale, double-blind, according to the method described by Frei *et al.*<sup>[19]</sup> in 1984. Severity of damage were ranked from zero to four (zero: No damage, 1: Minimum damage, 2: Mild damage, 3: Average damage, 4: Severe damage). Scoring was performed in five microscopic fields of each cut, randomly, with magnification of × 100.

### Statistical analysis

All values were given as mean ± SEM. Statistical analysis was carried out using SPSS 21, One-way analysis of variance followed by Duncan *post hoc* test. Statistical *P*-value less than 0.05 was considered significant.

## RESULTS

### Biochemical measurement

Paraquat compared to control and sham groups significantly (*P* < 0.05) increased serum level of liver factors including ALT, AST and ALP, Bilirubin, MDA and TOC; while serum level of Total Protein, Albumin and TAC were considerably lower in group receiving Paraquat (Tables 1 and 2).

Paraquat treatment groups with aqueous extract of Salep at doses of 80, 160 and 320 mg/kg significantly decreased serum level of ALT, AST, ALP, Bilirubin, MDA and TOC and significantly increased elevated Total Protein, Albumin and TAC serum level as compared to Paraquat treatment group alone (Tables 1 and 2). Aqueous extract of Salep at doses of 40 mg/kg made no significant changes in serum level of mentioned biochemical parameters while the greatest effect is related to the dose of 320 mg/kg of Salep.

### Histopathological examination

Microscopic examination of liver tissue of control and sham groups showed that liver tissue structure was normal and healthy (normal structure of lobules with



**Table 1** The serum levels of liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), bilirubin, malondialdehyde and total oxidant capacity in different study groups

Group/parameter	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Bilirubin (mg/dL)	MDA (nmol/L)	TOC (IU/mL)
Control	183.4 ± 2.19	95.4 ± 2.52	138.4 ± 4.55	0.8 ± 0.02	0.13 ± 0.005	0.18 ± 0.01
Sham	185.5 ± 2.19	94.7 ± 3.00	138.9 ± 5.01	0.8 ± 0.02	0.13 ± 0.007	0.18 ± 0.01
Paraquat at 2 mg/kg	563.2 ± 11.43 <sup>a</sup>	265.5 ± 7.48 <sup>a</sup>	736.1 ± 4.21 <sup>a</sup>	2.4 ± 0.05 <sup>a</sup>	3.36 ± 0.06 <sup>a</sup>	2.02 ± 0.05 <sup>a</sup>
Paraquat + Salep at 40 mg/kg	536.4 ± 14.44 <sup>b</sup>	252.5 ± 6.25 <sup>b</sup>	730.0 ± 9.30 <sup>b</sup>	2.3 ± 0.04 <sup>b</sup>	3.27 ± 0.03 <sup>b</sup>	1.98 ± 0.05 <sup>b</sup>
Paraquat + Salep at 80 mg/kg	517.2 ± 7.30 <sup>b,c</sup>	234.7 ± 7.14 <sup>b,c</sup>	709.0 ± 9.14 <sup>b,c</sup>	2.0 ± 0.04 <sup>b,c</sup>	2.92 ± 0.13 <sup>b,c</sup>	1.77 ± 0.05 <sup>b,c</sup>
Paraquat + Salep at 160 mg/kg	460.5 ± 12.01 <sup>b,c</sup>	209.4 ± 4.55 <sup>b,c</sup>	626.4 ± 6.74 <sup>b,c</sup>	1.7 ± 0.03 <sup>b,c</sup>	2.48 ± 0.05 <sup>b,c</sup>	1.55 ± 0.04 <sup>b,c</sup>
Paraquat + Salep at 320 mg/kg	376.5 ± 12.07 <sup>b,c</sup>	166.0 ± 3.75 <sup>b,c</sup>	428.1 ± 7.25 <sup>b,c</sup>	1.1 ± 0.03 <sup>b,c</sup>	1.22 ± 0.04 <sup>b,c</sup>	1.20 ± 0.03 <sup>b,c</sup>

<sup>a</sup>Significant difference between Sham and Paraquat-exposed Group; <sup>b</sup>Significant difference between Sham and Paraquat + Salep treated Group; <sup>c</sup>Significant difference between Paraquat-exposed and Paraquat + Salep Treated Group (Based on Duncan's test). The means are presented in the form of Mean ± SEM. *P* < 0.05 is considered statistically significant. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; MDA: Malondialdehyde; TOC: Total oxidant capacity.

**Table 2** The serum levels of albumin, total protein and total antioxidant capacity in different study groups

Group/parameter	Total protein (g/dL)	Albumin (g/dL)	TAC (IU/mL)
Control	8.0 ± 0.19	5.0 ± 0.13	1.11 ± 0.02
Sham	7.9 ± 0.26	5.1 ± 0.18	1.6 ± 0.04
Paraquat at 2 mg/kg	4.2 ± 0.09 <sup>a</sup>	2.5 ± 0.06 <sup>a</sup>	0.40 ± 0.02 <sup>a</sup>
Paraquat + Salep at 40 mg/kg	4.1 ± 0.05 <sup>b</sup>	2.6 ± 0.5 <sup>b</sup>	0.43 ± 0.02 <sup>b</sup>
Paraquat + Salep at 80 mg/kg	5.0 ± 0.10 <sup>b,c</sup>	3.1 ± 0.04 <sup>b,c</sup>	0.66 ± 0.03 <sup>b,c</sup>
Paraquat + Salep at 160 mg/kg	5.7 ± 0.09 <sup>b,c</sup>	3.6 ± 0.07 <sup>b,c</sup>	0.82 ± 0.05 <sup>b,c</sup>
Paraquat + Salep at 320 mg/kg	6.6 ± 0.11 <sup>b,c</sup>	5.0 ± 0.07 <sup>b,c</sup>	1.10 ± 0.03 <sup>b,c</sup>

<sup>a</sup>Significant difference between Sham and Paraquat-exposed Group; <sup>b</sup>Significant difference between Sham and Paraquat + Salep treated Group; <sup>c</sup>Significant difference between Paraquat-exposed and Paraquat + Salep treated Group (Based on Duncan's test). The means are presented in the form of Mean ± SEM. *P* < 0.05 is considered statistically significant. TAC: Total antioxidant capacity.

normal central venous, sinusoids and Kupffer cells and normal distribution of glycogen and lack of lymphocytic infiltration and congestion in the blood vessels) (Figure 1A-D).

In study group 3 (rats were given Paraquat alone), microscopic observation revealed hepatocyte necrosis, degenerative changes, proliferation and activation of Kupffer cells (sporadically), increased infiltration of inflammatory cells around the portal vein and in sinusoid space, formation of fibrotic inflamed bridges between liver lobules, and sever cellular ballooning and blood congestion in the sinusoids. In this group, progressive liver fibrosis had occurred as evidenced by presence of collagen fibers in the liver parenchyma, the portal space and around the central vein in the centrilobular region (Figure 1E-J).

Treatment with aqueous extract of Salep reduced the damaging effect of Paraquat on liver tissue. This reduction in destructive effect of Paraquat on liver tissue was mild with Salep at doses of 80 mg/kg, moderate at doses of 160 mg/kg and highest at doses of 320 mg/kg as compared to study group that received Paraquat alone (Figure 1K-R). A microscopic observation of liver tissue of rats under study have been brought to a quantitatively in Table 3.

## DISCUSSION

Oxidative stress has a key role in triggering Paraquat-mediated liver injury<sup>[20]</sup>. Paraquat causes oxidative stress *via* modulation of redox cycling, generation of free radicals and reduction of endogenous antioxidant levels<sup>[21-23]</sup>. Furthermore, generation of nitric oxide and reactive oxygen species like superoxide also play a crucial role in Paraquat - induced hepatotoxicity<sup>[24]</sup>. Salep could have protective effect against chemical induced liver injury *via* reinforcing endogenous antioxidant systems and free radical scavenging<sup>[12,16]</sup>. As liver is one of the major sites of Paraquat toxicity, this study was done in order to evaluate protective potential of Salep against Paraquat- induced liver injury.

Remarkable increase of ALT, AST, ALP and bilirubin and significant decrease of total protein and Albumin levels were observed in Paraquat - exposed group in comparison with control group, which confirmed the hepatotoxic potential of Paraquat. These results were in concurrence with previous studies on evaluation of Paraquat induced liver toxicity, which showed increase in serum level of liver enzymes<sup>[21,25]</sup>. Significant reduction of increased level of ALT, AST, ALP and bilirubin and marked increased in level of Albumin and Total Protein in Paraquat + Salep treated groups showed that Salep

**Table 3** The effect of aqueous extract of Salep roots on Paraquat - induced rat liver injury

Group/damage score	Control	Sham	Paraquat 2 mg/kg	Paraquat + Salep at 40 mg/kg	Paraquat + Salep at 80 mg/kg	Paraquat + Salep at 160 mg/kg	Paraquat + Salep at 320 mg/kg
Portal congestion and inflammation							
Score 0	8	8	0	0	0	0	1
Score 1	0	0	0	0	0	0	5
Score 2	0	0	0	1	2	3	1
Score 3	0	0	2	2	2	1	1
Score 4	0	0	6	5	4	4	0
Necrosis							
Score 0	8	8	0	0	0	0	1
Score 1	0	0	0	0	0	1	4
Score 2	0	0	1	2	2	2	3
Score 3	0	0	2	1	2	2	0
Score 4	0	0	5	5	4	3	0
Interstitial infiltration of inflammatory cells							
Score 0	8	8	0	0	0	0	1
Score 1	0	0	0	0	0	1	4
Score 2	0	0	1	1	1	3	2
Score 3	0	0	1	2	3	2	1
Score 4	0	0	6	5	4	2	0

Zero: No damage; 1: Minimum damage; 2: Mild damage; 3: Average damage; 4: Severe damage. Scoring was performed in five microscopic fields of each cut, randomly, with magnification of  $\times 100$ .

could have protective effect against Paraquat-mediated hepatic injury in dose dependent manner. These results are supported by a previous report which also revealed the protective effect of Salep on liver function<sup>[12]</sup>.

Liver pathological examination showed hepatocyte necrosis, proliferation and activation of Kupffer cells, increased infiltration of inflammatory cells around the portal vein and in sinusoid space, formation of fibrotic inflamed bridges between liver lobules, and sever cellular ballooning and blood congestion in the sinusoids in Paraquat - exposed group, which was in accordance with previous reports<sup>[3,20]</sup>. Remarkable recovery toward normal liver histology in Paraquat + Salep treated groups also favored the protective activity of Salep against Paraquat-induced liver injury.

As oxidative stress has a crucial role in Paraquat-induced liver injury, in this study we evaluated serum level of TOC, which precisely shows the oxidant status of blood, and TAC, as indicator of blood, cells and tissues defense system against free radicals, measures the antioxidant capacity of all antioxidants in a biological sample and not just the antioxidant capacity of a single compound. Measurement of TAC can provide information on overall antioxidant status, which may include those antioxidants not yet recognized or not easily measured<sup>[20,26]</sup>. Significant augmentation of TOC and reduction of TAC were observed in Paraquat-exposed groups, which confirmed the role of free radical generation and attenuation of antioxidant level in Paraquat-mediated hepatic injury. Significant reduction of TOC and marked increased of TAC in Paraquat + Salep treated groups demonstrated that Salep could have protective effects against Paraquat toxicity by possessing antioxidant activity. These results were supported by a previous study done by Pourahmad

*et al*<sup>[12]</sup>. The antioxidant effect of Salep could be due to the presence of flavonoids and polyphenols such as Quercetin, Ferulic Acid and Glucomannan<sup>[11,16,18]</sup>. The two latter components of Salep could also reduce serum level of liver enzymes such as ALT and AST<sup>[27,28]</sup>. Zhang *et al*<sup>[16]</sup> showed that Quercetin could have hepatoprotective and antioxidant activity by decreasing lipooxygenase, free radical scavenging, enhancing the expression of antioxidant transcription factor and antioxidant enzyme such as Thioredoxin and Peroxiredoxin.

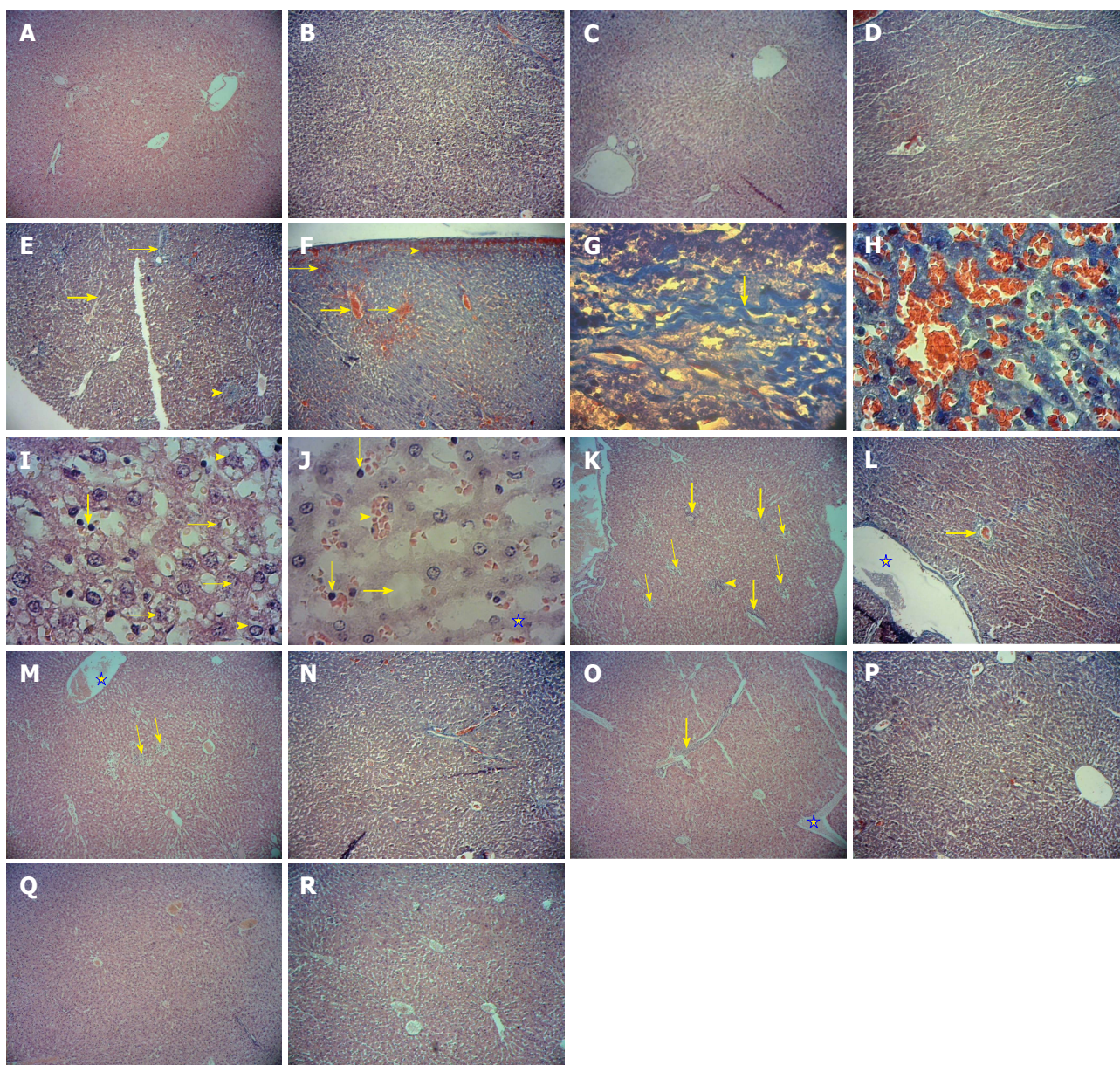
Furthermore, part of Paraquat hepatotoxicity is related to lipid peroxidation due to free radical generation including Oxygen Reactive Species<sup>[24]</sup>. MDA works as an indicator of lipid peroxidation and oxidative stress assessment<sup>[29]</sup>. MDA level was significantly augmented in Paraquat - exposed group which was in accordance with previous studies<sup>[6]</sup>. Serum level of MDA was substantially decreased in Paraquat + Salep treated groups as compared with Paraquat - exposed group in dose dependent manner. This result also supported the protective activity of Salep against oxidative stress and lipid peroxidation caused by Paraquat.

In conclusion, based on our results, it could be concluded that Salep possesses remarkable hepatoprotection activity against Paraquat-induced liver injury and could reduce the damaging effect of Paraquat on liver by having antioxidant activity and reducing lipid peroxidation and oxidative stress. Further studies are required to evaluate protective and antioxidant effect of Salep in human.

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**Figure 1 Microscopic views of the liver tissue in study Groups.** Five micron paraffin sections were prepared, stained employing the haematoxylin and eosin stain and histological and pathological changes were studied using a light microscope. A: Control: With a natural structure (Hematoxylin-eosin, 40 × magnification); B: Control: With a natural structure and leukocyte infiltration and congestion cannot be seen (Masson trichrome, 40 × magnification); C: Sham: With a natural structure and Central venous congestion cannot be seen (Hematoxylin-eosin, 40 × magnification); D: Sham: With a natural structure and Collagen fibers cannot be seen. (Masson trichrome, 40 × magnification); E: Paraquat 2 mg/kg: Formation of fibrotic inflamed bridges between liver lobules (thin arrow), the loss of cellular order toward the center (wide arrow), accumulation of collagen fibers and inflammatory cells around the centrilobular vein (arrowhead) (Masson trichrome, 40 × magnification); F: Paraquat 2 mg/kg: Enlarged and congested centrilobular vein (wide arrow), congestion in the sinusoids (thin arrow) (Masson trichrome, 40 × magnification); G: Paraquat 2 mg/kg: Accumulation and progressive of collagen fibers in the liver parenchyma (Masson trichrome, 400 × magnification); H: Paraquat 2 mg/kg: Sever congestion in the sinusoids (Masson trichrome, 400 × magnification); I: Paraquat 2 mg/kg: Sever cellular ballooning (arrowhead), degenerative changes (thin arrow), proliferation and activation of Kupffer cells (wide arrow), (Hematoxylin-eosin, 400 × magnification); J: Paraquat 2 mg/kg: Activation of Kupffer cells (thin arrow), sever congestion in the sinusoids (arrowhead), degenerative changes (asterisk), enlargement of sinusoids space (wide arrow), (Hematoxylin-eosin, 400X magnification); K: Paraquat + Salep at 40 mg/kg: Infiltration of inflammatory cells around the centrilobular vein (wide arrow), Infiltration of inflammatory cells around the portal space (arrowhead), degenerative changes (thin arrow), (Hematoxylin-eosin, 40 × magnification); L: Paraquat + Salep at 40 mg/kg: Enlargement and congested centrilobular vein (asterisk), accumulation of collagen fibers around the portal space (wide arrow) (Masson trichrome, 40X magnification); M: Paraquat + Salep at 80 mg/kg: Degenerative changes (thin arrow), congested centrilobular vein (asterisk) (Hematoxylin-eosin, 40 × magnification); N: Paraquat + Salep at 80 mg/kg: Decreased infiltration of inflammatory cells around the portal, decreased congestion in the sinusoids and more regular cellular order toward the center (Masson trichrome, 40 × magnification); O: Paraquat + Salep at 160 mg/kg: Infiltration of inflammatory cells around the portal space (wide arrow), congested centrilobular vein (asterisk), (Hematoxylin-eosin, 40 × magnification); P: Paraquat + Salep at 160 mg/kg: More regular cellular order toward the center, more decreased congestion of sinusoids and more decreased infiltration of inflammatory cells in the liver parenchyma (Masson trichrome, 40 × magnification); Q: Paraquat + Salep at 320 mg/kg: Its tissues seem relatively healthy, without any certain pathological changes (Hematoxylin-eosin, 40 × magnification); R: Paraquat + Salep at 320 mg/kg: Its tissues seem relatively healthy, without any certain pathological changes (Masson trichrome, 40 × magnification).



## COMMENTS

### Background

Paraquat is a common herbicide used in agriculture and could cause severe damage to the lungs, liver and other tissues in mammals. Oxidative stress has a key role in triggering Paraquat-mediated hepatotoxicity. Salep could have protective effect against chemical induced hepatotoxicity via reinforcing endogenous antioxidant systems and free radical scavenging.

### Research frontiers

In the present study, the authors found that Salep possesses remarkable hepatoprotection activity against Paraquat-induced liver injury and could reduce damaging effect of Paraquat on liver by having antioxidant activity and reducing lipid peroxidation and oxidative stress.

### Innovations and breakthroughs

This is the first study evaluating the effect of Salep on Paraquat-induced liver injury. This study investigates the protective and antioxidant effect of salep on liver damage caused by Paraquat. The results of current study demonstrated that Salep could ameliorate paraquate-mediated liver injury by having antioxidant activity and reducing lipid peroxidation and oxidative stress.

### Applications

Salep aqueous extract could reduce damaging effect of Paraquat on liver tissue by having significant antioxidant activity. Therefore, the results of this study showed that Salep can be introduced as an alternative to chemical agents as potential therapeutic strategies for Paraquate-induced liver injury.

### Terminology

Oxidative stress is essentially an disturbance in balance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidant which could cause tissue damage including liver. Lipid peroxidation is a crucial step in the pathogenesis of several disease states in adult and infant patients. Lipid peroxidation is a process mainly caused by the effect of reactive oxygen species including hydroxyl radical and hydrogen peroxide. These reactive oxygen species readily attack the polyunsaturated fatty acids of the cell membrane, initiating a self-propagating chain reaction. The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are dangerous for the viability of cells, even tissues.

### Peer-review

The paper by Atashpour *et al* has an interesting rationale and a good background. The results presented are consistent with the effects of the different components of the Salep.

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**Clinical Trials Study**

# Phase 3 trial of first generation protease inhibitor therapy for hepatitis C virus/human immunodeficiency virus coinfection

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**Institutional review board statement:** The study was performed in the NIH AIDS Clinical Trials Group network (ACTG, National Institutes of Health Registration number NCT01482767) with enrollment of participants at 42 sites across the United States. The study was conducted with approval of

Institutional Review Boards (IRB) at each individual site.

**Clinical trial registration statement:** This study is registered at ClinicalTrials.gov. The registration identifier is NCT01482767.

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

**Conflict-of-interest statement:** Sherman KE serves on a Merck Advisory Board (paid to institution); the other authors declare no conflict of interests.

**Data sharing statement:** No additional data are available.

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## Abstract

### AIM

To evaluate efficacy/safety of hepatitis C virus (HCV) protease inhibitor boceprevir with pegylated interferon (PEG-IFN) alfa and weight-based ribavirin (RBV) in a phase 3 trial.

### METHODS

A prospective, multicenter, phase 3, open-label, single-arm study of PEG-IFN alfa, weight-based RBV, and boceprevir, with a PEG-IFN/RBV lead-in phase was performed. The HCV/human immunodeficiency virus coinfecting study population included treatment naïve (TN) and treatment experienced (TE) patients. Treatment duration ranged from 28 to 48 wk dependent upon response-guided criteria. All patients had HCV Genotype 1 with a viral load > 10000 IU/mL. Compensated cirrhosis was allowed. Sample size was determined to establish superiority to historical (PEG-IFN plus RBV) rates in sustained viral response (SVR).

### RESULTS

A total of 257 enrolled participants were analyzed (135 TN and 122 TE). In the TN group, 81.5% were male and 54.1% were black. In the TE group, 76.2% were male and 47.5% were white. Overall SVR12 rates (HCV RNA < lower limit of quantification, target not detected, target not detected) were 35.6% in TN and 30.3% in TE. Response rates at SVR24 were 28% in TN and 10% in TE, and exceeded those in historical controls. The highest rate was observed in TN non-cirrhotic participants (36.8% and the lowest in TE cirrhotics (26.3%). Cirrhotic TN participants had a 27.8% SVR12 rate and 32.1% of TE non-cirrhotics achieved SVR12. Significantly lower response rates were observed among black participants; in the TE, SVR12 was 39.7% in white participants but only 13.2% of black subjects ( $P = 0.002$ ). Among the TN, SVR12 was 42.1% among whites and 27.4% among blacks ( $P = 0.09$ ).

### CONCLUSION

The trial met its hypothesis of improved SVR compared to historical controls but overall SVR rates were low. All-oral HCV treatments will mitigate these difficulties.

**Key words:** Human immunodeficiency virus; Hepatitis C virus; Boceprevir; Pegylated interferon alfa; Ribavirin

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**Core tip:** Approval of first generation hepatitis C virus (HCV) protease inhibitors has initiated a change in care of HCV infected patients. Phase 2 trials in HCV/human immunodeficiency virus coinfecting patients have suggested improved efficacy and tolerability for regimens that combined pegylated interferon (PEG-IFN) + ribavirin (RBV) with either boceprevir or telaprevir. We evaluated an HCV treatment regimen using a first generation HCV protease inhibitor (boceprevir) with PEG-IFN, and weight-based RBV in a phase 3 treatment trial, including HCV

treatment-naïve and treatment-experienced coinfecting subjects. While sustained viral response rates were low overall they did exceed historical PEG-IFN/RBV rates. Use of new interferon-free direct acting antiviral agents modalities in this population is indicated.

Sherman KE, Kang M, Sterling R, Umbleja T, Marks K, Kiser JJ, Alston-Smith B, Greaves W, Butt AA. Phase 3 trial of first generation protease inhibitor therapy for hepatitis C virus/human immunodeficiency virus coinfection. *World J Hepatol* 2017; 9(4): 217-223 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i4/217.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i4.217>

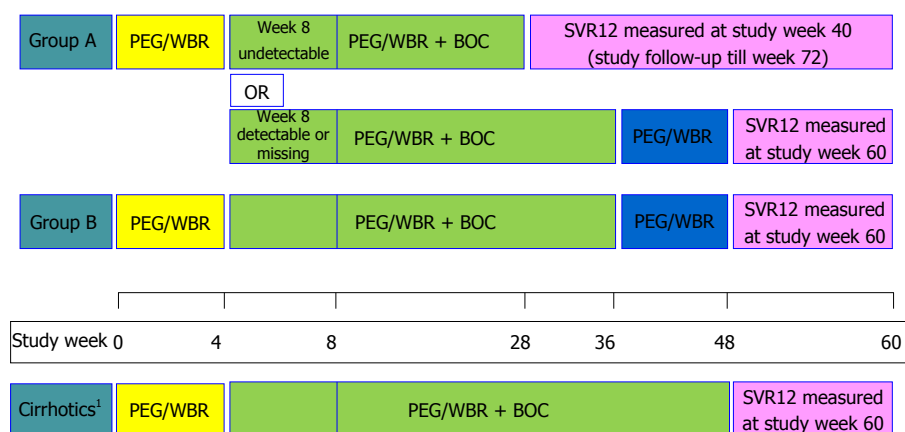
## INTRODUCTION

Hepatitis C virus (HCV) coinfection is a major cause of morbidity and mortality among those with human immunodeficiency virus (HIV) infection<sup>[1-4]</sup>. Prior to the emergence of new HCV targeted direct acting antiviral agents (DAAs) in 2011, response to standard therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) was poor, both in terms of efficacy and medication tolerability<sup>[5]</sup>. The approvals of first generation serine protease inhibitors of HCV replication initiated a revolution in terms of the care and management of HCV infected patients. Phase 2 trials in HCV/HIV coinfecting patients suggested improved efficacy with moderate drug tolerability for treatment regimens that combined either boceprevir or telaprevir with PEG-IFN + RBV<sup>[6,7]</sup>. In an effort to define treatment efficacy with response- and cirrhosis-guided regimens in HCV/HIV coinfecting, we conducted a prospective, multicenter, open-label Phase 3 trial in both HCV treatment naïve and treatment experienced participants with comparison to historical controls in the same clinical trials network.

## MATERIALS AND METHODS

The study was performed in the NIH AIDS Clinical Trials Group network (ACTG, National Institutes of Health Registration number NCT01482767) with enrollment of participants at 42 sites across the United States. All participants provided informed consent and the study was conducted with approval of Institutional Review Boards at each site. The study was monitored by an independent, NIH-chartered data safety and monitoring board.

The overall study design is shown in Figure 1. Briefly, treatment naïve (TN) participants (Group A) were treated with PEG-IFN alfa 2b 1.5 µg/kg subcutaneously with weight-based ribavirin (800-1400 mg/d) for 4 wk (lead-in). Then boceprevir 800 mg tid was added to the treatment regimen. Cirrhotic participants received 44 wk of triple therapy. Among non-cirrhotics, the week 8 serum HCV RNA was used to determine total duration of therapy. Those who had undetectable HCV RNA at week



**Figure 1 Overall study design.** Group A refers to treatment naïve participants while Group B refers to treatment experienced participants. PEG/WBR treatment is pegylated-interferon alpha 2b (PEG-IFN) and weight-based ribavirin (WBR). <sup>1</sup>Cirrhotic participants received 44 wk of triple therapy. SVR12: HCV RNA < LLOQ, target not detected at 12 wk post treatment discontinuation; BOC: Boceprevir; SVR: Sustained viral response; HCV: Hepatitis C virus; LLOQ: Lower limit of quantification.

8 completed therapy at week 28. Those with detectable HCV RNA at week 8 received 32 wk of triple therapy followed by 12 additional weeks of double-drug therapy with PEG-IFN/RBV. Treatment experienced participants (TE) (Group B) also had lead-in followed by 32 wk of triple therapy and 12 wk of PEG-IFN/RBV double therapy if non-cirrhotic, or by 44 wk of triple therapy if cirrhotic. Treatment was to be discontinued due to failure if: (1) HCV RNA  $\geq 100$  IU/mL at week 12; (2) detectable HCV RNA at week 24; or (3) confirmed HCV RNA > 1000 IU/mL any time after week 12. HCV RNA was determined to be undetectable if below the lower limit of quantification (LLOQ) and target not detected (TND) by Roche COBAS<sup>®</sup> TaqMan<sup>®</sup> HCV Test v2.0.

Key inclusion criteria included HCV genotype 1 with HCV RNA  $\geq 10000$  IU/mL. All participants underwent either liver biopsy or non-invasive marker (FibroSure<sup>®</sup>) testing to determine whether or not cirrhosis was present. Cirrhotics were confirmed to have stage A Child-Pugh disease. HIV RNA viral load was required to be < 50000 copies/mL for participants not on antiretroviral therapy, or less than 50 copies/mL for those on an approved antiretroviral regimen. A CD4<sup>+</sup> T-cell count of > 200 cells/mm<sup>3</sup> was also required within 42 d of study entry. Approved regimens included efavirenz, raltegravir, lopinavir/ritonavir, atazanavir/ritonavir or darunavir/ritonavir plus a dual nucleoside reverse transcriptase inhibitor backbone that did not include zidovudine or didanosine. Key exclusion criteria were those with mixed HCV genotypes, prior use of HCV protease or polymerase inhibitors or the presence of decompensated liver disease. Also excluded were other known causes of significant liver disease including HBV, HAV, hemochromatosis, or alpha-1 antitrypsin deficiency.

Data were centrally submitted and analyzed using SAS 9.4 (SAS Institute, Cary, NC, United States). The key outcome measure was sustained viral response in each study group and how the estimates compared to those in historical controls from a prior study of PEG-IFN plus RBV therapy (ACTG 5178). The study was powered

to conclude that sustained viral response (SVR) is greater than 28% in TN and 10% in treatment experienced participants, based on A5178 results on HCV genotype 1 participants. The SVR proportions were estimated with two-sided 95% Wilson confidence intervals (CI), and Fisher's exact tests were conducted for comparisons between groups. The analyses included all participants who met the eligibility criteria and initiated the study treatment.

## RESULTS

The baseline characteristics of the TN and TE participants as well as the historical controls are shown in Table 1. A total of 257 enrolled participants were analyzed: 135 TN (Group A) and 122 TE (Group B). The study included primarily middle-age males. There was a high representation of black/African-American participants, and this was accompanied by a similarly high percentage of IL28b genotypes carrying the "T" allele. Median CD4 counts were above 600 cell/mm<sup>3</sup> in both groups, corresponding to the high rate of active antiretroviral therapy (> 95%). There were more participants with cirrhosis in TE than in TN, in both A5294 and historical controls.

Overall SVR12 (HCV RNA < LLOQ, TND (target not detected) at 12 wk post treatment discontinuation) rates were 35.6% (95%CI: 28.0-43.9%) in TN and 30.3% (95%CI: 22.9%-39.0%) in TE (Table 2). Rates of response exceeded SVR24 in historical controls: 28% in TN and 10% in TE. The highest rate was observed in TN non-cirrhotic participants (36.8%, 95%CI: 28.6%-45.8%) and the lowest in TE cirrhotic participants (26.3%, 95%CI: 15.0%-42.0%). Cirrhotic TN participants had a 27.8% (95%CI: 12.5%-50.9%) SVR12 rate and 32.1% (95%CI: 23.1%-42.7%) of TE non-cirrhotics achieved SVR12. Race was a significant factor in treatment outcome. Indeed, among TE, SVR12 was noted to occur in 39.7% of white participants but in only 13.2% of those identified as black ( $P = 0.002$ ). Among TN, SVR12 was 42.1% among whites and 27.4% among blacks ( $P =$



**Table 1 Demographic and laboratory characteristics *n* (%)**

Characteristic	A5294		Historical controls	
	Treatment naïve ( <i>n</i> = 135)	Treatment Exp ( <i>n</i> = 122)	Treatment naïve ( <i>n</i> = 183)	Treatment Exp ( <i>n</i> = 87)
Age (yr)				
Median	51	53	48	48
Q1, Q3	44, 57	49, 57	41, 52	42, 51
Sex				
Male	110 (81.5)	93 (76.2)	151 (82.5)	74 (85.1)
Female	25 (18.5)	29 (23.8)	32 (17.5)	13 (14.9)
IV drug history				
Never	71 (52.6)	70 (57.4)	73 (39.9)	39 (44.8)
Currently	0	0	4 (2.2)	1 (1.1)
Previously	64 (47.4)	52 (42.6)	106 (57.9)	47 (54.0)
Race				
Asian	2 (1.5)	2 (1.6)	1 (0.5)	0
Black or African American	73 (54.1)	53 (43.4)	91 (49.7)	24 (27.6)
White	57 (42.2)	58 (47.5)	79 (43.2)	59 (67.8)
American Indian	0	2 (1.6)	3 (1.6)	1 (1.1)
More than One Race	2 (1.5)	2 (1.6)	5 (2.7)	0
Unknown	1 (0.7)	5 (4.1)	4 (2.2)	3 (3.4)
BMI (kg/m <sup>2</sup> )				
Median	26.3	27.5	25.7	26
Q1, Q3	22.6, 29.6	25.0, 31.0	22.9, 29.4	23.6, 30.1
Missing	1	0	0	0
IL28b genotype (RS 12979860)				
c/c	32 (25.2)	31 (27.2)	38 (33.9)	19 (31.1)
c/t	61 (48.0)	39 (34.2)	51 (45.5)	30 (49.2)
t/t	34 (26.8)	44 (38.6)	23 (20.5)	12 (19.7)
Missing	8	8	71	26
CD4 (cells/mm <sup>3</sup> )				
Median	646	621.5	495	520
Q1, Q3	462, 818	488.5, 858.5	373, 697	368, 706
Missing	2	2	0	0
HIV RNA quantitation				
Unquantifiable	133 (100.0)	113 (92.6)	129 (70.5)	71 (81.6)
Quantifiable	0	9 (7.4%)	54 (29.5)	16 (18.4)
Missing	2	0	0	0
HCV RNA (log <sub>10</sub> IU/mL)				
Median	6.7	6.9	6.5	6.6
Q1, Q3	6.2, 7.1	6.5, 7.3	6.1, 6.8	6.3, 7.0
Missing	1	0	0	0
Cirrhosis				
Yes	18 (13.3)	38 (31.1)	20 (10.9)	18 (20.7)
No	117 (86.7)	84 (68.9)	163 (89.1)	69 (79.3)
Baseline cART regimen				
No ART	2 (1.5)	6 (4.9)	40 (21.9)	11 (12.6)
EFV + 2 NRTIs	58 (43.0)	51 (41.8)	NA	NA
RAL + 2 NRTIs	47 (34.8)	45 (36.9)	NA	NA
LPV/RTV + 2 NRTIs	4 (3.0)	4 (3.3)	NA	NA
ATV/RTV + 2 NRTIs	18 (13.3)	10 (8.2)	NA	NA
DRV/RTV + 2 NRTIs	6 (4.4)	6 (4.9)	NA	NA

NA: A5178 did not have cART restrictions; Exp: Experienced; Q1, Q3: First quartile (Q1), third quartile (Q3); BMI: Body mass index; rs: Reference sequence for single nucleotide polymorphism; ART: Antiretroviral therapy; EFV: Efavirenz; NRTIs: Nucleoside reverse transcriptase inhibitors; RAL: Raltegravir; LPV: Lopinavir; RTV: Ritonavir; ATV: Atazanavir; DRV: Darunavir; cART: Combination antiretroviral therapy.

0.09). Treatment discontinuation rates were high in all groups and were attributed to a mix of treatment failure per HCV viral load criteria or due to adverse events. Among TN, there was one death unrelated to the study, 42 (31%) treatment failures leading to early discontinuation, and additional 22 (16%) premature treatment discontinuations due to adverse events. In TE, there were 52 treatment failures (43%), additional 16 (13%) premature treatment discontinuations due to adverse events, and no deaths. The most commonly

reported adverse events of grade 3 or higher included hematologic laboratory events (44% in TN and 48% in TE), and general body (chills, fatigue, pain, weight loss; 23% in TN and 22% in TE), gastrointestinal (4% in TN and in 3% TE) and neurologic (7% in TN and 5% in TE) symptoms. HIV breakthrough was rare and only two study participants (both on raltegravir regimen) met predetermined criteria for this event.

Among TN, the highest SVR rates were observed among participants whose cART regimen included rito-

**Table 2 Sustained viral response rates**

A5294 participants (n = 257)	% SVR12
Treatment naïve (n = 135)	35.6
Non-cirrhotic (n = 117)	36.8
Cirrhotic (n = 18)	27.8
Treatment experienced (n = 122)	30.3
Non-cirrhotic (n = 84)	32.1
Cirrhotic (n = 38)	26.3

navir - boosted atazanavir with a 2 nucleoside/nucleotide backbone. Overall SVR12 rate in this group (n = 18) was 61.1% (95%CI: 38.6%-79.7%) which was significantly higher than SVR12 rates among participants receiving other cART regimens combined (P = 0.018) in a post-hoc analysis. However, we note that this was an exploratory analysis on a small subset not adjusted for baseline co-variables, and this effect was not observed in TE.

## DISCUSSION

HCV/HIV coinfection remains a serious medical problem characterized by a high global disease burden (4-5 million) of patients who are at risk for increased fibrotic progression, cirrhosis, and hepatocellular carcinoma<sup>[8]</sup>. Coinfected patients also have significant non-hepatic complications including increased cardiovascular risk<sup>[9]</sup>. Therefore, HCV cure is a priority in the management of coinfecting HCV/HIV patients. The emergence of new DAAs for HCV has been a rapid and turbulent process which followed years of stagnation in the field. It is not surprising that new therapeutic regimens have been under investigation, even as earlier regimens were entering confirmatory clinical trials. The primary Phase 2 trial for boceprevir/PEG-IFN/RBV was initiated in 2010 and results were reported in July 2013<sup>[6]</sup>. Planning for the Phase 3 trial reported in this publication began in 2011, and the study completed in early 2015. During this brief interlude, even more effective, shorter duration regimens were studied and brought to the marketplace.

Despite this rapid advancement in therapy, the Phase 3 trial met its primary goals and moved the field forward in a number of key aspects. First, it again demonstrated the importance of Phase 3 trials which often reveal efficacy levels that fall short of their Phase 2 predecessors. The Phase 2 HCV/HIV coinfection trial of the boceprevir/PEG-IFN/RBV regimen yielded an SVR rate of 63%. This is significantly higher than what we observed in the Phase 3 trial which enrolled a population more representative of the United States HCV/HIV population at large in terms of racial distribution. Indeed, the proportion of black participants in this study (49%) is higher than the imputed racial distribution of HCV/HIV coinfecting patients in United States (23%-33%) based upon a 2002 analysis<sup>[10]</sup>. It also exceeds the black representation in the previously reported Phase 2 trial<sup>[6]</sup>. Our treatment population was more male, more non-white, with a higher representation of the IL28b T allele and with more advanced fibrosis/cirrhosis than the population enrolled

in the previously reported Phase 2 study. Our findings of a lower SVR in this population is similar to that reported in "real world" analyses using first generation protease inhibitors<sup>[11]</sup>.

Interestingly, we observed a higher SVR12 among treatment-naïve subjects whose cART regimen consisted of ritonavir boosted atazanavir + a dual NRTI backbone. Pharmacokinetic data indicates that boceprevir AUC was reduced 32% when administered with ritonavir-boosted darunavir while atazanavir AUC decreased only 5%<sup>[12]</sup>. While we cannot categorically state that this difference affected overall SVR, we suspect it represents an important factor in treatment outcomes among treatment naïve patients. The lack of this finding in treatment experienced participants may represent the overall decreased effectiveness of the PEG-IFN component in that group which masks more subtle effects related to HCV protease inhibitor pharmacokinetics.

Interferon-based therapy is difficult to tolerate and this is clearly demonstrated by the high drop-out rate seen in our study cohort. Though some guidelines and insurers still encourage use of PEG-IFN in some treatment groups, this approach may be particularly detrimental in the HIV-infected patient where tolerability to interferon-based regimens seems to be lower than that observed in comparable Phase 3 trials in monoinfected patients.

Though the treatments utilized in this Phase 3 multicenter trial will not be utilized in general practice, our study provided several important principles and observations that may guide future trials in the field. First, we provide additional support to the concept that Phase 3 trials represent a more accurate representation of true response rates compared to Phase 2 trials. We also note that outcomes in HCV/HIV coinfecting patients may be related to the background HIV antiretroviral regimen and that this effect may be a drug effect rather than a class effect. Finally, we note the systematic delays in initiation of clinical trials for those with underlying HIV infection vs those without HIV. Phase 3 trials of first generation HCV protease inhibitors lagged significantly behind drug approvals in HCV monoinfected patients. More recent drug development programs have attempted to remedy this situation, but the HIV research community should remain vigilant to reduce this bias going forward, particularly in rapidly moving developmental fields.

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## COMMENTS

### Background

Hepatitis C virus (HCV) coinfection is a major cause of morbidity and mortality among those with human immunodeficiency virus (HIV) infection. Prior to the emergence of new HCV targeted direct acting antiviral agents in 2011, response to standard therapy with pegylated interferon (PEG-IFN) and ribavirin (RBV) was poor, both in terms of efficacy and medication tolerability. The approvals of first generation serine protease inhibitors of HCV replication initiated a revolution in terms of the care and management of HCV infected patients. Phase 2 trials in HCV/HIV coinfecting patients suggested improved efficacy with moderate drug tolerability for treatment regimens that combined either boceprevir or telaprevir with PEG-IFN + RBV. In an effort to define treatment efficacy with response- and cirrhosis- guided regimens in HCV/HIV coinfecting, the authors conducted a prospective, multicenter, open-label Phase 3 trial in both HCV treatment naïve and treatment experienced participants with comparison to historical controls in the same clinical trials network.

### Research frontiers

The treatment of hepatitis C is a rapidly moving and dynamic field. Introduction of new agents has led to expansion of indications prior to completion of comprehensive Phase 3 trials in some cases. This study provides data regarding a large Phase 3 trial of a first generation protease inhibitor of HCV which was utilized in combination with PEG-IFN and RBV in HCV/HIV coinfecting patients.

### Innovations and breakthroughs

This is the largest study to investigate the efficacy and safety of this first generation protease inhibitor therapy in HCV/HIV coinfecting patients. The treatment was not optimal, but it did meet criteria for treatment success compared to historical controls treated with PEG-IFN plus RBV.

### Applications

While this study demonstrates efficacy of a first generation HCV protease inhibitor in the treatment of HCV/HIV coinfecting patients, the regimen is unlikely to be widely used due to rapid development of all-oral regimens that have supplanted the use of PEG-IFN-based regimens. The importance of conducting Phase 3 trials was emphasized by the lower rates of efficacy than were observed in Phase 2 trials that included highly selected patients.

### Terminology

Treatment naïve patients are those who have never been treated with a hepatitis C active agent while treatment experienced are those who may have been exposed to interferon or PEG-IFN with or without RBV in the past. Therapies for HIV are collectively called cART which includes combinations of drugs used for antiretroviral therapy.

## Peer-review

The authors report data on efficacy and safety of HCV protease inhibitor boceprevir with PEG-IFN alfa and weight-based RBV in a phase 3 trial in patients with HCV plus HIV. The result, in terms of RBV, is similar to that reported by other studies in the real world and reflects the limits of this treatment. The authors, correctly, described the chronology of their trial, born before the entry in the clinical practice of the new treatments.

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## Robotic liver surgery is the optimal approach as bridge to transplantation

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### Abstract

The role of minimally invasive liver surgery as a bridge to transplantation is very promising but still underestimated. However, it should be noted that surgical approach for hepatocellular carcinomas (HCC) is not merely a technical or technological issue. Nowadays, the epidemiology of HCC is evolving due to the increasing role of non-alcoholic fatty-liver-disease, and the emerging concerns on direct-acting antivirals against hepatitis C virus in terms of HCC incidence. Therefore, a fully multidisciplinary study of the cirrhotic patient is currently more important than ever before, and the management of those patients should be reserved to tertiary referral hepatobiliary centers. In particular, minimally invasive approach to the liver showed several advantages compared to the classical open procedure, in terms of: (1) the small impact on abdominal wall; (2) the gentle manipulation on the liver; (3) the limited surgical trauma; and (4) the respect of venous shunts. Therefore, more direct indications should be outlined also in the Barcelona Clinic Liver Cancer model. We believe that treatment of HCC in cirrhotic patients should be reserved to tertiary referral hepatobiliary centers, that should offer patient-tailored approaches to the liver disease, in order to provide the best care for each case, according to the individual comorbidities, risk factors, and personal quality of life expectations.

**Key words:** Hepatocellular carcinomas; Liver transplant; Robotic surgery; Bridge to transplantation; Da Vinci; Barcelona Clinic Liver Cancer; Patient safety

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**Core tip:** We read with great interest the manuscript

by Dr. Memeo *et al.* The role of minimally invasive liver surgery as a bridge to transplantation is very promising but still underestimated. In particular, minimally invasive approach to the liver showed several advantages compared to the classical open procedure in cirrhotic patients, and currently it deserves more direct indications that should be outlined also in the Barcelona Clinic Liver Cancer model.

Magistri P, Tarantino G, Ballarin R, Coratti A, Di Benedetto F. Robotic liver surgery is the optimal approach as bridge to transplantation. *World J Hepatol* 2017; 9(4): 224-226 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i4/224.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i4.224>

## TO THE EDITOR

We read with great interest the paper by Memeo *et al.*<sup>[1]</sup>, recently published on *World Journal of Hepatology* and titled "Innovative surgical approaches for hepatocellular carcinoma". In their well written and complete analysis of surgical planning and treatment for hepatocellular carcinomas (HCC), the authors affirm that the well-known advantages of minimally invasive liver surgery (MLS) compared to the classic "open" approach (OLS) may result in an easier access to the abdomen in case of future liver transplantation (LT). We completely agree and compliment them for highlighting this issue, which is currently underestimated. In July 2014 we started a robotic program at University of Modena and Reggio Emilia and in a period of two years 69 procedures have been performed. A total of 47 robotic liver procedures were ruled out, and among those 24 resection for HCC in cirrhotic patients. In this cohort of patients there were no conversions to laparotomy, mean operative time was 318 min (docking time included), and the mean in-hospital stay was 5.1 d. No readmission nor recurrences were observed. Our robotic cohort of HCC patients is included in an ongoing study funded by "Regione Emilia Romagna" (Regional Public Health System) that aims to investigate the role of robotic surgery in bridging patients with HCC to LT. Up to now, in our Institution two patients successfully underwent LT after MLS and four are on the waiting list.

The robotic platform is expanding its field of application on liver surgery for HCC including the so-called "difficult segments", and should be considered as a valuable tool for bridging patients to LT<sup>[2-6]</sup>. Although OLS has been classically limited to a strictly selected population of patients, several studies demonstrated that MLS is safe, feasible and particularly effective for parenchyma-sparing procedures, as needed in cirrhotic patients<sup>[7]</sup>. However, it should be noted that surgical approach for HCC is not merely a technical or technological issue. Nowadays, the epidemiology of HCC is evolving due to the increasing role of non-alcoholic fatty-liver-disease and direct-acting antivirals against

hepatitis C virus<sup>[8]</sup>. Therefore, a fully multidisciplinary study of the cirrhotic patient is currently more important than ever before, and the management of those patients should be reserved to tertiary referral hepatobiliary centers. Moreover, it should be taken into account that the intraoperative management as well is not only a matter of individual ability to perform certain procedures. MLS seems more effective than OLS in patients affected by HCC within a cirrhotic liver due to several reasons. First of all, in a setting of reduced liver function and reduced functional reserve, we can benefit from less impact on the abdominal wall, gentle manipulation on the liver, respect of the venous shunts and limited surgical trauma. In addition, the perioperative perspiration is consistently less with MLS compared to OLS: Consequently, fluids administration can be more conservative since generous substitutions are not needed. Finally, a better control of post-operative pain and early mobilization of the patient after MLS reduce respiratory complications by enhancing respiratory movements<sup>[9]</sup>. Currently, there is no formal evidence of the superiority of robotic approach vs conventional laparoscopy and also oncological results are similar<sup>[10]</sup>. The correct timing and criteria for choosing between liver resection or LT is still debated, and optimizing organ allocation is still our priority<sup>[11]</sup>. MLS offers an opportunity to safely treat HCC patients even with a Child A-B cirrhotic liver, with lower rates of overall morbidity when compared to OLR, and lower incidence of local recurrence when compared to radiofrequency ablation<sup>[12]</sup>. In conclusion, minimally invasive liver procedures can be considered as an independent field of surgery, with particular indication for Child A and B patients and parenchyma-sparing procedures, that should be better classified in the classical Barcelona Clinic Liver Cancer model<sup>[13-15]</sup>.

We compliment again the Authors for their work and their effort as a referral center of technological innovation to improve both surgical performances and patients' safety. We believe that a modern hepatobiliary center should offer patient-tailored approaches to the liver disease, in order to provide the best care for each case, according to the individual comorbidities, risk factors and personal quality of life expectations.

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## Adverse effects of oral antiviral therapy in chronic hepatitis B

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### Abstract

Oral nucleoside/nucleotide analogues (NAs) are currently the backbone of chronic hepatitis B (CHB) infection treatment. They are generally well-tolerated by patients

and safe to use. To date, a significant number of patients have been treated with NAs. Safety data has accumulated over the years. The aim of this article is to review and update the adverse effects of oral NAs. NAs can cause class adverse effects (*i.e.*, myopathy, neuropathy, lactic acidosis) and dissimilar adverse effects. All NAs carry a "Black Box" warning because of the potential risk for mitochondrial dysfunction. However, these adverse effects are rarely reported. The majority of cases are associated with lamivudine and telbivudine. Adefovir can lead to dose- and time-dependent nephrotoxicity, even at low doses. Tenofovir has significant renal and bone toxicity in patients with human immunodeficiency virus (HIV) infection. However, bone and renal toxicity in patients with CHB are not as prominent as in HIV infection. Entecavir and lamivudine are not generally associated with renal adverse events. Entecavir has been claimed to increase the risk of lactic acidosis in decompensated liver disease and high Model for End-Stage Liver Disease scores. However, current studies reported that entecavir could be safely used in decompensated cirrhosis. An increase in fetal adverse events has not been reported with lamivudine, telbivudine and tenofovir use in pregnant women, while there is no adequate data regarding entecavir and adefovir. Further long-term experience is required to highlight the adverse effects of NAs, especially in special patient populations, including pregnant women, elderly and patients with renal impairment.

**Key words:** Nucleoside/nucleotide analogues; Adverse events; Lamivudine; Chronic hepatitis B; Side effects; Safety; Telbivudine; Hepatitis B infection; Adefovir; Entecavir; Adverse effects; Tenofovir; Hepatitis B virus

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**Core tip:** Extrahepatic effects of nucleotide analogues (*i.e.*, myopathy, nephropathy, bone disorders) are more commonly indicated in current reports. Some of these adverse events can be attributed to their effect of causing mitochondrial dysfunction. These adverse events are named as "class effects" and mostly associated



with lamivudine and telbivudine treatment. Adefovir is a well-known nephrotoxic agent. Nephrotoxic and bone density loss effects of tenofovir in patients with chronic hepatitis B (CHB) are not as clear as in those with human immunodeficiency virus infection. Serum creatinine, phosphorus and creatine kinase levels should be monitored. Safety profile is a major issue that should not be ignored in the treatment of CHB.

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## INTRODUCTION

Chronic hepatitis B (CHB) infection is one of the major causes of chronic liver diseases and affects an estimated 350 to 400 million people worldwide<sup>[1]</sup>. Up to 15%-40% of patients with CHB are at risk of developing complications including cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC)<sup>[2]</sup>. Prevention of disease progression and disease-related complications is the main goal of treatment in CHB and achieved by suppression of hepatitis B virus (HBV) DNA replication<sup>[2]</sup>. Because CHB requires long-term treatment in the majority of patients, the safety profiles of drugs become important in addition to their antiviral activities. Two different groups of antiviral agents have been approved for the treatment of CHB: Conventional or pegylated interferons (IFN or Peg-IFN), and oral nucleoside/nucleotide analogues (NAs)<sup>[2-4]</sup>. IFN/Peg-IFNs have some disadvantages, including severe side effects, aggravation of decompensated cirrhosis and autoimmune diseases. NAs have become currently the backbone of CHB treatment because they have been well tolerated by patients for decades without severe side effects<sup>[5]</sup>. There are currently five NAs approved for the treatment of CHB and they are classified into two groups: Nucleoside analogues (lamivudine, telbivudine and entecavir) and nucleotide analogues (adefovir dipivoxil and tenofovir dipivoxil fumarate)<sup>[6]</sup>. To date, a significant number of patients have been treated with NAs. Therefore, experience with the efficacy, resistance and safety profile of NAs has increased over the years. The aim of this article is to provide a review of the adverse effects of oral NAs in light of the current data.

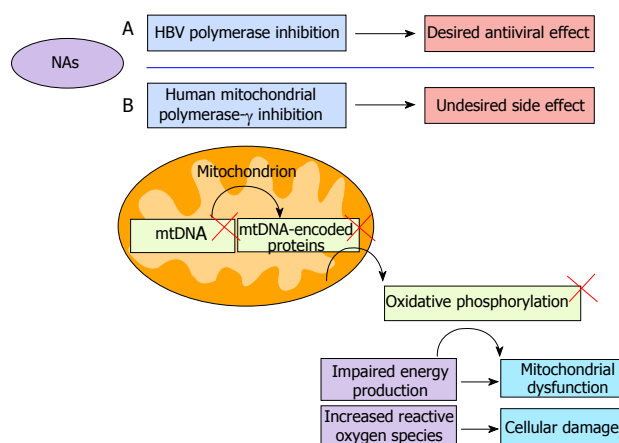
All five NAs have a favorable safety profile<sup>[7]</sup>. However, undesired extrahepatic adverse events may occur during the treatment of CHB infection. The most common extrahepatic adverse events are renal dysfunction, decreased bone mineral density and some neurological findings. Because hepatitis B infection itself may lead to extrahepatic organ involvement<sup>[5]</sup>, determining the source of extrahepatic manifestations may be difficult sometimes during the treatment of CHB. Extrahepatic adverse events may result from mitochondrial toxic effect

of NAs. These adverse effects are generally named as "class effects"<sup>[8]</sup>.

## CLASS EFFECTS OF NAs

NAs suppress viral replication by the inhibition of the HBV polymerase enzyme. As NAs structures were similar to natural nucleosides, some of these agents can also inhibit human mitochondrial polymerase- $\gamma$  and cause mitochondrial toxicity<sup>[3,5,9]</sup>. Mitochondrial toxicity was first noticed during human immunodeficiency virus (HIV) treatment with antiretroviral therapy. Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) are activated by phosphorylation in the cell, and then inhibit HIV reverse transcriptase. Additionally, these drugs also inhibit a human polymerase- $\gamma$  enzyme, which is responsible for the production of mitochondrial DNA (mtDNA) content. mtDNA-encoded proteins are present in multiple copies in each mitochondrion and responsible for encoding enzyme subunits of the respiratory chain function. Respiratory chain function is required for numerous metabolic pathways, including oxidative synthesis of ATP and synthesis of DNA. The depletion of mtDNA-encoded proteins results in mitochondrial dysfunction that causes impaired oxidative phosphorylation. The other result of human mitochondrial polymerase- $\gamma$  inhibition is increased reactive oxygen species that cause cellular damage (Figure 1)<sup>[5,8,10]</sup>. The close relation between NRTIs and mitochondrial toxicity have been described in many reports<sup>[5,8,11]</sup>. Because NAs lead to a minimal mitochondrial polymerase- $\gamma$  inhibition, NAs-associated mitochondrial toxicity cases have been rarely reported. All NAs carry a warning of mitochondrial toxicity as part of their prescribing information<sup>[5,8]</sup>. The clinical manifestations of mitochondrial toxicity include hematologic disorders, peripheral neuropathy, skeletal and cardiac myopathy, pancreatitis, hepatic failure and lactic acidosis<sup>[8,11]</sup>.

The most remarkable examples of mitochondrial toxicity were reported with clevudine therapy. Clevudine is a thymidine-nucleoside analogue approved in South Korea and the Philippines for the treatment of CHB. Although no mitochondrial dysfunction findings had been detected in preclinical studies, multi-center international phase III studies were terminated due to the emergence of clevudine-associated myopathy cases. Clevudine had been shown to be peripherally phosphorylated by mitochondrial thymidine kinase and to accumulate in cells rich in mitochondria<sup>[5]</sup>. South Korea revoked its approval because of indirect adverse effects<sup>[12-14]</sup>. The emergence of an association between clevudine and myopathy served as a reminder that all NAs have a potential risk for mitochondrial toxicity. Among the NAs, lamivudine and telbivudine are the agents most frequently reported to be associated with myopathy and peripheral neuropathy (Table 1). Long-peripheral neurons were more susceptible to mitochondrial toxic effect of NAs due to length-dependent effect<sup>[15]</sup>. Xu *et al*<sup>[16]</sup> performed muscle and nerve biopsy in the 6 cases



**Figure 1 Effects of nucleos(t)ide analogues.** A: NAs show antiviral effect by inhibition of hepatitis B virus (HBV) polymerase; B: NAs also inhibits human mitochondrial polymerase- $\gamma$  enzyme. Thus, mitochondrial DNA (mtDNA) can not be synthesized. Oxidative phosphorylation is impaired. There are two consequences of this: Impaired energy production and increased reactive oxygen species that cause cellular damage. NAs: Nucleos(t)ide analogues.

of NAs-associated myopathy or neuropathy and revealed similar changes in all the muscle and nerve biopsy samples of the patients in light or electronic microscopy and showed the decrease of the mitochondrial DNA by the quantitative real-time PCR in the affected muscle. Although an association between telbivudine and mitochondrial toxicity was not detected *in vitro* studies<sup>[12]</sup>, telbivudine-associated myopathy and creatine kinase (CK) elevations have been reported repeatedly in real-life patients after phase studies. Myopathy may be accompanied by neuropathy in some of patients given telbivudine or lamivudine for the treatment of CHB infection. In one study, 3 of 6 patients with lamivudine or telbivudine-associated myopathy had a complaint of numbness in the distal end of limbs, suggesting peripheral neuropathy. The presence of neuropathy was confirmed by the electrophysiological studies and nerve biopsies by the study team<sup>[16]</sup>. Neuropathy cases have been reported more commonly in patients who have been treated with a combination therapy of telbivudine and Peg-IFN alfa-2a. Combination therapy provided a rapid reduction in HBV DNA level compared to telbivudine or Peg-IFN alfa-2a monotherapy. However, the risk of peripheral neuropathy has been reported to increase up to 20% in combination with Peg-IFN<sup>[10,12,15,17]</sup>.

Myopathy is characterized by CK elevation alongside muscle pain and weakness. CK elevations are among the well-described adverse effects of NAs, but they are not specific for myopathy and may be associated with strenuous exercise and many other illnesses. CK elevations may occur in patients treated with all approved NAs for CHB. However, the incidence of myopathy is very low during the treatment with adefovir, entecavir and tenofovir, and similar to comparative groups. The causal relationship has not been elucidated as of yet<sup>[3,18]</sup>. Myopathy cases can be seen in every age group (25–82 years). There is no difference between male and female

patients in terms of myopathy incidence. The mean onset time of myopathy from the initiation of NAs was reported as 6.4 mo, but it can occur even if in the 5<sup>th</sup> year of treatment. Myopathy cases had been mostly reported from the South Korea and China, but the association between myopathy and race remains unclear<sup>[19]</sup>.

## LAMIVUDINE

Lamivudine is the first oral NA approved by the United States Food and Drug Administration (FDA) for the treatment of CHB in 1998 at a dose of 100 mg/d. It is an analogue of cytidine [2',3'-dideoxy-3'thiacytidine (3TC)] and phosphorylated to its active triphosphates form by intracellular deoxycytidine kinase enzyme. The active anabolite prevents HBV replication by competitively inhibiting viral reverse transcriptase and terminating proviral DNA chain extension<sup>[20]</sup>. Lamivudine has been the most experienced oral antiviral in CHB patients<sup>[8,20]</sup>. It can be used effectively in a broad range of patients, with minimal adverse effects<sup>[21]</sup>. However, long-term treatment of lamivudine is associated with high rates of drug resistance, which lead to virological relapse and biochemical flare<sup>[1-3,8]</sup>. Therefore, lamivudine is recommended as a second-line therapy for the treatment of CHB<sup>[1,2]</sup>.

Long-term lamivudine treatment was generally well-tolerated by CHB patients<sup>[21,22]</sup>. In the GLOBE trial, a large, multi-center phase III study, of the 1367 CHB patients who received telbivudine and lamivudine, adverse events were reported in 23% of the lamivudine recipients, similar to the findings for the telbivudine recipients (29%). The most common adverse events were upper respiratory tract infection (16.2%), nasopharyngitis (13.1%), headache (13.4%) and fatigue (12.1%). Of the patients, 6% (44) experienced serious adverse events<sup>[23]</sup>. The primary adverse event was reported as hepatic flares due to emergence of lamivudine-resistant HBV with prolonged treatment. After 4 years, hepatic decompensation and other severe adverse effects increased among patients with lamivudine resistance<sup>[24]</sup>. In an Asian study by Leung *et al.*<sup>[22]</sup>, 12% ( $n = 7$ ) of patients treated with lamivudine experienced severe side effects. Most of these were increased transaminase and CK levels, and resolved spontaneously. Increased alanine aminotransferase (ALT) levels were generally associated with emergence of YMDD mutant strains and had no clinical importance. In another study conducted among 998 patients with hepatitis B e antigen (HBeAg)-positive compensated liver disease who were treated with lamivudine for up to 6 years, lamivudine demonstrated a good safety profile, with only a 5% rate of severe adverse events<sup>[24]</sup>. Similarly, lamivudine has been found to be effective in HBV DNA decrease, ALT normalization and histological improvement, and it was well-tolerated by patients with cirrhosis. Lamivudine had been used in patients with acute or fulminant hepatitis without any adverse event, and led to fast recovery and increased

**Table 1** Characteristics of approved oral antiviral drugs for chronic hepatitis B treatment

NAs (approval year)	Class effect	Renal effect	Most common adverse events	Laboratory monitoring	Rare severe adverse reactions	Pregnancy category	Detection in breastfeeding
Lamivudine (1998)	Myopathy and neuropathy cases were reported	No significant effect	Upper respiratory tract infection, nasopharyngitis, headache and fatigue ALT flairs CK elevation may occur (usually not requiring cessation of drug)	Serum ALT and bilirubin	Rhabdomyolysis, acute dystonia, pancreatitis Rare lactic acidosis	C	Yes
Telbivudine (2006)	Myopathy and neuropathy cases were reported (especially in combination with Peg- IFN)	Nephroprotective effect Increase in GFR	Upper respiratory tract infection, nasopharyngitis, headache and fatigue Increased incidence of CK elevation (usually asymptomatic and self-limiting, not required cessation of drug)	CK level Serum lactate	Lactic acidosis	B	Yes
Adefovir (2002)	Very rare, No increased incidence of myopathy compared to placebo	Clinically significant nephrotoxicity Decrease in GFR	Pharyngitis, asteni, headache, abdominal pain, flu-like symptoms and nausea	Serum creatinine and phosphate level	Hypophosphatemia Fanconi syndrome	C	Unknown, not recommend for use
Entecavir (2005)	Very rare, No increased incidence of mitochondrial toxicity in combination of entecavir with other NAs and IFN	No decrease in GFR	Headache, upper respiratory tract infection, cough, nasopharyngitis, fatigue, dizziness, upper abdominal pain and nausea	Serum lactate	Lactic acidosis	C	Unknown, not recommend for use
Tenofovir (2008)	Very rare, No increased incidence of myopathy compared to placebo	May decrease GFR, clinically insignificant Nephrotoxic in HIV patients Hypophosphatemia	Headache, nasopharyngitis, back pain, nausea Bone mineral density loss (more prominent in HIV patients)	Serum creatinine and phosphate level BMD		B	Yes

NAs: Nucleos(t)ide analogues; ALT: Alanine aminotransferase; CK: Creatine kinase; IFN: Interferon; GFR: Glomerular filtration rate; HIV: Human immunodeficiency virus; BMD: Bone mineral density.

survival<sup>[25]</sup>.

Lamivudine has a good safety profile in different patient populations having some comorbid diseases. It is the most experienced drug for preemptive treatment of hepatitis B infection in solid-organ recipient and immunosuppressive patients<sup>[1]</sup>. There are limited data for experiences with the other NAs<sup>[26]</sup>. Although highly potent oral NAs with high genetic barriers to antiviral resistance, such as entecavir and tenofovir, have become the current preferred regimen, lamivudine remains a therapeutic option for hepatitis B prophylaxis since it is the most cost-effective choice for these patients<sup>[27,28]</sup>. Lamivudine has been well tolerated by patients receiving immunosuppressive treatment. In a systematic review investigating the preventive effect of lamivudine on chemotherapy - induced hepatitis B-related morbidity and mortality in hepatitis B surface antigen (HBsAg)-positive patients with cancer, none of the eight studies that recorded safety profile of lamivudine reported any significant adverse events<sup>[29]</sup>. Lamivudine has also been

used safely in children without any serious side effects. In one study, only slight and transient increase of ALT levels were reported in 6.8% of children with CHB, without any complaint or clinical findings<sup>[30]</sup>.

Serious adverse events have rarely been reported with lamivudine treatment<sup>[31,32]</sup>. Lamivudine-induced rhabdomyolysis is one of them and characterized by a triad of muscle weakness, myalgia and abnormal laboratory findings including CK elevation, increased urine and blood myoglobin level, and acute renal injury. Tubular damage and obstruction is considered the main reason underlying pathogenesis<sup>[31-33]</sup>. Clinical and laboratory findings improve generally within a few days after cessation of the drug. However, in one case, rhabdomyolysis relapsed after readministration of lamivudine for HBV infection prophylaxis and resolved completely after discontinuation of the drug again<sup>[34]</sup>. The mortality rate was reported to be high in patients who developed rhabdomyolysis and may be reduced by the early recognition of the disease and fluid resuscitations<sup>[31]</sup>.

Lamivudine-induced acute dystonic reaction was reported in 2 patients, and the acute dystonia resolved after discontinuing the lamivudine therapy<sup>[35]</sup>. Lamivudine-associated ichthyosiform eruptions and pancreatitis cases have been reported in the literature<sup>[25,36-38]</sup>.

## TELIVUDINE

Telbivudine is a thymidine nucleoside analogue which selectively inhibits HBV DNA synthesis. It was approved in 2006 for the treatment of CHB patients at a dose of 600 mg/d. Telbivudine is a more potent NA against HBV compared to lamivudine and adefovir<sup>[3,39]</sup>. However, high resistance rates limit the use of telbivudine as the firstline therapy<sup>[2,3]</sup>. Upper respiratory tract infection, nasopharyngitis, fatigue and headache were reported as the most frequent adverse events associated with telbivudine use. Adverse events' frequencies were found to be similar in lamivudine and telbivudine groups. However, Grade 3/4 increase in CK level occurred more commonly in patients given telbivudine (12.9% vs 4.1%), but these were not associated with musculoskeletal adverse events and no rhabdomyolysis cases were detected during the study period<sup>[23]</sup>. CK elevations were generally self-limiting and asymptomatic. Discontinuation of telbivudine was not required in most of the cases. Telbivudine-associated myopathy and CK elevations have been reported in several studies<sup>[12,40-42]</sup>. Zou *et al.*<sup>[41]</sup> conducted a prospective study to investigate clinical features and risk factors of telbivudine-associated myopathy and CK elevations. The serum CK levels of 200 patients treated with telbivudine were analyzed. The 3-year cumulative incidence of CK elevations was considerably high (84.3%). Nine patients (5%) experienced myopathy and were required to discontinue telbivudine therapy in 3 of those. None of the patients developed rhabdomyolysis. CK elevations were reported to occur in males more often than in females and in those with HBeAg negativit and aged < 45 years. In another study in which 105 patients given telbivudine were evaluated for adverse reactions, 5 presented serious adverse events. There was nervous system damage in 3 of the cases and cardiac arrhythmia in 1 case. All 5 patients had elevated CK enzymes. Therefore, it is recommended that CHB patients treated with telbivudine should be monitored closely for musculoskeletal symptoms and CK enzyme levels<sup>[3]</sup>.

Some infrequent but serious side effects were reported in previous studies. Lactic acidosis is one of them and it was reported also in patients treated with all the other nucleos(t)ide analogues<sup>[43]</sup>. It results from mitochondrial dysfunction or loss due to the inhibitor activity of telbivudine on human mitochondrial DNA polymerase- $\gamma$ . A few lactic acidosis cases depending on telbivudine therapy were reported in the literature. The symptoms of patients were anorexia, nausea, vomiting, muscle pain and weakness in upper and lower extremities. The laboratory tests revealed elevated serum CK levels and hyperlactatemia<sup>[43]</sup>. One patient's complaints

continued even after the withdrawal of telbivudine treatment, and the patient recovered after venovenous hemodiafiltration. To diagnose hyperlactatemia, the patients should be monitored by periodic (3-6 mo interval) lactate measurements, in addition to the CK monitoring.

The mechanism of adverse events associated with telbivudine use has not yet been defined. Because adverse events may occur in multiple organs including muscles, nervous and cardiac systems, Zhang *et al.*<sup>[42]</sup> suggested that the mechanism is associated with cell energy metabolism. Deficiency in manufacture of the energy molecule ATP and, therefore, inadequate supplementation of substrate for oxidative phosphorylation causes mitochondrial damage. Highly energy-dependent organs such as nerves, heart and muscles are the most susceptible to mitochondrial dysfunction. Telbivudine leads to adverse events in these organs. However, to establish a link between adverse events and mitochondrial disease, muscle biopsy and DNA studies should be done<sup>[42]</sup>.

Synergistic effect can occur in case of simultaneous use of two drugs. A study comparing telbivudine and lamivudine combination and lamivudine monotherapy reported that the addition of telbivudine to lamivudine treatment did not increase the toxic adverse effects<sup>[44]</sup>. However, the combination of telbivudine with Peg-IFN caused peripheral neuropathy in 17.0% of patients. For this reason, telbivudine should not be recommended in combination with Peg-IFN<sup>[8]</sup>.

## ADEFOVIR DIPIVOXIL

Adefovir dipivoxil is an oral prodrug of the nucleotide analogue adefovir, approved for CHB treatment at 10 mg/d dose in 2002. It was used initially in patients with HIV infection, but its use was abandoned due to the fact that higher doses of adefovir led to nephrotoxicity<sup>[8]</sup>. Adefovir improves histological, biochemical and virological outcomes in CHB patients with lamivudine resistance. The rates of adverse events in patients given adefovir are similar to those given placebo<sup>[45-48]</sup>. The most common adverse events were pharyngitis, asteni, headache, abdominal pain, flu-like symptoms and nausea<sup>[45]</sup>. In a randomized controlled study, adverse events were similar in two groups, but headache and abdominal pain occurred more frequently in the adefovir group than in the placebo group. However, these adverse events did not lead to discontinuation of the study drug<sup>[48]</sup>. Adefovir is associated with dose-dependent renal toxicity. The nephrotoxic effect of adefovir was discussed in the section below on "Renal Safety of NAs".

Myopathy cases were reported in CHB patients given adefovir treatment, but its incidence was similar to patients receiving placebo<sup>[12]</sup>. Adefovir-related lactic acidosis may occur when combined with other NAs<sup>[49]</sup>. The development of resistance to adefovir therapy is another undesirable event. Drug resistance was reported in 26% of CHB patients treated with adefovir, after



5 years<sup>[8]</sup>. The resistance rate of adefovir in patients with lamivudine resistance who were given adefovir add-on lamivudine rescue therapy was 6% at the end of 5 years<sup>[50]</sup>. To optimize therapy in lamivudine-resistant patients, it is recommended not to discontinue lamivudine therapy for a while after initiating adefovir<sup>[8]</sup>.

## ENTECAVIR

Entecavir is a highly selective guanosine nucleoside analogue, approved by the FDA at a dose 0.5 mg in treatment naive and 1 mg/d in lamivudine-resistant CHB patients in 2005<sup>[3,51]</sup>. It inhibits three steps of viral replication, which involves HBV polymerase priming, reverse transcription of the pre-genomic messenger RNA and synthesis of the positive-stranded HBV DNA<sup>[3]</sup>. Entecavir is a well-tolerated antiviral agent in CHB patients, with rates of adverse events similar to placebo or lamivudine therapy. In a comparative study, the adverse event rate was found to be similar in patients given entecavir monotherapy to those given combination of entecavir and IFN<sup>[52]</sup>. Long-term use was reported to be associated with a very low rate of side effects. Adverse events were not dose-related; their frequencies were similar between 0.5 or 1 mg doses of entecavir<sup>[51,53]</sup>. The most frequent adverse events in clinical trials were headache (17%-23%), upper respiratory tract infection (18%-20%), cough (12%-15%), nasopharyngitis (9%-5%), fatigue (10%-13%), dizziness (9%), upper abdominal pain (9%) and nausea (6%-8%). Most of these adverse effects were mild or moderate severity and did not require discontinuation of the drug<sup>[51,54]</sup>. Severe adverse events accounted for 7%-10% and discontinuation of therapy accounted for 1%-2% of patients<sup>[51]</sup>. In a randomized controlled study, severe adverse events occurred in 4.7% of pediatric patients ( $n = 8$ ), and only one of them discontinued entecavir due to headache. This adverse event was not attributed to the study drug<sup>[54]</sup>. Although preclinical data reported an association between long-term entecavir use and carcinogenicity, to date, no evidence has been detected regarding occurrence of cancer due to entecavir therapy<sup>[55]</sup>.

The FDA requires all approved NAs to include a "Black Box" warning in their product label regarding potential mitochondrial toxicity<sup>[56]</sup>. Entecavir is the most innocent antiviral agent leading to mitochondrial toxicity among the effective therapies in CHB treatments. In long-term cell culture studies, entecavir has been observed to have very low potential for mitochondrial toxicity in *in vitro* cultures studies at the highest levels tested, 300  $\mu\text{mol/L}$ . Combination of entecavir with the other NAs also did not cause an increase in the risk of other drugs<sup>[8,57]</sup>. Entecavir-associated myopathy and peripheral neuropathy cases were very rarely reported in the literature<sup>[3,15,19]</sup>. Although a study reported similar CK elevation rates with both telbivudine and entecavir therapy, there were not many studies supporting this<sup>[58]</sup>. In a meta-analysis, six randomized controlled trials involving

555 patients treated with telbivudine and entecavir for 24 or 52 wk were evaluated. Both drugs had similar antiviral and biochemical effects. However, the entecavir group was reported have greater safety than the telbivudine group, in terms of adverse events<sup>[59]</sup>. In another meta-analysis comparing the effects of telbivudine and entecavir in HBeAg-positive CHB patients, thirteen trials (3925 patients in total) were evaluated. Adverse effects were reported in 10 trials and CK elevations in 5 trials. The rates of increased CK were found to be statistically higher in the telbivudine group than in the entecavir group<sup>[60]</sup>.

Lactic acidosis can also occur during treatment with NAs as a result of mitochondrial toxicity. US prescribing information for entecavir and the other NAs carries a warning regarding the risk of lactic acidosis in CHB patients treated with NAs<sup>[61-64]</sup>. Entecavir is a good option for the treatment of CHB patients with decompensated cirrhosis because of the rapid effect on HBV decline and low resistance rates. However, it was suggested that a high Model for End-Stage Liver Disease (MELD) score that is used to detect highly impaired liver function can be associated with lactic acidosis in patients receiving entecavir<sup>[49]</sup>. One retrospective study identified 5 cases of lactic acidosis among 16 entecavir-recipient CHB patients with cirrhosis. One of them died, and the lactic acidosis resolved within 4-5 d after withdrawal of entecavir in the remaining 4 cases. All patients who developed lactic acidosis had a MELD score of at least 20 (22-38), whereas the patients who did not develop lactic acidosis had a MELD score below 18. A significant ( $P = 0.002$ ) correlation was seen between the MELD score and the development of lactic acidosis<sup>[49]</sup>. However, a small retrospective study did not find an increased risk of lactic acidosis in the CHB patients with decompensated liver disease and high MELD scores during entecavir treatment, compared to those who have non-HBV-related decompensated liver disease and similar clinical features<sup>[65,66]</sup>. Entecavir has been reported to have a high safety profile in decompensated patients and recommended as one of the first-line treatment choices of CHB patients with decompensated liver disease in an Asian-Pacific consensus statement<sup>[67,68]</sup>. Nevertheless, the patients should be monitored cautiously for the risk of lactic acidosis during the treatment and entecavir should be suspended in the case of suspected lactic acidosis<sup>[49,66]</sup>.

Patients with severe acidosis complained of nausea, dyspnea and weakness, and showed a reduced general physical condition, impaired consciousness and tachypnea. In addition, 2 of 3 patients with severe acidosis suffered from paresthesia and the remaining 1 patient developed hepatic steatosis typical for mitochondrial toxicity. ALT flares, potentially leading to decompensated hepatic disease, can be another serious health problem in a patient given entecavir for CHB. In clinical trials, ALT flare had been reported to occur in a small percentage of patients treated with entecavir and to resolve even if the treatment continued. In an open-label study evaluating

the safety and tolerability of entecavir, Grade 3 and 4 adverse events were detected in 19% of the patients, with only 4% of them possibly related to entecavir. These Grade 3 and 4 adverse events were myalgia, neuropathy, increased lipase, increased creatinine and lactate, CK elevation, decreased bicarbonate and pancreatitis. Entecavir treatment was discontinued in only 1% of cases due to adverse events. ALT flares were reported in 3% of the patients during the treatment, and were associated with inhibition of viral replication, at least 2 log<sub>10</sub> decrease of HBV DNA<sup>[68]</sup>. In a multicenter European study investigating the incidence and outcome of ALT flares during long-term entecavir in CHB, 729 patients treated with entecavir for a median of 3.5 years were evaluated. Flares were classified as host-induced (preceded by HBV DNA decline), virus-induced (HBV DNA increase) or indeterminate (stable HBV DNA). A total cumulative incidence of ALT flare was 6.3% (30) at year 5. Of them, 12 were host-induced and associated with biochemical remission. HBeAg and HBsAg seroconversion was observed in only these host-induced flares. Virus-induced flares were reported to be associated with entecavir resistance and non-compliance to the therapy<sup>[69]</sup>. Therefore, long-term use of entecavir is generally safe and associated with low rates of serious adverse events, and discontinuation of the treatment is rarely required. ALT flares were low in patients receiving entecavir and generally associated with the improvement of liver disease. In current guidelines, entecavir is also recommended as treatment and prophylaxis of CHB infection in patients with renal transplant due to being an agent without signs of nephrotoxicity<sup>[2]</sup>.

## TENOFOVIR

Tenofovir disoproxil fumarate (TDF) is a prodrug of tenofovir that has been approved as a nucleotide analogue by the United States FDA for use in HIV infection in 2001 and in CHB infection in 2008 at a dose of 300 mg<sup>[8]</sup>. TDF is converted to tenofovir by hydrolysis and then phosphorylated by cellular enzymes to tenofovir diphosphate. It inhibits (potentially) HBV DNA polymerase and reverse transcriptase. Tenofovir, one of the main components in antiretroviral regimens, plays a key role in HIV treatment. It is also a highly potent inhibitor of HBV DNA replication and recommended as a first-line treatment choice in CHB by the current clinical guidelines due to the absence of resistance to the drug<sup>[1,70]</sup>. The molecular structure and general safety profile of tenofovir is similar to adefovir, but nephrotoxicity has not been a major problem with tenofovir at therapeutic doses. Therefore, it can be used at higher doses compared to adefovir and leads to more effective responses in HBV DNA decline. The nephrotoxic effect of tenofovir is discussed in detail in the below section on "Renal Safety of NAs".

In phase III studies of tenofovir, the adverse event profiles were similar to those in the comparative arm of adefovir. The most frequent adverse events were head-

ache, nasopharyngitis, back pain and nausea. Treatment-related adverse events were detected in 6% of patients, serious adverse events in 4% and adverse events that required discontinuation of tenofovir in less than 1%<sup>[8,55]</sup>. A 3-year, prospective real-world study (Vireal group) reported 68 adverse events in 41 (9.3%) patients among a total of 440 patients receiving tenofovir. Adverse events occurring in more than one patient were renal disorders ( $n = 11$ ), abdominal pain ( $n = 8$ ), asthenia ( $n = 7$ ), nausea ( $n = 6$ ), vomiting ( $n = 5$ ) and diarrhea ( $n = 5$ ). Nine of the 16 serious side effects were reported to be tenofovir-related (visual impairment, nausea, asthenia gait disturbance, weight loss, depression, muscular weakness, muscular pain and psoriasis)<sup>[71]</sup>.

Osteomalacia can occur during long-term tenofovir treatment. In randomized clinical trials, a great loss of bone mineral density (BMD) had been well-described in patients with HIV infection treated with tenofovir<sup>[55,72-74]</sup>. However, tenofovir-related bone fractures were not reported in patients with HBV mono-infection<sup>[55]</sup>. During the 3-year prospective follow-up, fractures were observed in 1% of 375 HBeAg-negative and 266 HBeAg-positive patients, but none were related to tenofovir<sup>[75]</sup>. The primary responsible mechanism for bone density loss is believed to be related with inhibitory effects of HIV proteins or immune status in osteoblasts and an increased osteoclastic activity. Modifying effects of tenofovir on osteoblast gene expression and function was the other mechanism defined in recent reports<sup>[72]</sup>. The exact mechanism of bone toxicity in CHB is not clear. Possibly, proximal tubular damage caused by TDF therapy leads to hypophosphatemia and, indirectly, to inadequate mineralization of bone matrix<sup>[3]</sup>. There have been case reports regarding tenofovir-associated osteomalacia. A recent study including 170 patients with CHB infection compared patients treated with tenofovir ( $n = 122$ ) and control patients ( $n = 48$ ) in terms of bone health<sup>[72]</sup>. The prevalence of BMD loss in patients receiving tenofovir was similar to those who were not exposed to tenofovir. Tenofovir was reported to be associated with a lower T score only in the hips. Additionally, in the study, there was no significant correlation between duration of exposure to tenofovir and reduction in BMD at any side. The risk factors for reduction in BMD other than tenofovir exposure were the known classical factors including advancing age, lower body mass index and smoking<sup>[72-74]</sup>. A large retrospective study including 53500 subjects in Hong Kong (46454 untreated and 7046 treated) investigated renal and bone events in CHB patients with and without NAs. The patients treated with NAs had similar risk of hip fracture, spine fracture and all fracture, compared to untreated CHB patients. Treatment with nucleotide analogues, compared to nucleoside analogues, was found to increase only the risk of hip fracture but not the other side fracture, and the overall fracture rate was low<sup>[76]</sup>. Additionally, BMD reduction was demonstrated to remain constant on a plateau from year 4 through year 7 of tenofovir treatment, for both hip and lumbar spine<sup>[77]</sup>. Thus, we may conclude that

BMD reduction is not a progressive event and is detected in the first years of treatment<sup>[78]</sup>. These are important findings due to CHB infection requiring lifelong treatment in the majority of patients because the discontinuation of NAs after sustained viral response have a high risk of relapse. Tenofovir can be preferred and used safely in CHB patients in the long-term. Nevertheless, BMD should be periodically performed in patients with CHB infection treated with tenofovir<sup>[79]</sup>. Osteoporotic patients, especially with advanced age and smoking history, should be monitored more closely and, if required, consulted with a physical rehabilitation specialist.

## RENAL SAFETY OF NAs

The adverse effect of NAs on renal function is an important issue that should be carefully evaluated, since HBV infection alone carries an increased risk of renal impairment<sup>[80]</sup>. All NAs are excreted through kidneys in unchanged forms and some of them are associated with dose-dependent nephrotoxicity<sup>[3]</sup>. Nephrotoxicity results from proximal tubular damage and presents with elevated serum creatinine, proteinuria, nephrogenic diabetes insipidus, hypophosphatemia or the more severe form, Fanconi syndrome<sup>[15]</sup>. Mauss *et al.*<sup>[81]</sup> reported a milder decrease in renal function with CHB therapy irrespective of medications. Comorbidities such as diabetes, hypertension and underlying chronic renal disease may also contribute to the nephrotoxic effect of NAs and aggravate renal dysfunction. In a study analyzing effects of NAs and comorbidities on renal function in 4178 CHB patients, age, diabetes, chronic renal disease, renal transplantation and simultaneous administration of diuretics were found to be independent risk factors for the rapid progression of renal disease<sup>[81]</sup>.

Renal toxicity is the most noticeable side effect of adefovir. It is generally dose- and time-dependent, and reversible with dose-adjustment or discontinuation of the drug<sup>[15,45,82-84]</sup>. In the majority of studies, nephrotoxicity was defined as an increase  $\geq 0.5$  mg/dL from baseline in serum creatinine or a serum phosphorus value of  $< 1.5$  mg/dL on two consecutive occasions<sup>[83]</sup>. In previous studies, including randomized controlled ones, adefovir at 30 mg/d was reported to be nephrotoxic, but adefovir at 10 mg/d was well tolerated and did not lead to an increase in renal dysfunction compared to placebo<sup>[45,85]</sup>. In a study including a total of 515 patients with CHB, three groups who were treated placebo ( $n = 170$ ), adefovir dipivoxil at 10 mg ( $n = 172$ ) or adefovir dipivoxil at 30 mg ( $n = 173$ ) were compared in terms of response to the treatment and adverse events rates<sup>[45]</sup>. The safety profile was similar in two groups, the placebo group and the adefovir dipivoxil at 10 mg per day group. There was no significant change in median serum creatinine level at wk 48 of the treatment in these groups. However, 8% of the 30-mg group experienced an increase from baseline of 0.5 mg/dL (44  $\mu$ mol/L) or greater in the serum creatinine level. The prolonged use of adefovir carries an extra risk of renal dysfunction. The incidences

of increased creatinine level and hypophosphatemia were reported to be increased with longer usage of adefovir, even in patients receiving standard low-dose drug.

In recent years, Fanconi syndrome cases due to long-term use of adefovir have been increasingly reported, especially in East Asian populations<sup>[83]</sup>. Fanconi syndrome is defined as hypophosphatemia and a slight increase in serum creatinine, resulting in proximal renal tubular dysfunction. Additionally, osteomalacia may develop secondary to hypophosphatemia. The patient's main symptoms can be muscular weakness and bone pain involving the knees, ankles and ribs. Clinicians should be aware of this potential complication and monitor periodically the renal function and serum phosphate level in any patient receiving adefovir<sup>[83,86]</sup>. In a current meta-analysis, including seven randomized controlled trials, four cohort studies and six single-arm studies, adefovir treatment was not found to be associated with increased nephrotoxicity in the randomized controlled trials. However, the cohort studies showed an increased nephrotoxicity risk in patients given adefovir, and the single-arm studies revealed an approximately 1.7-fold increased risk of renal dysfunction in patients given adefovir compared to those treated with all other NAs<sup>[82]</sup>. The authors drew attention to the differences between the risk of nephrotoxicity in randomized controlled trials and cohort studies and emphasized that since the randomized controlled trials were small-sized and short observational studies, the safety data may be inadequate and that these studies may have underestimated the adverse events. Current evidence indicated an increased risk of nephrotoxicity in CHB patients treated with adefovir.

The mechanism of adefovir nephrotoxicity was poorly understood. Nephrotoxicity may result from the apoptotic or mitochondrial toxic effect of adefovir in the renal tubular epithelium. The deterioration of the balance between the active adefovir uptake from blood into proximal tubular cells, the secretion into urine, and accumulation in proximal tubular cells represent the primary mechanism of tubular toxicity.

Fanconi syndrome is a rare but serious adverse effect of adefovir treatment. Fanconi syndrome is characterized by proximal renal tubular toxicity and leads to increased urinary excretion of amino acids, uric acid, bicarbonate, glucose and phosphate, and impaired re-absorption of these solutes. Clinical manifestations in adults include polyuria, polydipsia, dehydration and osteomalacia<sup>[87]</sup>. There are a significant number of cases of adefovir-associated Fanconi syndrome in the literature. Most cases occurred after prolonged use of the drug and resolved after cessation of adefovir or switching to another NA. The lowest dose of adefovir (10 mg) can also lead to Fanconi syndrome<sup>[88]</sup>. Normalization of creatinine level may require more than 1 year. In a retrospective case series study including 35 patients with Fanconi syndrome, hypophosphatemia, increased urinary phosphate excretion and elevated alkaline phosphatase were detected in all patients.

Although serum phosphate levels rapidly increased, especially within the 4 wk after adefovir discontinuation, serum creatinine levels did not decrease to normal range even 1 year after discontinuation of therapy<sup>[88]</sup>. Fanconi syndrome was rare in CHB patients treated with tenofovir; it has been reported especially in cases of HIV-HBV coinfection<sup>[87,89-91]</sup>.

Despite tenofovir being a higher dose preparation (300 mg/d) that has similar molecular structure with adefovir, renal toxicity has been less commonly detected<sup>[3]</sup>. In animal studies, tenofovir was reported to be associated with renal dysfunction<sup>[3,84]</sup>. The mechanism of nephrotoxicity is poorly understood, but it may involve proximal tubular damage, mitochondrial toxicity and apoptosis<sup>[8,92]</sup>.

Tenofovir has been shown to have a potential nephrotoxic effect in patients with HIV infection who were treated for an especially extended period. However, in clinical trials, nephrotoxicity does not seem to be a major problem in HBV monoinfection<sup>[3,55,93]</sup>. Increases in serum creatinine of > 0.5 mg/dL were reported to be detected in 1% of patients and remained stable over 4 years in less than 1% of patients, with increased serum creatinine levels of 0.5 mg/dL<sup>[93]</sup>. Nevertheless, renal functions and serum phosphate should be monitored regularly in patients treated with tenofovir<sup>[3]</sup>.

In a study conducted by the Vireal group, a slight decrease of mean glomerular filtration rate (GFR) was reported during tenofovir therapy. Median change in creatinine clearance and serum creatinine level remained stable over time. Of the patients, 15% ( $n = 65$ ) had a decline in GFR of  $\geq 20\%$  and 6% ( $n = 26$ ) had a decline in GFR of  $\geq 30\%$  compared to baseline. Tenofovir treatment was discontinued in 23 patients due to adverse events. Seven of them were associated with renal disorders ( $n = 3$ , renal failures;  $n = 2$ , renal impairments;  $n = 2$ , renal tubular disorders)<sup>[71]</sup>. Patients who have an underlying renal impairment or HIV coinfection and those who receive a nephrotoxic drug are at increased risk of nephrotoxicity. In a study comparing tenofovir and entecavir in the same number of patients, diabetes and transplantation but not tenofovir treatment were found to be associated with increased risk of renal impairment<sup>[94]</sup>. A significant number of studies reported that tenofovir did not lead to clinically relevant changes in renal function<sup>[79,95]</sup>.

In a prospective open-label study, conducted by Heathcote *et al.*<sup>[75]</sup>, creatinine and creatinine clearance were reported to remain stable during a 3-year period, with a change in creatinine of 0.02 mg/dL at week 144. Two patients experienced a 0.5 mg/dL increase in creatinine and 4 patients a reduction in serum phosphorus < 2 mg/dL. All patients remained in the study and continued the tenofovir therapy. The long-term follow-up results of tenofovir therapy support the previous data. At year 6, less than 1.5% experienced impairment in renal function ( $\geq 0.5$  mg/dL increase in serum creatinine from baseline, phosphorus < 2 mg/dL, or CrCL < 50 mL/min) with tenofovir treatment<sup>[55]</sup>. Recently, Buti

*et al.*<sup>[77]</sup> reported 7<sup>th</sup> year results of tenofovir treatment for CHB. Of 585 patients, 21 (3.6%) experienced renal function impairment. A serum creatinine increase  $\geq 0.5$  mg/dL above baseline were confirmed in only 10 patients (1.7%). The patients who did and did not develop renal insufficiency were statistically different in terms of mean age (47 years vs 40 years;  $P = 0.003$ ), baseline mean creatinine clearance (98.5 mL/min vs 117.4 mL/min;  $P = 0.003$ ) and main serum phosphate (2.8 mg/dL vs 3.3 mg/dL;  $P = 0.002$ ). Despite the absence of significant evidence that tenofovir is a nephrotoxic agent, possible proximal tubular damage should still be kept in mind<sup>[3]</sup>. The patients with normal renal function or mild renal impairment who have no increased risk for renal toxicity should be monitored every 6 mo for serum creatinine and phosphorus. The patients with impaired renal function or underlying comorbidities that show increased renal failure may be monitored more frequently<sup>[96]</sup>. Dose-adjustment should be made according to the renal impairment<sup>[3]</sup>.

Tenofovir safety was also similar in elderly and younger patients<sup>[59]</sup>. There is little experience with tenofovir treatment in renal transplantation. One study reported 7 HBV-positive organ transplant recipients ( $n = 3$ , kidney;  $n = 1$ , liver;  $n = 3$ , hearts) who were safely and effectively treated with tenofovir. No adverse events or kidney rejection were observed. There were no statistically significant changes in renal functions<sup>[97]</sup>.

In contrast to the nucleotide analogues, nucleoside analogues are not generally associated with renal adverse events. Increase in serum creatinine was reported in less than 1% of patients treated with entecavir<sup>[49]</sup>. In the study of Tsai *et al.*<sup>[98]</sup>, entecavir and telbivudine were found to be associated with GFR improvement. Despite the absence of strong evidence, the current guidelines recommend entecavir as the best option in renal transplant recipients due to lack of data demonstrating a major renal toxicity with entecavir<sup>[2,99-101]</sup>.

Interestingly, telbivudine improves renal functions<sup>[3,8,81]</sup>. Several real-life studies have shown that treatment with telbivudine increases GFR in CHB patients. The GLOBE study and long-term extension studies had revealed that long-term telbivudine treatment was associated with a sustained improvement in renal function in patients with compensated and decompensated cirrhosis who had an increased risk of renal impairment<sup>[23,102]</sup>. Gane *et al.*<sup>[102]</sup> indicated an improvement in renal function with telbivudine treatment by the calculation of GFR using the Modification of Diet in Renal Disease, Chronic Kidney Disease Epidemiology Collaboration, and Cockcroft-Gault methods. The increment of GFR was also shown in patients at increased risk for renal impairment: +17.2% in patients with baseline GFR of 60-89 mL/min per 1.73 m<sup>2</sup>, +11.4% in patients older than 50 years and +7.2% in cirrhotic patients. Additionally, improved renal function has been reported to be maintained for 4-6 years. In a study investigating the renoprotective effect of telbivudine on patients receiving adefovir-based combination therapy, combination of adefovir



and telbivudine was found to have a more protective effect on renal functions than the combination of adefovir and entecavir, combination of adefovir and lamivudine, adefovir alone or entecavir alone<sup>[79]</sup>. Preemptive telbivudine use was reported to prevent renal deterioration caused by cisplatin-based chemotherapy in patients with advanced HCC<sup>[103]</sup>. Additionally, telbivudine is recommended in the prophylactic treatment of CHB in patients with renal transplant due to its renoprotective effect on transplanted patients<sup>[2]</sup>. Telbivudine is a good option, especially in patients with renal impairment or in those with risk factors for renal disease.

All NAs are cleared by kidneys and their dosage should be adjusted in patients with creatinine clearance below 50 mL/min<sup>[104]</sup>. To minimize the risk of nephrotoxicity, simultaneous administration of the other nephrotoxic drugs should be avoided. Secondly, all patients with CHB infection who are treated with adefovir or tenofovir should be regularly monitored for serum creatinine and phosphate levels and drug dose should be modified if creatinine increases by more than 0.5 mg/dL above baseline or phosphate level decreases below 2.0 mg/dL, to the needed dose<sup>[8]</sup>.

## SAFETY IN PREGNANCY

Mother-to-child-transmission remains the main route of hepatitis B acquisition, especially in endemic countries<sup>[105]</sup>. Despite postnatal use of immune globulin and vaccine, mother-to-child transmission of HBV infection still occurs. Intrauterine transmission is considered the main reason underlying immunoprophylaxis failures<sup>[2,106]</sup>. High HBV DNA levels and HBeAg-positive status are the most important risk factors for perinatal HBV transmission. Thus, reducing maternal HBV DNA level has become the main preventive measure of perinatal mother-to-child transmission<sup>[106]</sup>. Current guidelines recommend initiating NAs in pregnant females with high HBV DNA levels (above  $> 10^{6-7}$  IU/mL) at 28-32 wk of gestation and cessation of NAs after delivery or 4-12 wk after delivery in females who do not have a risk for ALT flares and pre-existing advanced liver fibrosis/cirrhosis<sup>[2,105]</sup>.

Two of five NAs approved for the treatment of CHB, telbivudine and tenofovir, are classified as category B in the United States FDA Pregnancy Categories (meaning that no risk was observed in animal studies; however, there are no adequate and well-controlled studies performed in pregnant women). The other three NAs, lamivudine, entecavir and adefovir, are classified as category C (meaning that an adverse effect on the fetus have been shown in animal studies, but there are no adequate studies in humans)<sup>[107]</sup> (Table 1). Prospective studies have revealed that fetal abnormality rates in mothers treated with NAs is low, and similar to those in the general population<sup>[3]</sup>. Lamivudine is the most experienced NA in pregnancy and it has been used safely in preventing mother-to-child transmission of HIV infection for 2 decades<sup>[2]</sup>. In randomized controlled studies, lamivudine has been shown to be effective in

preventing mother-to-child-transmission when used in the third trimester of pregnancy and early postnatal period. There was no significant difference in the incidence of fetal adverse effects between lamivudine and placebo groups<sup>[108,109]</sup>. The Antiretroviral Pregnancy Registry (APR) provides updated fetal safety data on various drugs used in pregnancy, and includes data from January 1989 to date. Up to 31 July 2015, APR reported newborn defect rates as 3.1% during the first trimester of 4566 pregnant women and 2.9% during the second/third trimester of 7263 pregnant women who were exposed to lamivudine. These rates were not different from those reported in the general population<sup>[110]</sup>. However, lamivudine administration, even if for short-term use such as during pregnancy, has a risk of selecting resistant strains due to poor antiviral activity<sup>[106]</sup>. Current guidelines do not recommend lamivudine as first-line therapy for the treatment of CHB infection in pregnant women<sup>[1,2]</sup>.

Tenofovir is recommended in current guidelines for preventing mother-to-child transmission in pregnant women with high viremia based on its potent antiviral activity, high barrier to resistance and being safe<sup>[1,2]</sup>. Data on tenofovir safety has been usually obtained from patients with HIV infection. It has been safely used in pregnant women with HIV infection for a relatively long time. APR reported newborn defect rates as 2.3% during the first trimester of 2608 pregnant women and 2.1% during the second/third trimester of 1258 pregnant women, which is similar to the rates in the general population. In a retrospective study, conducted in 45 HBeAg-positive pregnant women with high HBV DNA levels, tenofovir was found to be effective in preventing vertical transmission and no significant fetal adverse events were observed<sup>[111]</sup>. The other multi-center prospective observational study reported tenofovir to be more effective than lamivudine in preventing vertical transmission<sup>[112]</sup>. These data are supported by other studies<sup>[113]</sup>.

Telbivudine has greater potency than lamivudine in decreasing HBV DNA level and it is recommended by current guidelines in the prevention of mother-to-child transmission of HBV infection. Use of telbivudine during the second/third trimester of pregnancy was reported to be effective and safe. Compared to placebo, no serious adverse events were found in telbivudine-treated mothers and their infants<sup>[3,12]</sup>. Despite the relatively low resistance rate compared to lamivudine, telbivudine resistance may occur during therapy<sup>[105]</sup>. There are no adequate and well-controlled studies on the safety profile of entecavir and adefovir in pregnant women infected with CHB<sup>[15]</sup>.

Breast-feeding is discouraged during maternal NAs treatment due to the uncertain safety on infants<sup>[1,2]</sup>. Lamivudine is concentrated in breast milk. However, its amount in infants exposed to lamivudine during breast-feeding is accepted to be insignificant (approximately 2% of the recommended daily treatment dose)<sup>[114]</sup>. Similarly, tenofovir concentrations in breast milk have

been reported, but infants are exposed to a small amount because its oral bioavailability is limited<sup>[1]</sup>. There is no adequate evidence to recommend the use of entecavir and adefovir during the breast-feeding period<sup>[110,111]</sup>. Lamivudine or tenofovir is regarded as the choice in breastfeeding mothers who needed to receive treatment for HBV infection.

## CONCLUSION

In light of the current data, the treatment of CHB seems to be a life-long therapy. Thus, the long-term safety of the drugs is one of the main factors that influence treatment decision. To date, five oral NAs have been approved for the treatment of CHB. All NAs are generally safe and well-tolerated by CHB patients. All NAs carry a "Black Box" warning about mitochondrial dysfunction. The majority of mitochondrial toxicity cases are associated with lamivudine and telbivudine and generally present as myopathy, neuropathy or lactic acidosis. No increased incidence of myopathy was reported with adefovir, tenofovir and entecavir treatment, compared to placebo. Adefovir is a well-known nephrotoxic agent and may cause renal proximal tubular dysfunction. Fanconi syndrome cases have been increasingly reported in long-term adefovir therapy. Tenofovir has potential nephrotoxic and bone density loss effects, especially in patients with HIV coinfection. Entecavir and lamivudine are not generally associated with renal adverse events. Interestingly, telbivudine has the effect of improving renal function. Serum creatinine, phosphorus and CK levels should be monitored, especially in patients treated with adefovir and tenofovir. Since BMD reduction may occur during tenofovir treatment, BMD measurements should be periodically performed. Although entecavir is suggested to be associated with lactic acidosis in CHB patients with high MELD scores, its use in compensated and decompensated cirrhotic patients were reported to be safe. Safety profile is a major issue that should not be ignored in the treatment of CHB. Further studies should be done to clarify the adverse effects of NAs and determine follow-up timing and frequency, especially in selected patient populations including those with HIV-coinfection or renal impairment, and pregnant or breastfeeding women.

Prolonged treatment experience can still reveal some unknown adverse effects of drugs. Clinical trial data in different patient populations continue to accumulate in the literature. This review contains updated comprehensive data about the safety profile of NAs used in CHB.

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## Role of surgical resection for non-colorectal non-neuroendocrine liver metastases

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### Abstract

It is widely accepted that the indications for hepatec-

tomy in colorectal cancer liver metastases and liver metastases of neuro-endocrine tumors result in relatively better prognoses, whereas, the indications and prognoses of hepatectomy for non-colorectal non-neuroendocrine liver metastases (NCNNLM) remain controversial owing to the limited number of cases and the heterogeneity of the primary diseases. There have been many publications on NCNNLM; however, its background heterogeneity makes it difficult to reach a specific conclusion. This heterogeneous disease group should be discussed in the order from its general to specific aspect. The present review paper describes the general prognosis and risk factors associated with NCNNLM while specifically focusing on the liver metastases of each primary disease. A multidisciplinary approach that takes into consideration appropriate timing for hepatectomy combined with chemotherapy may prolong survival and/or contribute to the improvement of the quality of life while giving respite from systemic chemotherapy.

**Key words:** Non-colorectal non-neuroendocrine liver metastasis; Metastatic liver tumor; Hepatectomy; Gastric cancer liver metastasis; Gastrointestinal stromal tumor liver metastasis; Breast cancer liver metastasis; Melanoma liver metastasis; Sarcoma liver metastasis; Renal cell carcinoma liver metastasis; Ovarian cancer liver metastasis

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**Core tip:** Previous studies reported that the results of hepatectomy for non-colorectal, non-neuroendocrine liver metastasis (NCNNLM) showed an acceptable prognosis in the heterogeneous disease group. However, considering the indication of hepatectomy for NCNNLM, it is important to define the features of each primary disease. The present review paper describes the general prognosis and risk factors associated with NCNNLM, specifically focuses on liver metastasis associated with each primary disease. A multidisciplinary

approach that takes appropriate timing for hepatectomy combined with chemotherapy into consideration may prolong survival and/or contribute to the improvement of the quality of life, while taking time off from systemic chemotherapy.

Takemura N, Saiura A. Role of surgical resection for non-colorectal non-neuroendocrine liver metastases. *World J Hepatol* 2017; 9(5): 242-251 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i5/242.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i5.242>

## INTRODUCTION

Metastatic disease from solid organ tumors occurs frequently in the liver. Presently, surgical resection has been widely accepted as a treatment for colorectal cancer liver metastases<sup>[1,2]</sup> and liver metastases of neuro-endocrine tumors<sup>[3,4]</sup>, providing a relatively better prognosis, whereas, the indications and prognosis of hepatectomy for non-colorectal non-neuroendocrine liver metastases (NCNNLM) remain controversial owing to the rarity of the disease. The biological behavior of NCNNLM varies depending on its primary origin. Discussion of this heterogeneous disease group should be performed in the order from its general to specific aspects. To date, no prospective randomized study has been conducted in this limited field; therefore, in this report we provide a general review of large cohort retrospective studies on hepatectomy for NCNNLM and a more specific review on hepatectomy for liver metastases from different primaries.

## LITERATURE AND RESEARCH

In this report, we reviewed the literature reporting NCNNLM in a large number of patients and their specific primaries. More precisely, we reviewed articles in the English literature that included  $\geq 100$  cases with NCNNLM and relatively large case series for the specific primary (for liver metastases from gastric cancer, breast cancer, and melanoma, reports that included  $\geq 40$  cases were reviewed because of the limited availability of cases in many studies). Using the results reported in the selected literature, the survival outcomes and statistically significant risk factors that impacted survival by multivariate analysis (univariate analysis for some report) were evaluated.

### **Prognosis and risk factors after hepatectomy for NCNNLM**

Along with increased evidence of prolonged survival by hepatectomy in patients with colorectal and neuro-endocrine liver metastases, Schwartz *et al*<sup>[5]</sup> initially categorized NCNNLM and reviewed the literatures in 1995, followed by the analysis of prognosis in a large cohort study by Harrison *et al*<sup>[6]</sup> in 1997. Many validation studies were performed in other patient cohorts that are

summarized in Table 1<sup>[7-16]</sup>. In the present report, we reviewed the 10 largest studies, each with  $\geq 100$  patients who underwent hepatectomy for NCNNLM. In this cohort, the 3- and 5-year overall survival rates were reported as 34%-57% and 19%-42%, respectively, with median survival times of 23-49 mo. The 3- and 5-year disease-free survival rates were 21%-37% and 18%-29%, respectively, with median disease-free survival times of 10-21 mo. The postoperative mortality and morbidity rates were reported 0%-5% and 18%-33%, respectively. In these cohort studies, the reported negative risk factors for survival were the margin status in six studies<sup>[8-11,15,16]</sup>; primary tumor type in four<sup>[8,10,11,15]</sup>; shorter disease-free interval between primary tumor resection and hepatectomy<sup>[8,10,15]</sup> and extrahepatic disease<sup>[10,12,16]</sup> in three; postoperative complications<sup>[14,16]</sup>, larger hepatic metastasis in diameter<sup>[12,13]</sup>, and squamous cell histology<sup>[10,15]</sup> in two; and age<sup>[10]</sup>, major hepatectomy<sup>[10]</sup>, minor hepatectomy<sup>[15]</sup>, synchronous metastasis<sup>[11]</sup>, lymphovascular invasion<sup>[13]</sup>, stromal tumor histology<sup>[15]</sup> and  $> 3$  liver metastases<sup>[16]</sup> in one (Table 1). Negative risk factors for recurrence were extrahepatic disease<sup>[12,16]</sup> in two studies; and primary tumor<sup>[8]</sup>, disease-free interval<sup>[8]</sup>, larger hepatic metastasis in diameter<sup>[12]</sup>, blood transfusion<sup>[14]</sup>, preoperative chemotherapy<sup>[14]</sup>,  $> 3$  liver metastases<sup>[16]</sup>, and residual tumor<sup>[16]</sup> in one. Patients with liver metastases from breast cancer showed significantly better survival in three studies<sup>[10,11,15]</sup>, whereas those with liver metastases from genitourinary tumor liver showed better survival in one<sup>[11]</sup>, and patients with liver metastases from melanoma showed poorer survival compared to other primaries in two studies<sup>[10,15]</sup> (Table 2).

As previously mentioned, the type of primary origin was one of the greatest predictors of survival in patients with this heterogeneous disease. Among the 10 largest studies, the most dominant primary origin was the breast<sup>[7,10,13,15]</sup> and genitourinary<sup>[8,11,12,16]</sup> in four studies and gastrointestinal tract in two<sup>[9,14]</sup>. Elias *et al*<sup>[7]</sup> and Yedibela *et al*<sup>[9]</sup> commented that the resection of liver metastases from gastrointestinal adenocarcinoma correlated with a poor prognosis; however, a more recent report by Takemura *et al*<sup>[14]</sup> showed acceptable prognosis after resection of liver metastases from gastrointestinal carcinoma in their largest cohort with a median survival time of 33.5 mo after hepatectomy. As Yedibela *et al*<sup>[9]</sup> and Groeschl *et al*<sup>[13]</sup> reported that in the more recent years, patients undergoing hepatectomy for NCNNLM appeared to have longer survival compared to previous years, advances in chemotherapy regimens might contribute to prolong survival after the resection of NCNNLM. Adam *et al*<sup>[10]</sup> developed a risk model based on their results of multivariate prognostic factor analysis, which was validated by Lendoire *et al*<sup>[11]</sup>. Their risk model can efficiently stratify the patients into groups; however, the prognosis of each group differed between the two studies depending on the heterogeneous backgrounds of the patient. To facilitate discussion, the prognosis of each primary disease after hepatectomy for NCNNLM has been discussed separately in following section.



**Table 1** Summary of studies each of which included  $\geq 100$  patients who underwent hepatectomy for non-colorectal non-neuroendocrine liver metastases (overall survival)

Ref.	Year	Period	No. of patients	Primary tumor (GI/breast/GU/ melanoma/sarcoma/others)	MST (mo)	3-ysr (%)	5-ysr (%)	Factors associated with worse overall survival
Elias <i>et al</i> <sup>[7]</sup>	1998	1984-1996	120 <sup>1</sup>	(22/35/31/10/13/9)	NR	NR	36 <sup>2</sup>	NR
Yedibela <i>et al</i> <sup>[9]</sup>	2005	1978-2001	150 <sup>1</sup>	(50/24/11/5/15/45)	23 <sup>2</sup>	NR	26 <sup>2</sup>	Margin status (R1,2)
Weitz <i>et al</i> <sup>[8]</sup>	2005	1981-2002	141	(12/29/50/17/0/33)	42	57	NR	Primary tumor type, disease-free interval $\leq 24$ mo, margin status (R1,2)
Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	1452	(314/460/332/148/0/198)	35	49	36	Age, primary tumor (ocular melanoma, non-breast), squamous tumor, disease-free interval, extrahepatic disease, major hepatectomy, margin status (R1,2)
Lendoire <i>et al</i> <sup>[11]</sup>	2007	1989-2006	106	(7/19/40/6/23/11)	27	34	19	Primary tumor (non-breast, non-GU), synchronous metastasis, margin status (R1,2)
O'Rourke <i>et al</i> <sup>[12]</sup>	2008	1986-2006	102	(27/11/31/20/3/10)	42	56	39	Diameter of liver metastasis $> 5$ cm, extrahepatic nodal disease
Groeschl <i>et al</i> <sup>[13]</sup>	2012	1990-2009	420	(13/15/92/31/98/71)	49	50	31	Diameter of liver metastasis $\geq 5$ cm, lymphovascular invasion
Takemura <i>et al</i> <sup>[14]</sup>	2013	1993-2009	145	(91/30/12/1/8/3)	42	55	41	Postoperative complication
Hoffmann <i>et al</i> <sup>[15]</sup>	2015	2001-2012	150	(30/42/33/15/9/21)	46	NR	42	Primary tumor (melanoma, non-breast), interval $< 24$ mo, squamous tumor, non-stromal tumor, minor hepatectomy, margin (R2)
Schiergens <i>et al</i> <sup>[16]</sup>	2016	2003-2013	167	(43/16/61/8/25/14)	35	49	NR	$> 3$ liver metastases, extrahepatic disease, residual tumor (R1,2), major complications

<sup>1</sup>Patients with neuroendocrine tumors were excluded; <sup>2</sup>Results including neuroendocrine tumors. GI: Gastrointestinal; GU: Genitourinary; MST: Median survival time; ysr: Year survival rate; NR: Not reported.

**Table 2** Summary of studies each of which included  $\geq 100$  patients who underwent hepatectomy for non-colorectal non-neuroendocrine liver metastases (disease-free survival)

Ref.	Year	No. of patients	MDFST (mo)	3-ydfrs (%)	5-ydfrs (%)	Factors associated with worse disease-free survival
Elias <i>et al</i> <sup>[7]</sup>	1998	120 <sup>1</sup>	NR	NR	28 <sup>2</sup>	NR
Yedibela <i>et al</i> <sup>[9]</sup>	2005	150 <sup>1</sup>	NR	NR	NR	NR
Weitz <i>et al</i> <sup>[8]</sup>	2005	141	17	30	NR	Primary tumor, disease-free interval $\leq 24$ mo
Adam <i>et al</i> <sup>[10]</sup>	2006	1452	13	27	21	NR
Lendoire <i>et al</i> <sup>[11]</sup>	2007	106	NR	NR	NR	NR
O'Rourke <i>et al</i> <sup>[12]</sup>	2008	102	18	37	27	Diameter of liver metastasis $> 5$ cm, extrahepatic nodal disease
Groeschl <i>et al</i> <sup>[13]</sup>	2012	420	NR	NR	NR	NR
Takemura <i>et al</i> <sup>[14]</sup>	2013	145	10	21	18	Blood transfusion, preoperative chemotherapy
Hoffmann <i>et al</i> <sup>[15]</sup>	2015	150	21	36	29	NR
Schiergens <i>et al</i> <sup>[16]</sup>	2016	167	15	NR	NR	$> 3$ liver metastases, extrahepatic disease, residual tumor (R1,2)

<sup>1</sup>Patients with neuroendocrine tumors were excluded; <sup>2</sup>Results including neuroendocrine tumors. MDFST: Median disease-free survival time; ydfrs: Year disease-free survival ratio; NR: Not reported.

## LIVER METASTASES FROM GASTROINTESTINAL PRIMARY TUMORS

### Gastric cancer liver metastases

In the present report, we reviewed the largest 8 studies, each with  $\geq 40$  patients who underwent hepatectomy for liver metastases from gastric cancer. In this series, the 3- and 5-year overall survival rates were reported as 14%-51% and 9%-42%, respectively, with median survival times of 12-41 mo (Table 3)<sup>[10,17-23]</sup>. Among these studies, the negative risk factors for survival were multiple liver metastases in three studies<sup>[18,20,23]</sup>; larger hepatic metastasis in diameter<sup>[19,21]</sup> and serosal invasion

of primary gastric cancer<sup>[19,21]</sup> in two; and synchronous hepatic metastases<sup>[17]</sup>,  $> 3$  liver metastases<sup>[21]</sup> and  $> 2$  positive regional lymph node metastases of primary gastric cancer<sup>[23]</sup> in one (Table 3). The results of hepatectomy for liver metastasis from gastric cancer are influenced by the statuses of both the primary cancer and liver metastasis. The recent meta-analysis of gastric cancer liver metastases revealed that the surgical resection of liver metastases from gastric cancer was associated with a significantly improved survival and among the patients who underwent surgical resection, patients with solitary hepatic metastasis demonstrated a significantly prolonged survival compared to patients with

**Table 3** Summary of studies each of which included  $\geq 40$  patients who underwent hepatectomy for liver metastasis from gastric cancer

Ref.	Year	Period	No. of patients	MST (mo)	3-yr (%)	5-yr (%)	Factors associated with worse overall survival
Ambiru <i>et al</i> <sup>[17]</sup>	2001	1975-1999	40	12	NR	18	Synchronous metastasis
Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	64	15	NR	27	NR
Cheon <i>et al</i> <sup>[18]</sup>	2008	1995-2005	41	18	32	21	Multiple liver metastases
Takemura <i>et al</i> <sup>[19]</sup>	2012	1993-2011	64	34	50	37	Serosal invasion of primary gastric cancer, maximum hepatic metastasis diameter > 5 cm
Aizawa <i>et al</i> <sup>[20]</sup>	2014	1997-2010	53	27	NR	18	Multiple liver metastases
Kinoshita <i>et al</i> <sup>[21]</sup>	2014	1990-2010	256	31	42	31	Serosal invasion of primary gastric cancer, > 3 liver metastases, maximum hepatic metastasis diameter > 5 cm
Tiberio <i>et al</i> <sup>[22]</sup>	2015	1997-2011	53	13	14	9	NR <sup>2</sup>
Oki <i>et al</i> <sup>[23]</sup>	2015	2000-2010	69	41	51	42	Multiple liver metastases, > 2 positive regional lymph node metastases of primary gastric cancer

<sup>1</sup>As a part of the report of on-colorectal non-neuroendocrine liver metastases; <sup>2</sup>Only risk factors including palliative patients were reported. MST: Median survival time; yr: Year survival rate; NR: Not reported.

**Table 4** Summary of studies with relatively large cohort of patients who underwent hepatectomy for liver metastasis from gastrointestinal stromal tumors

Ref.	Year	Period	No. of patients underwent hepatectomy	MST (mo)	3-yr (%)	5-yr (%)	3-yPFS (%)	No. of patients with TKI	Factors associated with worse overall survival
DeMatteo <i>et al</i> <sup>[26]</sup>	2001	1982-2000	34 <sup>1</sup>	39 <sup>1</sup>	50 <sup>1</sup>	30 <sup>1</sup>	45 <sup>1</sup>	NR	Interval from primary tumor diagnosis $\leq 24$ mo <sup>2</sup>
Nunobe <i>et al</i> <sup>[27]</sup>	2005	1984-2003	18	36	64	34	NR	3 (17%)	NR
Xia <i>et al</i> <sup>[28]</sup>	2010	2005	19	33 (mean)	90	NR	NR	19 (100%)	Non-surgical therapy <sup>2</sup>
Turley <i>et al</i> <sup>[29]</sup>	2012	1995-2010	39	Not reached at 5 yr	68	NR	NR	27 (73%) <sup>3</sup>	Non-TKI therapy, extrahepatic disease
Bauer <i>et al</i> <sup>[30]</sup>	2014	Until 2011	104	96	NR	NR	NR	> 84%	Male <sup>4</sup> , R2 resection <sup>4</sup> , progression disease to TKI at the time of surgery <sup>4</sup> , extrahepatic disease <sup>4</sup>
Du <i>et al</i> <sup>[31]</sup>	2014	NR	19	Not reached	NR	NR	88 (2-yr)	19 (100%)	Non-surgical therapy <sup>2</sup>
Seesing <i>et al</i> <sup>[32]</sup>	2016	1999-2014	48	90	80	76	67	42 (88%)	Margin status (R1,2)

<sup>1</sup>Including gastrointestinal sarcoma; <sup>2</sup>Copmarison to the non-operation group; <sup>3</sup>Excluding two patients lost to follow-up; <sup>4</sup>Results including resections of extrahepatic metastasis. GIST: Gastrointestinal stromal tumor; MST: Median survival time; yr: Year survival rate; PFS: Progression-free survival; TKI: Tyrosine kinase inhibitor; NR: Not reported.

multiple hepatic metastases<sup>[24]</sup>. Compared to colorectal liver metastasis, reports on aggressive repeat hepatectomy have been highly limited<sup>[25]</sup>, which might be owing to the frequent occurrence of extrahepatic recurrence such as peritoneal seeding and lymph node recurrence. However, advancements in effective chemotherapy regimens can expand not only the prognosis but also the surgical indications for hepatectomy in patients with liver metastasis from gastric cancer and colorectal liver metastases alike.

#### Gastrointestinal stromal tumors liver metastases

The 7 largest studies on the hepatectomy for liver metastases from gastrointestinal stromal tumors (GIST) reported 50%-90% and 30%-76% overall 3- and 5-year survival rates, respectively, with median survival times of 33-96 mo (Table 4)<sup>[26-32]</sup>. Non-surgical therapy<sup>[28,31]</sup>, positive resection margin<sup>[30,32]</sup>, and extrahepatic disease<sup>[29,30]</sup> in two studies each and a disease free interval  $\leq 24$  mo<sup>[26]</sup>, absence of tyrosine kinase inhibitor (TKI) therapy<sup>[29]</sup>, male patients<sup>[30]</sup> and progression disease to

TKI therapy at the time of surgery<sup>[30]</sup> were the factors associated with worse survival (Table 4). Different from other NCNNLMs, the emergence of TKI dramatically changed the treatment and prognoses of patients with advanced GIST. The role of surgical resection in the treatment of metastatic GIST had remained unclear in the initial era of treatment with TKI<sup>[33]</sup>; however, recent reports showed evidence that surgical resection combined with TKI offered better prognosis than TKI monotherapy<sup>[29,31,32]</sup>. As Bauer *et al*<sup>[30]</sup> reported progression disease to TKI therapy at the time of surgery, an urgent issue to debate is the appropriate duration of preoperative therapy to minimize the risk of acquiring secondary mutations responsible for TKI resistance<sup>[26,29]</sup>.

#### Other gastro-intestinal primary tumor liver metastases

Pertaining to reports of liver resection for other gastrointestinal primary liver metastases that rarely indicated hepatectomy, esophagus and pancreas cancer liver metastasis showed dismal prognosis with a median overall survival time of 7-20 mo<sup>[10,16,34,35]</sup>. In the mean-

**Table 5** Summary of studies with relatively large cohort of patients who underwent hepatectomy for liver metastases from gastrointestinal primaries other than gastric cancer and gastrointestinal stromal tumors

Disease	Ref.	Year	Period	No. of patients	MST (mo)	3-ysr (%)	5-ysr (%)	Factors associated with worse overall survival
Peri-ampullary	De Jong <i>et al</i> <sup>[34]</sup>	2010	1993-2009	40	17 [23 (intestinal), 13 (pancreaticobiliary)]	18	NR	Intestinal type (ampullary or duodenal) tumors
Ampullary	Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	15	38	NR	46	NR
Small bowel	Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	28	58	NR	49	NR
Pancreas	Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	40	20	NR	25	NR
	Schiergens <i>et al</i> <sup>[16]</sup>	2016	2003-2013	19	7	17	NR	NR
Esophagous	Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	20	16	32	NR	NR
	Ichida <i>et al</i> <sup>[35]</sup>	2013	2003-2005	5	13	NR	NR	NR

<sup>1</sup>As a part of the report of on-colorectal non-neuroendocrine liver metastases. MST: Median survival time; yrs: Year survival rate; NR: Not reported.

**Table 6** Summary of studies with  $\geq 40$  patients who underwent hepatectomy for liver metastasis from breast cancer

Ref.	Year	Period	No. of patients	MST (mo)	3-ysr (%)	5-ysr (%)	MDFS (mo)	Factors associated with worse overall survival
Pocard <i>et al</i> <sup>[36]</sup>	2000	1988-1997	52	42	49	NR	NR	Disease free interval $\leq 48$ mo (univariate)
Elias <i>et al</i> <sup>[37]</sup>	2003	1986-2000	54	34	50	34	NR	Hormone receptor-negative
Adam <i>et al</i> <sup>[38]</sup>	2006	1984-2004	85	32	NR	37	20	Poor response to preoperative chemotherapy, R2, no repeat hepatectomy
Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	454	45	NR	41	NR	NR
Hoffman <i>et al</i> <sup>[39]</sup>	2010	1999-2008	41	58	68	48	34	Positive resection margin, disease-free interval $< 24$ mo
Abbott <i>et al</i> <sup>[40]</sup>	2012	1997-2010	86	57	NR	44	14	ER-negative, disease progression before hepatectomy
Groeschl <i>et al</i> <sup>[13]</sup>	2012	1990-2009	115	52	52	27	22	NR
Mariani <i>et al</i> <sup>[41]</sup>	2013	1988-2007	51	91	NR	NR	NR	Non-hepatectomy <sup>3</sup> , bone metastasis <sup>4</sup>
Hoffmann <i>et al</i> <sup>[15]</sup>	2015	2001-2012	42	63	NR	53	NR	NR
Sadot <i>et al</i> <sup>[42]</sup>	2016	1991-2014	69 <sup>2</sup>	50 <sup>2</sup>	NR	38 <sup>2</sup>	29	Lymph node metastasis in the primary tumor, absence of trastuzumab therapy, multiple liver metastases

<sup>1</sup>As a part of the report of on-colorectal non-neuroendocrine liver metastases; <sup>2</sup>Including 18 patients who underwent percutaneous ablation therapy;

<sup>3</sup>Comparison to the non-operation group; <sup>4</sup>Comparison including patients without hepatectomy. MST: Median survival time; yrs: Year survival rate; NR: Not reported.

while, intestinal type primary tumors such as duodenal, ampullary and small intestinal cancer showed relatively better prognosis with median survival times of 23-58 mo<sup>[10,34]</sup> (Table 5).

## LIVER METASTASES FROM BREAST CANCER

The largest 10 studies, each with  $\geq 40$  patients who underwent hepatectomy for liver metastases from breast cancer were reviewed. In this series, the 3- and 5-year overall survival rates were 49%-68% and 27%-53%, respectively, with median survival times of 41-115 mo (Table 6)<sup>[10,13,15,36-42]</sup>. The negative prognostic predictive factors were short disease-free interval<sup>[36,39]</sup>, negative expression of hormone receptors<sup>[37,40]</sup>, poor response to systemic chemotherapy before surgery<sup>[38,40]</sup>, and positive hepatic resection margin<sup>[38,39]</sup> in two studies; and the absence of repeat hepatectomy<sup>[38]</sup>, non-hepatectomy<sup>[41]</sup>, bone metastasis<sup>[41]</sup>, lymph node metastasis in the primary tumor<sup>[42]</sup>, absence of trastuzumab therapy<sup>[42]</sup>, and multiple liver metastases<sup>[42]</sup> in one (Table 6). Some prognostic factors of liver metastases from breast

cancer are unique and different from other NCNNLMs, which could indicate that the presence of hormone receptors and HER2 overexpression requires the use of chemotherapy and/or hormone therapy and influences patient survival. Neuman *et al*<sup>[43]</sup> suggested that the impact of local control for liver metastases from breast cancer was greatest in the presence of effective targeted therapy. Similar to other NCNNLMs, surgical resection before progression of disease even with chemotherapy might result in better outcomes of selected patients with liver metastases from breast cancer<sup>[40]</sup>. As Sadot *et al*<sup>[42]</sup> advocated in their study, hepatic resection for liver metastases from breast cancer might not confer a survival advantages; however, might allow time off from systemic chemotherapy.

## LIVER METASTASES FROM MELANOMA

The largest four studies, each with  $\geq 40$  patients who underwent liver resection for liver metastases from melanoma, reported an overall 5-year survival rate of approximately 7%-20% with a median survival time of 14-28 mo (Table 7)<sup>[10,44-46]</sup>. Short disease-free interval from the diagnosis of primary tumor<sup>[45]</sup>, positive resection

**Table 7 Summary of studies with  $\geq 40$  patients who underwent hepatectomy for liver metastasis from melanoma**

Ref.	Year	Period	No. of patients	Ocular/ cutaneous	MST (mo) (ocular/ cutaneous)	3-yr (%)	5-yr (%)	Factors associated with worse overall survival
Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	148	104/44	19/27	NR	21 (ocular)/22 (cutaneous)	NR
Pawlik <i>et al</i> <sup>[44]</sup>	2006	1988-2004	40	16/24	28 [29 (ocular)/24 (cutaneous)]	62 (ocular)/48 (cutaneous) (2-yr)	11 (21 (ocular)/0 (cutaneous))	Cutaneous melanoma, no preoperative chemotherapy (in cutaneous melanoma) (univariable)
Mariani <i>et al</i> <sup>[45]</sup>	2009	1991-2007	255 (R2 = 157)	255/0	14 (27 mo after R0 resection)	NR	7	Interval from primary tumor diagnosis $\leq 24$ mo, R1 and R2, number of the metastases $> 4$ , miliary disease
Mariani <i>et al</i> <sup>[46]</sup>	2016	2000-2013	70 (including 13 concomitant with RFA)	70/0	27 (hepatectomy), 28 (+RFA)	NR	NR	NR

<sup>1</sup>As a part of the report of on-colorectal non-neuroendocrine liver metastases. MST: Median survival time; ysr: Year survival rate; NR: Not reported.

**Table 8 Summary of studies with relatively large cohort of patients who underwent hepatectomy for liver metastasis from sarcoma**

Ref.	Year	Period	No. of patients	MST (mo)	3-yr (%)	5-yr (%)	Factors associated with worse overall survival
Lang <i>et al</i> <sup>[48]</sup>	2000	1982-1996	26 (including 9 second, 2 third resection)	32 (R0 first resection), 21 (R1,2 resection)	NR	13	NR
DeMatteo <i>et al</i> <sup>[26]</sup>	2001	1982-2000	56 <sup>1</sup>	39 <sup>1</sup>	50 <sup>1</sup>	30 <sup>1</sup>	Time to liver metastasis from the primary tumor diagnosis $\leq 24$ mo Non-GIST
Pawlik <i>et al</i> <sup>[49]</sup>	2006	1996-2005	53 (35Hx, 18RF + Hx, and 13RF), (including 36 GISTs)	47 <sup>2</sup>	65 <sup>2</sup>	27 <sup>2</sup>	Primary leiomyosarcoma
Marudanayagam <i>et al</i> <sup>[50]</sup>	2011	1997-2009	36 <sup>1</sup> (including 5 GISTs)	24	48	32	NR
Groeschl <i>et al</i> <sup>[13]</sup>	2012	1990-2009	98	72	60	32	Interval from primary tumor diagnosis $\leq 24$ mo, extrahepatic disease, positive margins
Zhang <i>et al</i> <sup>[51]</sup>	2015	2000-2009	27	NR	NR	46	

<sup>1</sup>Including some patients with GIST before 1993, GISTs were considered as leiomyosarcomas; <sup>2</sup>Including results of RF and patients with GIST; <sup>3</sup>As a part of the report of on-colorectal non-neuroendocrine liver metastases. GIST: Gastrointestinal stromal tumor; MST: Median survival time; ysr: Year survival rate; NR: Not reported; Hx: Hepatectomy; RF: Radiofrequency ablation.

margin<sup>[45]</sup>,  $> 4$  liver metastases<sup>[45]</sup>, miliary disease of the primary melanoma<sup>[45]</sup>, cutaneous melanoma<sup>[46]</sup>, and no preoperative chemotherapy were the risk factors predicting poor patients survival (Table 7). The metastatic pathway of ocular and cutaneous melanomas is different. Ocular melanoma often spreads hematogenously to the liver because there are no lymphatics in the uveal tract. In contrast, cutaneous melanomas potentially spread to the lung, lymph node and soft tissue, and infrequently to the liver<sup>[47]</sup>. Liver metastases from ocular melanoma often recur within the liver, whereas cutaneous melanoma is more likely to develop extrahepatic recurrence<sup>[44]</sup>. Surgical resection should be performed concomitantly with system in chemotherapy as part of a multidisciplinary approach because recurrent disease frequently develops after hepatectomy.

## LIVER METASTASES FROM SARCOMA

The six largest studies on the resection of liver metastases from sarcoma reported 50%-65% and 13%-46% overall 3- and 5-year survival rates, respectively, with median survival times of 24-72 mo (Table 8)<sup>[13,26,48-51]</sup>.

Negative risk factors for overall survival in this cohort were a time of  $< 24$  mo from the diagnosis of primary tumor to the time of liver metastasis<sup>[26,51]</sup>, non-GIST<sup>[49]</sup>, leiomyosarcoma<sup>[50]</sup>, extrahepatic disease<sup>[51]</sup>, and positive resection margins<sup>[51]</sup> (Table 8). These studies included some GIST patients particularly in the early study periods because GIST had been considered as leiomyosarcoma before around 1993. Repeat hepatic resection was reported in four studies. Lang *et al*<sup>[48]</sup> reported 9 second and 2 third cases of hepatectomy for intrahepatic recurrent sarcoma. Less sensitivity to chemotherapy might prompt the surgeon to conduct a repeat hepatectomy with R0 resection, resulting in a favorable outcome<sup>[48]</sup>.

## LIVER METASTASES FROM GENITOURINARY TUMORS

Genitourinary tumors mainly comprise renal cell carcinoma, gynecological carcinoma most commonly with ovarian cancer, and testicular cancer. In the present report, we have reviewed 6 studies pertaining to liver metastases from the renal cell carcinoma which reported



**Table 9** Summary of studies with relatively large cohort of the patients who underwent hepatectomy for liver metastasis from genitourinary primary tumor

Disease	Ref.	Year	Period	No. of patients	MST (mo)	3-yr (%)	5-yr (%)	Factors associated with worse overall survival
Renal cell carcinoma	Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	85	36	NR	38	NR
	Thelen <i>et al</i> <sup>[52]</sup>	2007	1988-2006	31	48	54	39	Resection margin (R1,2)
	Staehler <i>et al</i> <sup>[53]</sup>	2010	1995-2006	68	142	NR	62	High-grade primary renal cell carcinoma, performance status $\geq 1$ , lymph node status
	Ruys <i>et al</i> <sup>[54]</sup>	2011	1990-2008	29	33	47	43	Synchronous metastases, R1,2 resection margin (univariate)
	Hatzaras <i>et al</i> <sup>[55]</sup>	2012	1994-2011	43	Not reached	62	NR	Disease-free interval $\leq 12$ mo, extrahepatic disease (univariate)
Gynecologic primary Ovarian cancer	Schiergens <i>et al</i> <sup>[16]</sup>	2016	2003-2013	28	50	68	NR	NR
	Kamel <i>et al</i> <sup>[56]</sup>	2011	1990-2010	52	53	57	41	NR
	Merideth <i>et al</i> <sup>[57]</sup>	2003	1976-1999	26 <sup>2</sup>	26	NR	NR	Interval from the primary diagnosis < 12 mo, residual disease > 1 cm (univariate)
	Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	65	98	NR	50	NR
	Lim <i>et al</i> <sup>[58]</sup>	2009	2001-2008	14 <sup>2</sup>	Not reached	NR	51	Hematogenous liver metastasis < hepatic parenchymal metastasis from peritoneal seeding <sup>5</sup>
	Neumann <i>et al</i> <sup>[59]</sup>	2012	1991-2007	41	42(R0 resection)	NR	NR	R1,2 resection, pre-operative ascites, bilobular liver metastasis
	Niu <i>et al</i> <sup>[60]</sup>	2012	2000-2011	60	39	NR	30	R1,2 resection
	Kolev <i>et al</i> <sup>[61]</sup>	2014	1988-2012	27 <sup>3</sup>	56	NR	NR	Interval from the primary surgery $\leq 24$ mo, residual disease $\geq 1$ cm
	Bacalbasa <i>et al</i> <sup>[62]</sup>	2015	2002-2014	31 <sup>2,4</sup>	16 (metastasis from seeding), 13 (hematogenous)	NR	NR	No significant risk factor
	Schiergens <i>et al</i> <sup>[16]</sup>	2016	2003-2013	24	33	43	NR	NR
Testicular cancer	Hahn <i>et al</i> <sup>[63]</sup>	1999	1974-1996	57	NR	97 (2-yr)	NR	NR
	Adam <i>et al</i> <sup>[10]</sup>	2006	1983-2004	78	82	NR	51	NR

<sup>1</sup>As a part of the report of on-colorectal non-neuroendocrine liver metastases; <sup>2</sup>As a part of debulking surgery; <sup>3</sup>Hepatectomy as secondary cytoreduction; <sup>4</sup>Including 2<sup>nd</sup> ( $n = 15$ ), 3<sup>rd</sup> (3) and 4<sup>th</sup> (2) cytoreduction operations; <sup>5</sup>Only risk factors that included patients undergoing palliative treatment were reported. MST: Median survival time; yr: Year survival rate; NR: Not reported.

overall 3- and 5-year survival rate of 54%-68% and 38%-62%, respectively, with median survival times of 33-142 mo (Table 9)<sup>[10,16,52-55]</sup>. The negative prognostic risk factors were the resection margin<sup>[52,54]</sup>, high-grade tumor<sup>[53]</sup>, poor performance status<sup>[53]</sup>, lymph node metastasis<sup>[53]</sup>, synchronous metastasis<sup>[54]</sup>, short disease-free interval<sup>[55]</sup>, and extra hepatic disease<sup>[55]</sup> (Table 9). Staehler *et al*<sup>[53]</sup> is the first to advocate a favorable prognosis for hepatectomy in patients who underwent resection of liver metastases from renal cell carcinoma over the prognosis of patients who refused to undergo hepatectomy for metastatic renal cell carcinoma, albeit the requirement for further systemic treatment.

The nine largest studies pertaining to gynecological primary cancers, particularly with ovarian cancer, reported 5-year overall survival rates of 30%-51% with median survival times of 26-98 mo (Table 9)<sup>[10,16,56-62]</sup>. Factors associated with worse survival were shorter interval from the diagnosis of primary disease to metastasis<sup>[56,61]</sup>, residual tumor measuring > 1 cm<sup>[56,61]</sup>, hematogenous liver metastasis<sup>[57]</sup>, positive resection margins<sup>[59,60]</sup>, pre-operative ascites<sup>[59]</sup>, and bi-lobular hepatic metastasis<sup>[59]</sup> (Table 9). Owing to the unique features of ovarian cancer, hepatectomy was regarded as a part of cytoreductive surgery and concomitant chemotherapy, which has been accepted as the standard treatment for advanced ovarian cancer. In contrast to

other NCNNLMs, the resection of liver metastases from the peritoneal seeding showed better prognosis than resection of hematogenous liver metastases<sup>[57]</sup>.

Chemotherapy is highly effective in the treatment of testicular carcinoma; however, one-third of the patients either did not achieve complete responses or experienced relapses<sup>[63]</sup>. The limited studies involving treatment with sensitive chemotherapy and subsequent hepatectomy for testicular carcinoma have sufficiently demonstrated a favorable prognosis in patients who underwent this treatment regimen<sup>[63]</sup>.

## CONCLUSION

The clinical evidence accumulated with regards to NCNNLM has indicated the possibility of a chemotherapy-free period and a few studies have demonstrated a curing potential; however, almost all studies reviewed in the present report were conducted retrospectively in selected patients who underwent hepatic resection, which makes determining the absolute indications for hepatectomy in patients with NCNNLM challenging. Indications of hepatectomy for NCNNLM change according to the development of chemotherapy regimens. Strong and highly effective chemotherapy regimens might either expand the indications for hepatectomy or replace hepatectomy in this field. A multidisciplinary approach is

required for the treatment of patients with diseases that are otherwise difficult to treat.

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Retrospective Study

# Efficacy and safety of telaprevir- and simeprevir-based triple therapies for older patients with chronic hepatitis C

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## Abstract

### AIM

To evaluate and compare the efficacy and safety of telaprevir (TVR)- and simeprevir (SMV)-based triple therapies in elderly patients, specifically patients aged 66 years or older.

### METHODS

The present study enrolled 112 and 76 Japanese patients with chronic hepatitis C virus genotype 1b infection who were treated with a 12-wk TVR-based or SMV-based triple therapy, respectively, followed by a dual therapy that included pegylated interferon  $\alpha$  and ribavirin (RBV) for 12 wk. The patients were categorized into two groups according to age as follows: A younger group of patients aged  $\leq 65$  years old and an older group of patients aged  $> 65$  years old. Among the patients treated with TVR-based triple therapy, 34 patients were included in the older group. The median ages were 56 years (range: 28–65 years) in the younger group and 69 years (range: 66–81 years) in the older group. Among the patients treated with SMV-based triple therapy, 39 patients were included in the older group. The median ages were 59 years (range: 36–65 years) in the younger group and 71 years (range: 66–86 years) in the older group. The clinical, biochemical and virological data were analyzed before and during treatment.

### RESULTS

Among the patients treated with the TVR-based triple therapy, no significant difference in the sustained virological response (SVR) was found between the younger (80.8%) and older (88.2%) groups. The SVR rates for patients with the interleukin 28B (IL28B) (rs8099917) TG/GG-genotypes (73.9% and 60.0% in the younger and older groups, respectively) were significantly lower than for patients with the IL28B TT-genotype (86.3% and 92.9%, respectively). The cumulative exposure to RBV for the entire 24-wk treatment period (as a percentage of the target dose) was significantly higher in the younger group than in the older group (91.7% *vs* 66.7%, respectively,  $P < 0.01$ ), but the cumulative exposure to TVR was not significantly different between the younger and older groups (91.6% *vs* 81.9%, respectively). A multivariate analysis identified the TT-genotype of IL28B (OR = 8.160; 95%CI: 1.593–41.804,  $P = 0.012$ ) and the adherence of RBV ( $> 60\%$ ) (OR = 11.052; 95%CI: 1.160–105.273,  $P = 0.037$ ) as independent factors associated with the SVR. Adverse events resulted in discontinuation of the treatment in 11.3% and 14.7% of the younger and older groups, respectively. Among the patients treated with the SMV-based triple therapy, no significant difference in the SVR rate was found between the younger (81.1%) and older (82.1%) groups. The SVR rates for patients with the IL28B TG/GG-genotypes (77.8% and 64.7% in the younger and older groups, respectively) were significantly lower than for patients with the IL28B TT-genotype (88.2% and 100%, respectively). A multivariate analysis identified the TT-genotype of IL28B as an independent factor associated with the SVR (OR = 9.677; 95%CI:

1.114–84.087,  $P = 0.040$ ). Adverse events resulted in discontinuation of the treatment in 7.0% and 14.3% of patients in the younger and older groups, respectively.

### CONCLUSION

Both TVR- and SMV-based triple therapies can be successfully used to treat patients aged 66 years or older with genotype 1b chronic hepatitis C. Genotyping of the IL28B indicates a potential to achieve SVR in these difficult-to-treat elderly patients.

**Key words:** Telaprevir; Aged patients; Hepatitis C virus genotype 1b; Interleukin 28B; Simeprevir

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**Core tip:** We evaluated the efficacy and safety of telaprevir (TVR)- and simeprevir (SMV)-based triple therapies for elderly patients with chronic hepatitis C, especially patients aged 66 years or older, in a real-world clinical setting. In both the TVR and SMV groups, no significant differences in the SVR and adverse events resulting in treatment discontinuation were found between the younger (aged  $\leq 65$ ) and older (aged  $> 65$ ) patients. Both the TVR- and SMV-based triple therapies can be successfully used to treat patients aged 66 years or older with chronic hepatitis C virus genotype 1b infection. Genotyping of the interleukin-28B indicates a potential to achieve SVR in these difficult-to-treat elderly patients.

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## INTRODUCTION

Chronic hepatitis C virus (HCV) infections affect approximately 130–170 million people worldwide and are associated with an increased risk of developing liver cirrhosis and hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. In Japan, an estimated 1.5–2 million people are infected with HCV<sup>[3]</sup>. Most of infected patients in Japan are infected with genotype 1 HCV and are older than the infected patients in Europe and the United States<sup>[4]</sup>. Although older patients with chronic HCV infection have a higher risk of developing HCC than younger patients even at the same liver fibrosis stage<sup>[5]</sup>, older patients have been reported to show poor virological responses to antiviral treatments, especially postmenopausal women<sup>[6–8]</sup>. Because older patients often have reduced cardiovascular, pulmonary, and renal function and a decreased blood count, they are usually more susceptible to the toxic effects of antiviral treatments, which may lead to a

higher rate and severity of adverse events and a poor adherence to the treatment<sup>[4]</sup>. Therefore, an evaluation of the safety and efficacy of antiviral treatments, especially in elderly patients with chronic HCV infections, is still necessary.

Before the introduction of direct-acting antiviral agents (DAA), pegylated interferon (PegIFN)  $\alpha$  and ribavirin (RBV) were the standard of care for HCV genotype 1 infections. However, with the approval of telaprevir (TVR) that is an HCV non-structural (NS) 3/4A protease inhibitor, the optimum treatment regimen for chronic HCV genotype 1 infections was changed to a triple therapy with a protease inhibitor plus PegIFN  $\alpha$  and RBV for 24 wk<sup>[9]</sup>. The TVR-based triple therapy has achieved an improved sustained virological response (SVR) rate compared to PegIFN monotherapy or PegIFN  $\alpha$  plus RBV dual therapy<sup>[10,11]</sup>. However, the TVR-based triple therapy is associated with an increased rate and severity of adverse events, including pruritus, skin rash, anemia, and anorectal diseases, as well as increased rates of treatment discontinuation compared to patients receiving PegIFN  $\alpha$  plus RBV dual therapy<sup>[10,11]</sup>. Because of the increased risk and severity of adverse events associated with the TVR-based triple therapy, it is difficult to use this therapy in older patients, and, therefore, reports describing the safety and efficacy of TVR-based triple therapy in elderly patients are limited<sup>[4]</sup>.

Simeprevir (SMV) is a second-generation oral HCV NS3/4A protease inhibitor with antiviral activity against HCV genotype 1, 2, 4, 5 and 6 infections<sup>[12]</sup>. The QUEST 1 and QUEST 2 phase 3 clinical trials demonstrated the SVR rates of 80% and 81%, respectively, in patients treated with SMV-based triple therapy combined with PegIFN  $\alpha$  and RBV<sup>[13]</sup>. In Japan, 4 phase 3 clinical trials (CONCERTO) were conducted, and the SVR rates were 88.6% and 91.7% for treatment-naïve patients; 35.8%, 50.9% and 38.5% for non-responders; and 89.8% and 96.6% for patients that relapsed<sup>[14-16]</sup>. Although the SMV-based triple therapy shows a favorable efficacy without inducing severe dermatologic and hematologic toxicities, the safety and efficacy of the SMV-based triple therapy for elderly patients has not yet been fully evaluated. Therefore, in the present study, we aimed to assess the efficacy and safety of TVR- and SMV-based triple therapies in elderly patients, specifically patients aged 66 years or older, in a real-world clinical setting.

## MATERIALS AND METHODS

### Patients

This prospective and multicenter study enrolled 112 and 76 HCV genotype 1b Japanese patients who received 12 wk of TVR-based and SMV-based triple therapies, respectively, followed by a dual therapy that included PegIFN  $\alpha$  and RBV for 12 wk. Nine hospitals in Niigata, Japan, including Niigata University Hospital, participated in this study. The patients were categorized into two groups according to age as follows: A younger group

of patients aged  $\leq 65$  years old and an older group of patients aged  $> 65$  years old. Among the patients treated with the TVR-based triple therapy, 34 patients were included in the older group. The median ages were 56 years (range: 28-65 years) in the younger group and 69 years (range: 66-81 years) in the older group. Among the patients treated with the SMV-based triple therapy, the older group consists of 39 patients. The median ages were 59 years (range: 36-65 years) in the younger group and 71 years (range: 66-86 years) in the older group. Liver biopsy samples were obtained from 34 (30.6%) and 42 patients (55.2%) in the TVR and SMV groups, respectively. For each sample, the fibrosis stage (F0-4) and activity grade (A0-3) were evaluated according to the Metavir score<sup>[17]</sup>.

According to responses to prior treatments, relapse was defined as undetectable HCV during and at the end of treatment with positive HCV RNA detecting later on. Non-responder was defined as detectable HCV RNA for more than 24 wk. Patients with decompensated liver cirrhosis, hepatocellular carcinoma, co-infection with hepatitis B virus or human immunodeficiency virus, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, or Wilson's disease were excluded. Patients with uncontrollable diabetes mellitus, chronic renal failure, depression, and those with a history of alcohol abuse, were also excluded. Information regarding patient profiles was shown in Tables 1 and 2.

### Study design

All patients received a 12-wk triple therapy that included either TVR [1500 or 2250 mg/d; the initial dose of TVR was determined by each attending physician based on each patient's baseline characteristics such as bodyweight (BW)] (the dose of TVR was also reduced by each attending physician based on each patient's adverse events such as anemia, malaise, and anorexia) (Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan) or SMV (100 mg/d) (Sovriad; Janssen Pharmaceutical K.K., Tokyo, Japan) combined with PegIFN  $\alpha$ 2a (180  $\mu$ g/wk) (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) or PegIFN  $\alpha$ 2b (1.5  $\mu$ g/BW kg per week) (Peg-Intron; MSD, Tokyo, Japan) and RBV (600-1000 mg/d according to BW as follows:  $< 60$  kg: 600 mg/d; 60-80 kg: 800 mg/d;  $> 80$  kg: 1000 mg/d; if the patient's hemoglobin was  $< 13$  g/dL at the start of therapy, RBV was reduced by 200 mg) (Rebetol; MSD or Copegus; Chugai Pharmaceutical Co., Ltd.), followed by dual therapy of PegIFN  $\alpha$ 2a or PegIFN  $\alpha$ 2b with RBV for 12 wk.

This study was conducted in accordance with the Declaration of Helsinki. The study was reviewed and approved by the Niigata University Medical and Dental Hospital Institutional Review Board. Written informed consent was appropriately obtained from all of the individuals who enrolled in the study according to the institutional review board's approved protocols (approval No. 1474) at the Niigata University Medical and Dental Hospital.

**Table 1 Patient characteristics by age (telaprevir)**

Factors (median, range)	Patients aged < 66	Patients aged ≥ 66	P value
<i>n</i>	78	34	
Gender, <i>n</i> (male/female)	41/37	20/14	0.68
Age (yr)	56 (28-65)	69 (66-81)	< 0.001
Body weight (kg)	61.1 (35.0-97.4)	57.8 (41.0-74.8)	0.105
Body mass index (kg/m <sup>2</sup> )	22.7 (15.8-32.2)	22.9 (17.9-28.9)	0.892
Baseline HCV-RNA (log IU/mL)	6.7 (3.9-7.7)	6.7 (3.1-7.8)	0.766
White blood cell (/mm <sup>3</sup> )	5000 (1900-8720)	4500 (2700-7700)	0.245
Hemoglobin (g/dL)	14.0 (9.1-18.6)	13.5 (9.5-16.3)	0.121
Platelets (× 10 <sup>3</sup> /mm <sup>3</sup> )	15.8 (6.5-28.7)	13.4 (8.3-29.0)	0.068
Albumin (mg/dL)	4.1 (2.7-5.9)	3.9 (2.4-4.4)	0.007
AST (IU/L)	40 (17-249)	45 (20-163)	0.909
ALT (IU/L)	48 (15-278)	38 (15-189)	0.486
γ-GTP (IU/L)	39 (11-717)	25 (11-144)	0.034
Serum creatinine (mg/dL)	0.7 (0.4-1.2)	0.8 (0.4-1.0)	0.036
Estimated GFR (mL/min)	79.0 (44.0-134.0)	71.5 (39.0-101.9)	0.006
Prior treatment response, <i>n</i> (naïve/relapse/non-responder)	45/26/7	15/15/4	0.403
Liver histology (F0-2/3-4/ND)	21/6/51	4/3/27	0.348
IL28B SNP (rs8099917), <i>n</i> (TT/non-TT/ND)	51/22/5	28/5/1	0.235
HCV ISDR, <i>n</i> (0/1-3/4-/NT)	32/26/6/14	15/10/2/7	0.955
HCV Core 70, <i>n</i> (Wild/Mutant/ND)	46/18/14	18/10/6	0.751
HCV Core 91, <i>n</i> (Wild/Mutant/ND)	42/22/14	19/9/6	1
Serum CXCL10 (pg/mL)	510 (95-1794)	543 (118-1218)	0.445

GFR: Glomerular filtration rate; IL28B SNP: Interleukin-28B single nucleotide polymorphism; ND: Not determined; ISDR: Interferon sensitivity-determining region; HCV Core 70 or 91: At position 70 or 91 of the HCV core protein; CXCL10: Chemokine (C-X-C motif) ligand 10; HCV: Hepatitis C virus; AST: Aspartate transaminase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl-transpeptidase.

**Table 2 Patient characteristics by age (simeprevir)**

Factors (median, range)	Patients aged < 66	Patients aged ≥ 66	P value
<i>n</i>	37	39	-
Gender, <i>n</i> (%) (male/female)	19/18 (48.6)	14/25 (64.1)	0.123
Age (yr)	59 (36-65)	71 (66-86)	< 0.001
Body weight (kg)	62.0 (39.8-94.0)	56.0 (37.5-76.6)	0.011
Body mass index (kg/m <sup>2</sup> )	22.8 (17.2-30.3)	22.7 (17.8-32.1)	0.287
Baseline HCV-RNA (log IU/mL)	6.7 (5.4-7.8)	6.6 (4.7-7.6)	0.631
White blood cells (/mm <sup>3</sup> )	4620 (2600-7800)	4300 (2400-8100)	0.010
Hemoglobin (g/dL)	13.8 (11.0-16.7)	13.1 (9.8-16.8)	< 0.001
Platelets (× 10 <sup>3</sup> /mm <sup>3</sup> )	16.4 (8.7-28.8)	16.3 (7.3-31.7)	0.291
Albumin (mg/dL)	4.2 (2.8-4.8)	4.0 (3.1-4.6)	0.002
AST (IU/L)	45 (21-159)	34 (19-128)	0.056
ALT (IU/L)	42 (16-316)	29 (12-112)	0.006
γ-GTP (IU/L)	29 (13-260)	27 (9-171)	0.388
Serum creatinine (mg/dL)	0.70 (0.44-1.01)	0.70 (0.42-1.36)	0.689
Estimated GFR (mL/min)	78.7 (50.0-112.6)	77.4 (41.3-109.0)	0.221
Prior treatment response, <i>n</i> (naïve/relapse/non-responder)	20/10/7	13/16/10	0.197
Liver histology (F0-2/3-4/ND)	12/6/19	19/5/15	0.483
IL28B SNP (rs8099917), <i>n</i> (TT/non-TT/ND)	17/19/1	18/17/4	1
HCV ISDR, <i>n</i> (0/1-3/4-/ND)	9/13/5/10	11/12/2/14	0.044
HCV Core 70, <i>n</i> (Wild/Mutant/ND)	17/13/7	15/8/16	1
HCV Core 91, <i>n</i> (Wild/Mutant/ND)	18/12/7	18/5/16	0.385

GFR: Glomerular filtration rate; IL28B SNP: Interleukin-28B single nucleotide polymorphism; ND: Not determined; ISDR: Interferon sensitivity-determining region; HCV core 70 or 91: At position 70 or 91 of the HCV core protein; HCV: Hepatitis C virus; AST: Aspartate transaminase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl-transpeptidase.

### Laboratory and safety assessments

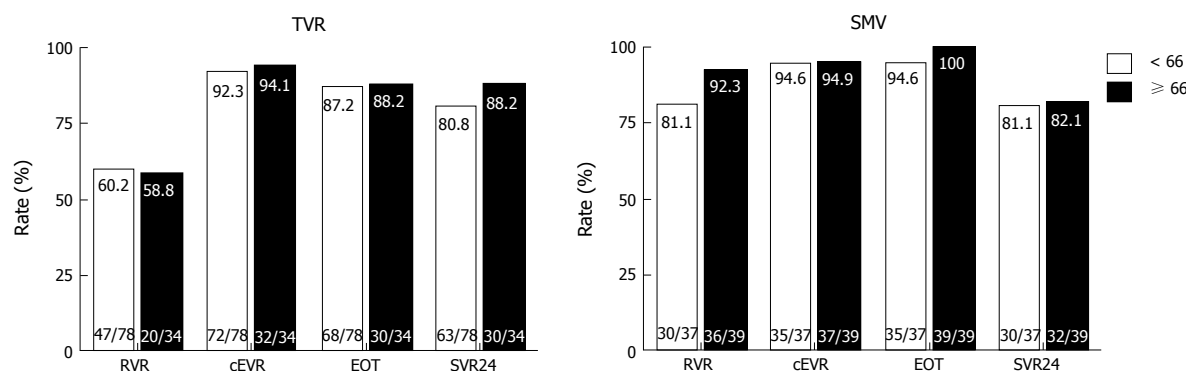
Laboratory and safety assessments were performed at initiation of treatment; at treatment weeks 2, 4, 8, 12, 16, 20 and 24; at the end of treatment; and at 12 and 24 wk after the end of treatment. Data on adverse events were collected, and physical examinations were

performed at each visit, if clinically indicated.

### Detection of HCV markers

The detection of HCV viremia was performed using a real-time polymerase chain reaction assay (COBAS TaqMan HCV test, Roche Diagnostic, Tokyo, Japan) with





**Figure 1** Rates of virological responses to telaprevir and simeprevir by age. Percentages indicate the proportion of patients with undetectable serum hepatitis C virus (HCV) RNA levels. Patient numbers are shown in parenthesis. TVR: Telaprevir; SMV: Simeprevir; RVR: Rapid virological response; cEVR: Complete early virological response; EOT: End of treatment response; SVR24: Sustained virological response defined as undetectable serum HCV RNA at 24 wk after the end of treatment.

a lower limit of quantitation of 15 IU/mL and a linear dynamic range of 1.2–7.8 log IU/mL. The number of amino acid substitutions in the interferon sensitivity-determining region (in the range of 2209–2248 in the HCV NS5A) was determined using a direct sequencing method as reported previously<sup>[18]</sup>. The core amino acid substitutions at positions 70 and 91 of the HCV genome were determined by direct sequencing as reported previously<sup>[19]</sup>.

### Treatment efficacy

SVR that is defined as undetectable serum HCV RNA at 24 wk after the end of treatment was successful treatment. Early virological responses during the first 12 wk of treatment were defined as rapid virological response (RVR), which was undetectable HCV RNA at week 4, and complete early virological response (cEVR), which was undetectable at week 12. End of treatment response (ETR) was defined as undetectable HCV RNA at the end of treatment. Relapse was defined as an ETR response but non-SVR.

### Interleukin 28B single-nucleotide polymorphism

Human genomic DNA was extracted from the peripheral blood. Single-nucleotide polymorphism (SNP) genotyping of the interleukin 28B (IL28B) (rs8099917) gene was performed using the TaqMan allelic discrimination demonstration kit (Applied Biosystems, Foster City, CA). The rs8099917 genotype was classified into the following 2 categories: TT (major genotype) and non-TT (minor genotype, TG or GG).

### Statistical analysis

Continuous data from patients are expressed as the median with the interquartile range. The significance of the differences was analyzed statistically by the  $\chi^2$ , Fisher's exact test, or Mann-Whitney *U* test, as appropriate, using SPSS software (Ver.18, SPSS Inc., Chicago, IL). To evaluate independent factors for predicting an SVR, variables that reached the  $P < 0.1$  level in the univariate tests were used as candidate factors in a multivariate logistic regression analysis. In all of the cases, the level of

significance was set as  $P$  value  $< 0.05$ .

## RESULTS

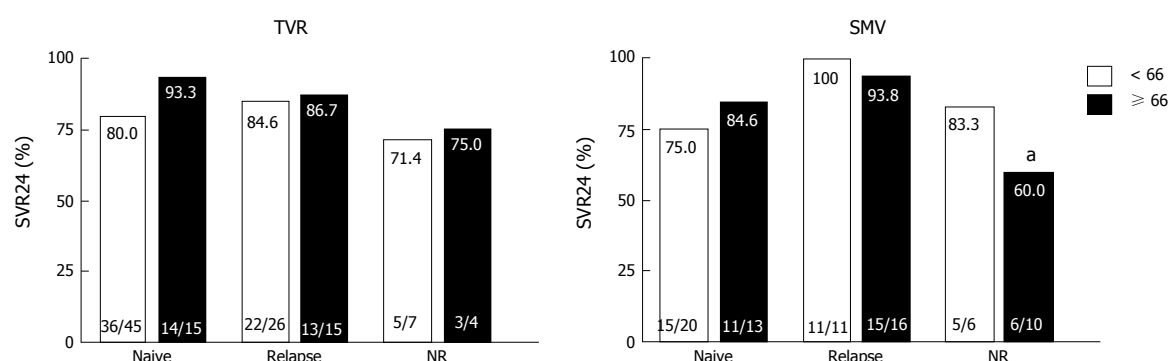
### Patient characteristics

The patient characteristics in the TVR group ( $n = 112$ ) and SMV group ( $n = 76$ ) are summarized by age in Tables 1 and 2. The analysis of the pretreatment factors revealed that serum albumin,  $\gamma$ -glutamyl-transpeptidase, and the estimated glomerular filtration rate in the older patients were significantly lower than those of the younger patients in the TVR group (Table 1). Pretreatment serum chemokine C-X-C motif ligand 10 (CXCL10) levels were not significantly different between the younger (543 pg/mL, range: 118–1218 pg/mL) and older (510 pg/mL, range: 95–1794 pg/mL) groups. In the SMV group, BW, white blood cell count, hemoglobin, serum albumin, and serum alanine aminotransferase (ALT) in the older patients were significantly lower than those of the younger patients (Table 2). No significant differences in the prior treatment response, HCV core 70/91 mutations, or IL28B SNPs were found between the younger and older group in both TVR and SMV groups.

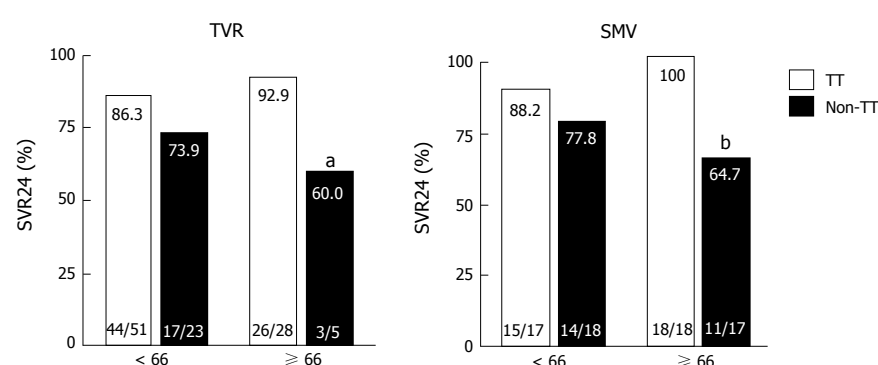
### Virological response and outcome

Figure 1 shows the virological responses by age. RVR, cEVR, ETR and SVR did not significantly differ between the younger and older patients in the TVR group (60.2% vs 58.8%, 92.3% vs 94.1%, 87.2% vs 88.2%, and 80.8% vs 88.2%, respectively). Similar to the TVR group, RVR, cEVR, ETR and SVR did not significantly differ between the younger and older patients in the SMV group (81.1% vs 92.3%, 94.6% vs 94.9%, 94.6% vs 100% and 81.1% vs 82.1%, respectively). In the older patients, SVR did not significantly differ between the TVR and SMV groups, although RVR was significantly higher in the SMV group than in the TVR group (92.3% vs 58.5%,  $P < 0.01$ ).

Figure 2 shows the virological responses according to prior treatment responses. In both the TVR and SMV groups, SVR did not significantly differ between the younger and older patients with the same treatment responses. In the older patients in the SMV group, SVR



**Figure 2** Rates of sustained virological response to telaprevir and simeprevir by prior treatment responses. Percentages indicate the proportion of patients with undetectable serum hepatitis C virus (HCV) RNA levels at 24 wk after the end of treatment. Patient numbers are shown in parenthesis. <sup>a</sup> $P = 0.033$  (compared to relapsers in the older patients). NR: Non-responders; TVR: Telaprevir; SMV: Simeprevir; SVR24: Sustained virological response defined as undetectable serum HCV RNA at 24 wk after the end of treatment.



**Figure 3** Rates of sustained virological response to telaprevir and simeprevir by interleukin 28B single-nucleotide polymorphism. Percentages indicate the proportion of patients with undetectable serum hepatitis C virus RNA levels at 24 wk after the end of treatment. Patient numbers are shown in parenthesis. TT, interleukin 28B (IL28B) (rs8099917) TT-genotype; non-TT, IL28B TG/GG-genotypes <sup>a</sup> $P = 0.038$  (compared to older patients with the IL28B TT-genotype). <sup>b</sup> $P = 0.005$  (compared to older patients with the IL28B TT-genotype). TVR: Telaprevir; SMV: Simeprevir; SVR24: Sustained virological response defined as undetectable serum HCV RNA at 24 wk after the end of treatment.

was significantly lower in the prior non-responders than the prior relapsers (60% vs 93.8%,  $P = 0.033$ ). Figure 3 shows the virological responses according to IL28B (rs8099917) SNP status. In the TVR group, the SVR rate for the older patients with the IL28B TT-genotype was significantly higher than for the older patients with the IL28B TG/GG-genotypes (92.9% and 60%,  $P = 0.038$ ). In the SMV group, the SVR rate for the older patients with the IL28B TT-genotype was also significantly higher than for the older patients with the IL28B TG/GG-genotypes (100% and 64.7%,  $P < 0.01$ ).

### Safety and tolerability

Treatment tolerability was summarized in Tables 3 and 4. In the TVR group, adverse events resulted in treatment discontinuation in 16.7% (13/78 cases) and 11.8% (4/34 cases) of patients in the younger and older groups, respectively. Although a greater number of older patients in the TVR group was treated with the lower initial dose of TVR (1500 mg/d) than the younger patients ( $P < 0.01$ )<sup>[20]</sup>, 9 patients (26.4%) discontinued TVR because of adverse events (four patients experienced skin rash, four patients experienced anemia, and one patient experienced renal dysfunction). However, the rate of dis-

continuation of TVR did not significantly differ between the younger and older patients (Table 3). The cumulative exposure to RBV for the whole 24-wk treatment period (as a percentage of the target dose) was significantly higher in the younger patients than in the older patients ( $79.3\% \pm 26.2\%$  vs  $62.7\% \pm 25.3\%$ ,  $P < 0.01$ ), but the cumulative exposure to TVR was not significantly different between the younger and older patients ( $88.8\% \pm 22.8\%$  vs  $83.5\% \pm 25.5\%$ ,  $P = 0.103$ ). Conversely, SMV was not discontinued in either the younger or older patients, although the rate of discontinuation of RBV was significantly higher in the older patients than the younger patients in the SMV group (58.9% vs 29.7%,  $P = 0.012$ ) because of anemia. Adverse events resulted in treatment discontinuation in 8.1% (3/37 cases) and 7.6% (3/39 cases) of patients in the younger and older groups, respectively.

### Predictive factors correlated with SVR24

To identify pretreatment and treatment factors that contribute to SVR, univariate and multivariate analyses were performed in the TVR and SMV groups including the following variables: Gender, age, body mass index, baseline HCV viral load, serum ALT, hemoglobin, platelet

**Table 3 Treatment tolerability (telaprevir)**

	Patients aged < 66	Patients aged ≥ 66	P value
Initial doses (median, range)			
PEG-IFN/BW (μg/kg per week)	1.48 (0.98-2.00)	1.49 (1.15-1.87)	0.859
TVR/BW (mg/kg per day)	33.0 (19.2-64.3)	29.2 (7.5-54.2)	0.044
TVR (2250 mg/1500 mg/others), <i>n</i>	55/23/0	11/21/2	< 0.001
RBV/BW (mg/kg per day)	11.4 (6.8-20.0)	11.4 (5.7-28.0)	0.103
Dose reduction, <i>n</i> (%)			
PEG-IFN	7 (8.9)	6 (17.6)	0.209
TVR	19 (24.3)	12 (35.3)	0.256
RBV	40 (51.2)	27 (79.4)	0.006
Discontinuation, <i>n</i> (%)			
PEG-IFN	13 (16.7)	4 (11.8)	0.580
TVR	12 (15.4)	9 (26.5)	0.192
RBV	12 (15.4)	7 (20.6)	0.585
Adherence, mean ± SD (%)			
PEG-IFN	88.2 ± 25.7	90.1 ± 19.8	0.606
TVR	88.8 ± 22.8	83.5 ± 25.5	0.103
RBV	79.3 ± 26.2	62.7 ± 25.3	< 0.001

PEG-IFN: Pegylated interferon; BW: Bodyweight; TVR: Telaprevir; RBV: Ribavirin.

**Table 4 Treatment tolerability (simeprevir)**

	Patients aged < 66	Patients aged ≥ 66	P value
Initial doses (median, range)			
PEG-IFNα2a (180/90) (μg/wk)	19/0	10/1	0.366
PEG-IFNα2b (120/100/80/others) (μg/wk)	2/16/5/1	0/25/5/1	0.422
SMV/BW (mg/kg per day)	1.6 (1.1-2.5)	1.8 (1.3-2.7)	0.011
RBV/BW (mg/kg per day)	11.6 (6.8-17.1)	12.3 (6.0-20.6)	0.166
Dose reduction, <i>n</i> (%)			
PEG-IFN	5 (13.5)	6 (15.3)	1
SMV	0	0	1
RBV	3 (8.1)	6 (15.3)	0.481
Discontinuation, <i>n</i> (%)			
PEG-IFN	5 (13.5)	5 (12.8)	1
SMV	2 (5.4)	2 (5.1)	1
RBV	11 (29.7)	23 (58.9)	0.012
Adherence, mean ± SD (%)			
PEG-IFN	93.6 ± 16.8	92.3 ± 19.5	0.592
SMV	98.1 ± 7.2	93.9 ± 18.1	0.079
RBV	91.0 ± 16.1	86.8 ± 20.2	0.126

PEG-IFN: Pegylated interferon; SMV: Simeprevir; BW: Bodyweight; RBV: Ribavirin.

counts, IL28B SNP, initial dose of TVR, TVR/BW (mg/kg per day), SMV/BW (mg/kg per day), dose reduction of treatments, and RVR (Tables 5 and 6). In the TVR group, the IL28B SNP significantly correlated with SVR according to the univariate analysis. A multivariate logistic regression analysis identified the IL28B TT-genotype (OR = 8.160; 95%CI: 1.593-41.804,  $P = 0.012$ ) and the adherence of RBV (> 60%) (OR = 11.052; 95%CI: 1.160-105.273,  $P = 0.037$ ) as independent factors associated with the SVR (Table 5). In the SMV group, the IL28B SNP and the absence of a dose reduction in PegIFN significantly correlated with SVR according to the univariate analysis. In the multivariate logistic regression analysis, the independent factors associated with the SVR were IL28B TT-genotype (OR = 9.677; 95%CI: 1.114-84.087,  $P = 0.040$ ) and the absence of a dose reduction in PegIFN (OR = 6.557; 95%CI: 1.328-32.377,

$P = 0.021$ ) (Table 6).

## DISCUSSION

In this study, we evaluated and compared the efficacy and safety of TVR- and SMV-based triple therapies in combination with PegIFN and RBV in elderly Japanese patients with chronic hepatitis C (CHC), specifically patients aged 66 years or older. The rate of SVR did not differ significantly between younger and older patients in either the TVR or the SMV groups. Among the older patients who were more difficult to treat, more patients carrying the IL28B TG/GG genotypes and prior non-responders were enrolled in the SMV group than the TVR group. However, the rate of SVR did not differ significantly between the TVR and SMV group, although the rates of RVR and relapse were significantly higher in

**Table 5 Univariate and multivariate analysis of factors contributing to SVR24 (telaprevir)**

Factors	Univariate analysis		Multivariate analysis	
	Odds ratio (95%CI)	P value	Odds ratio (95%CI)	P value
Age	1.012 (0.955-1.072)	0.689		
Gender (female)	0.784 (0.262-2.342)	0.663		
Body mass index (kg/m <sup>2</sup> )	1.074 (0.875-1.318)	0.495		
Prior treatment response (non-NR)	3.850 (0.830-17.861)	0.085		
Baseline HCV-RNA (log IU/mL)	1.264 (0.457-3.495)	0.652		
Baseline ALT (IU/mL)	1.008 (0.998-1.017)	0.105		
Baseline platelets ( $\times 10^4$ /mm <sup>3</sup> )	1.017 (0.906-1.142)	0.775		
Baseline hemoglobin (g/dL)	1.038 (0.736-1.464)	0.830		
IL28B SNP (TT)	6.700 (1.826-24.584)	0.004	8.160 (1.593-41.804)	0.012
Initial dose of TVR (2250 mg/d)	2.069 (0.670-6.553)	0.204		
TVR/BW (mg/kg per day)	0.938 (0.870-1.011)	0.093		
RBV/BW (mg/kg per day)	0.811 (0.617-1.066)	0.133		
PEG-IFN dose reduction (none)	2.134 (0.253-17.988)	0.486		
TVR dose reduction (none)	1.020 (0.281-3.703)	0.976		
RBV dose reduction (none)	1.548 (0.433-5.525)	0.501		
Adherence of RBV (> 60%)	6.873 (1.784-26.474)	0.005	11.052 (1.160-105.273)	0.037
RVR (none)	0.88 (0.123-1.216)	0.104		

HCV: Hepatitis C virus; ALT: Alanine aminotransferase; NR: Non-responder; IL28B SNP: Interleukin-28B single nucleotide polymorphism; TVR: Telaprevir; RVR: Rapid virological response; PEG-IFN: Pegylated interferon; BW: Bodyweight; RBV: Ribavirin.

**Table 6 Univariate and multivariate analysis of factors contributing to SVR24 (simeprevir)**

Factors	Univariate analysis		Multivariate analysis	
	Odds ratio (95%CI)	P value	Odds ratio (95%CI)	P value
Age	0.998 (0.942-1.058)	0.953		
Gender (female)	0.330 (0.083-1.314)	0.116		
Body mass index (kg/m <sup>2</sup> )	1.164 (0.934-1.450)	0.175		
Prior treatment response (non-NR)	2.955 (0.811-10.764)	0.101		
Baseline HCV-RNA (log IU/mL)	0.767 (0.328-1.791)	0.540		
Baseline ALT (IU/mL)	0.998 (0.985-1.012)	0.785		
Baseline platelets ( $\times 10^4$ /mm <sup>3</sup> )	1.082 (0.953-1.228)	0.224		
Baseline hemoglobin (g/dL)	1.257 (0.827-1.910)	0.285		
IL28B SNP (TT)	12.593 (1.516-104.576)	0.019	9.677 (1.114-84.087)	0.040
SMV/BW (mg/kg per day)	0.306 (0.054-1.742)	0.182		
RBV/BW (mg/kg per day)	1.085 (1.138-3.913)	0.501		
PEG-IFN dose reduction (none)	7.250 (1.712-30.700)	0.007	6.557 (1.328-32.377)	0.021
RBV dose reduction (none)	1.556 (0.470-5.160)	0.470		
RVR (none)	0.351 (0.075-1.637)	0.183		

HCV: Hepatitis C virus; ALT: Alanine aminotransferase; NR: Non-responder; IL28B SNP: Interleukin-28B single nucleotide polymorphism; SMV: Simeprevir; BW: Bodyweight; PEG-IFN: Pegylated interferon; RBV: Ribavirin; RVR: Rapid virological response.

the SMV group than the TVR group. When we performed univariate analyses of factors associated with SVR in all the enrolled patients, we did not find any significance in the type of treatment (TVR vs SMV) (OR = 1.115, 95%CI: 0.415-3.192,  $P = 0.787$ ). Ogawa *et al*<sup>[21]</sup> reported that the rates of SVR were similar for patients with HCV genotype 1b who were treated with TVR- and SMV-based triple therapies, although patients treated with TVR-based triple therapy had more frequent severe adverse events than those treated with SMV-based triple therapy. In this study, the rate of adverse events that resulted in treatment discontinuation did not differ between the younger and older patients in either the TVR or the SMV group, although a higher frequency and severity of adverse events have been reported in patients treated with TVR-based triple therapy compared to patients treated with PegIFN and RBV dual therapy<sup>[10,11]</sup>.

We found that both TVR- and SMV-based triple therapy were effective and tolerable among older patients aged 66 years or older.

In Japan, an estimated 1.5-2 million people are infected with HCV, and these patients are older than those infected in Europe and the United States<sup>[3,22]</sup>. However, previous studies describing the safety and efficacy of TVR- and SMV-based triple therapies, especially in elderly patients with CHC, are limited. One of the reasons may be that the inclusion criteria for clinical trials were usually set to a maximum age of 65 years<sup>[11,23]</sup>. Furusyo *et al*<sup>[4]</sup> reported that there were no differences in the efficacy, frequency and severity of adverse events between patients aged > 60 years and those aged  $\leq 60$  years who were treated with TVR-based triple therapy. Consistent with our study, they reported that a multivariate analysis revealed that the IL28B TT-genotype and the achievement of



RVR were independent factors associated with SVR. Although the decrease in hemoglobin was significantly higher in patients aged > 60 years compared to younger patients aged ≤ 60 years, the rate of adverse events that resulted in treatment discontinuation was similar between the two groups<sup>[4]</sup>. Abe *et al.*<sup>[23]</sup> also reported that in patients treated with TVR-based triple therapy, the SVR rate in patients aged > 65 years was similar to that of patients aged ≤ 65 years and that there was no notable increase of the rate of treatment discontinuation. In our study, the rate of adverse events that resulted in treatment discontinuation in the older patients was lower in the SMV group than in the TVR group, but the difference was not statistically significant. However, considering the risk of higher frequency and severity of adverse events associated with TVR-based triple therapy, we recommend the use of SMV rather than TVR.

The IL28B SNP genotype had a limited impact on the SVR rate with triple therapy in treatment-experienced patients<sup>[24]</sup>, and the strength of the association between the IL28B genotype and the treatment outcome was attenuated in the triple therapy compared to the dual therapy<sup>[23,25]</sup>. In the present study, the IL28B SNP genotype displayed a striking influence on the outcome of both TVR- and SMV-based triple therapy, especially in older patients. In the older patients carrying the IL28B TT-genotype, the rates of SVR were 92.9% and 100% in the TVR and SMV groups, respectively. In contrast, in the older patients carrying the IL28B TG or GG-genotype, the rates of SVR were significantly decreased to 60% and 64.7% in the TVR and SMV groups ( $P = 0.038$  and  $P < 0.01$ ), respectively. Although the substitutions in the core aa70 of the HCV genotype 1b were reported to be important predictors of the efficacy of dual therapy and triple therapy<sup>[26,27]</sup>, our study revealed that the substitutions in the HCV core aa70 were not associated with the achievement of SVR (data not shown). This discrepancy may be explained by the differences in the study population, as our study consisted of a relatively higher number of aged patients. We also measured serum CXCL10 in patients treated with TVR-based triple therapy because previous studies have reported that pretreatment serum CXCL10 concentrations were associated with early virological response and treatment efficacy in patients treated with this therapy<sup>[28,29]</sup>. However, we did not confirm the utility of pretreatment CXCL10 concentrations as a predictor of virological response in patients treated with TVR-based triple therapy.

The present study has a number of limitations. First, the sample size might have provided inadequate statistical power to detect definitive differences between the SVR and no-SVR data in both the older and younger patients. However, the best of our knowledge, this is the first study to compare the efficacy and safety of TVR- and SMV-based triple therapies for elderly patients aged 66 years or older. Second, we only investigated Japanese patients with the HCV genotype 1b. Among the Japanese population, the favorable IL28B SNP is

found in the majority of the population (approximately 75%)<sup>[4]</sup>. Therefore, our results may not be generalizable to other racial cohorts. Third, the older patients who enrolled in the study did not have any severe baseline complications, such as renal and hematological diseases. Therefore, the conclusions drawn regarding the safety of triple therapies may be limited. However, we believe that our selection of older patients for the triple therapies was appropriate and acceptable. Therefore, our findings regarding the absence of severe adverse events, even in the older patients, are important.

Treatment for CHC has been changing worldwide<sup>[30,31]</sup>, and IFN-free DAA combination therapies are now available in Japan. Although the majority of CHC patients are usually treated with IFN-free DAA combination therapies, PegIFN and RBV-based therapy may still have utility in a small number of patients who do not show a favorable effect after the treatment with IFN-free DAA therapies. Moreover, considering the effect of preventing HCC by an eradication of HCV, long-term prevention of HCC has been shown only through the use of IFN-based therapies thus far<sup>[32,33]</sup>. Therefore, we believe that the present study will provide useful information regarding antiviral treatment for older patients with CHC.

In conclusion, we found that both TVR- and SMV-based triple therapies can be successfully used to treat patients aged 66 years or older with genotype 1b CHC. The IL28B genotype indicates a potential to achieve SVR in these difficult-to-treat older patients.

## COMMENTS

### Background

In Japan, an estimated 1.5-2 million people are infected with hepatitis C virus (HCV), and these patients are older than those infected in Europe and the United States. However, previous studies describing the safety and efficacy of telaprevir (TVR)- and simeprevir (SMV)-based triple therapies, especially in elderly patients with chronic HCV infections, are limited.

### Research frontiers

The patients were categorized into two groups according to age as follows: a younger group of patients aged ≤ 65 years old and an older group of patients aged > 65 years old. The rate of sustained virological response (SVR) did not significantly differ between the younger and older patients in both the TVR and SMV groups. The rate of SVR did not significantly differ between the TVR and SMV group, although the rate of rapid virological response was significantly higher in the SMV group than the TVR group. The rate of adverse events resulted in treatment discontinuation did not differ between the younger and older patients in both TVR and SMV group, although a higher frequency and severity of adverse events has been reported in patients treated with TVR-based triple therapy compared to patients treated with pegylated interferon (PegIFN) and ribavirin (RBV) dual therapy.

### Innovations and breakthroughs

In this study, the authors found that both TVR- and SMV-based triple therapies can be successfully used to treat patients aged 66 years or older with genotype 1b chronic hepatitis C (CHC). The interleukin 28B genotype indicates a potential to achieve SVR in these difficult-to-treat elderly patients.

### Applications

Treatment for CHC has been changing worldwide, and interferon (IFN)-free direct-acting antiviral agents (DAA) combination therapies are now available

in. Although the majority of CHC patients are usually treated with IFN-free DAA combination therapies, PegIFN $\alpha$  and RBV-based therapy may still have utility in a small number of patients who do not show a favorable effect after the treatment with IFN-free DAA therapies. Importantly, HCV mutants that are resistant to multiple IFN-free DAA therapies have been shown to be sensitive to IFN-based therapies. Moreover, considering the effect of preventing HCC by an eradication of HCV, long-term prevention of HCC has been shown only through the use of IFN-based therapies thus far. Therefore, they believe that the present study will still provide useful information regarding antiviral treatment for older patients with CHC.

## Terminology

TVR: An HCV non-structural 3/4A (NS3/4A) protease inhibitor; SMV: A second-generation oral HCV NS3/4A protease inhibitor with antiviral activity against HCV genotype 1, 2, 4, 5, and 6 infections.

## Peer-review

The manuscript is well written and it is clear.

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Observational Study

# Malnutrition negatively impacts the quality of life of patients with cirrhosis: An observational study

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**Author contributions:** Higuera-de la Tijera F was the guarantor and designed the study; Rojas-Loureiro G, Servín-Caamaño A and Pérez-Reyes E participated in the acquisition, analysis and interpretation of data; Higuera-de la Tijera F wrote the manuscript; Servín-Abad L reviewed the final manuscript and revised the article critically for important intellectual content; all authors read and approved the final manuscript.

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## Abstract

### AIM

To verify how malnutrition is related to health-related quality of life (HRQL) impairment in patients with cirrhosis.

### METHODS

Data was retrospectively abstracted from medical records and obtained by direct interview. We included patients with cirrhosis from any etiology, evaluated at the Liver Clinic from Gastroenterology Department in a tertiary healthcare center, from June 2014 to June 2016. Child-Pugh score, data about complications, and demographic, clinical and anthropometric characteristics of patients were obtained. Nutritional status was evaluated by the Subjective Global Assessment (SGA). HRQL was evaluated through the Chronic Liver Disease Questionnaire. Patients were requested to assess their global HRQL with the following code: 0 = impairment



of HRQL, when it was compared with other healthy subjects; 1 = good HRQL, if it was similar to the quality of life of other healthy subjects. To compare the primary outcome between malnourished and well-nourished groups, the  $\chi^2$  test, Fisher's exact test or Student's *t*-test were used, based on the variable type. Associations between predictor variables and deterioration of HRQL were determined by calculating the hazard ratio and 95% confidence interval using Cox proportional hazards regression.

## RESULTS

A total of 127 patients with cirrhosis were included, and the mean age was  $54.1 \pm 12.3$  years-old. According to Child-Pugh scoring, 25 (19.7%) were classified as A (compensated), 76 (59.8%) as B, and 26 (20.5%) as C (B/C = decompensated). According to SGA, 58 (45.7%) patients were classified as well-nourished. Sixty-nine patients identified HRQL as good, and 76 patients (59.8%) perceived impairment of their HRQL. Multivariate analysis to determine associations between predictor variables and self-perception of an impairment of HRQL found strong association with malnutrition ( $P < 0.0001$ ). The most important impaired characteristics in malnourished patients were: Presence of body pain, dyspnea on exertion with daily activities, decreased appetite, generalized weakness, trouble lifting or carrying heavy objects, and decreased level of energy ( $P < 0.0001$ ).

## CONCLUSION

Malnutrition is a key factor related to impairment of HRQL in patients with cirrhosis.

**Key words:** Malnutrition; Subjective global assessment; Health-related quality of life; Cirrhosis; Chronic liver disease questionnaire

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**Core tip:** Several factors, particularly the severity of disease, development of ascites, need for paracentesis and history of hospitalization for any cause, are factors that worsen the health-related quality of life (HRQL) of patients with cirrhosis. Noteworthy malnutrition is a very important factor which impacts negatively on HRQL of patients suffering cirrhosis; clinicians must recognize it promptly and search for strategies to avoid this preventable comorbidity.

Rojas-Loureiro G, Servín-Caamaño A, Pérez-Reyes E, Servín-Abad L, Higuera-de la Tijera F. Malnutrition negatively impacts the quality of life of patients with cirrhosis: An observational study. *World J Hepatol* 2017; 9(5): 263-269 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i5/263.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i5.263>

## INTRODUCTION

Cirrhosis and its complications are important factors which contribute to mortality worldwide<sup>[1]</sup>. Compared with healthy people, the patients with compensated cirrhosis have five times more risk of non-survival, and those with decompensated cirrhosis have ten times more risk of non-survival during follow-up<sup>[2]</sup>.

Malnutrition is highly prevalent in cirrhotic patients. It is related to development of complications, or even death<sup>[3-5]</sup>.

Despite new treatment options for viral hepatitis, the high frequency of undiagnosed patients with chronic viral hepatitis and the increased incidence of metabolic syndrome with non-alcoholic steatohepatitis had led to the number of individuals progressing to cirrhosis being expected to increase until about 2030<sup>[6]</sup>. Moreover, despite increased knowledge of the pathogenesis of cirrhosis and major advances in the treatment, there remains a paucity of information related to health-related quality of life (HRQL) in these patients. Furthermore, the emotional impact of cirrhosis on individual's lives is rarely considered in clinical practice<sup>[7]</sup>.

HRQL is defined as the impact on three health domains regarding the patient's perception of their wellbeing: Physical, psychological, and social health. Measurement of HRQL requires administration of self-reported questionnaires<sup>[8,9]</sup>.

The Chronic Liver Disease Questionnaire (CLDQ) assesses HRQL in patients with chronic liver disease across diagnoses, at all stages of disease and treatment. The CLDQ is a 29-item self-reported questionnaire, with patient response options extending from 1 to 7 (all to none of the time). The CLDQ addresses the following domains that when combined give a composite score that indicates overall HRQL: Fatigue, activity, emotional function, abdominal pain, systemic symptoms, and anxiety. Mean domain scores and an overall quality of life score can be calculated, with higher scores representing better outcome<sup>[9,10]</sup>. Previous studies have confirmed how HRQL deteriorates from compensated to decompensated cirrhosis<sup>[11]</sup>.

Our aim in this study was to verify how malnutrition is related to HRQL impairment in patients with cirrhosis.

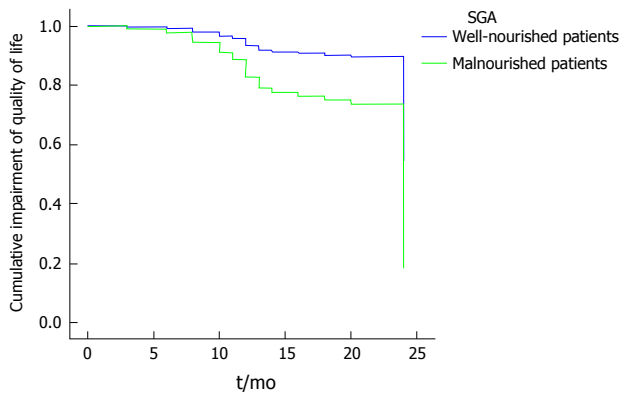
## MATERIALS AND METHODS

### Study design

We designed an observational analytic study. Data were retrospectively abstracted from medical records and obtained by direct interview. All study participants provided verbal informed consent prior to study enrollment.

### Patients

We included patients with cirrhosis from any etiology, who were evaluated at the Liver Clinic from Gastroenterology Department in a tertiary healthcare center,



**Figure 1** Kaplan-Meier curves showing the impairment of quality of life through the course of chronic liver disease, in patients with cirrhosis and malnutrition according to subjective global assessment. Malnourished patients had a worse quality of life during the follow-up in each visit to the physician, compared with those well-nourished patients.  $P < 0.0001$ . SGA: Subjective global assessment.

from June 2014 to June 2016. The Child-Pugh score was used to define compensated cirrhosis (Child-Pugh A) and decompensated cirrhosis (Child-Pugh B/C). We also collected data about complications of cirrhosis, including: Ascites, need of paracentesis, variceal bleeding, hepatic encephalopathy, and bacterial infection needing hospitalization. Patients with other chronic comorbidities, such as diabetes, chronic renal failure, heart or lung disease, neoplasms and acquired immunodeficiency syndrome, were excluded. We collected demographic, clinical and anthropometric characteristics of patients.

#### Anthropometric parameters

Weight, height, mid-arm circumference and triceps skinfold thickness were measured<sup>[12]</sup>. Body mass index (BMI) and ideal mid-arm muscle circumference were also calculated<sup>[13,14]</sup>.

#### Nutritional status

Nutritional status was evaluated by the Subjective Global Assessment (SGA)<sup>[4,5,15]</sup>. Patients were catalogued as well nourished, or moderately or severely malnourished. We chose the SGA for this study because of its being a simple bedside method recommended by the experts when other more accurate methods, such as phase angle or body cell mass measured by bioelectric impedance analysis, are not available to assess nutritional status.

#### HRQL

HRQL was evaluated through the CLDQ<sup>[10]</sup>. In addition, patients were requested to assess their global HRQL with the following coding system: 0 = impairment of HRQL, when it was compared with other healthy subjects; 1 = good HRQL, if it was similar to the quality of life of other healthy subjects.

#### Statistical analysis

Numeric variables were stated as mean and standard deviation (SD); categorical variables were stated as

proportions and percentages. To compare the primary outcome between malnourished and well-nourished groups, the  $\chi^2$  test, Fisher's exact test or Student's *t*-test were used, as appropriate. Associations between predictor variables and deterioration of quality of life were determined by calculating the hazard ratio (HR) and 95% confidence interval (CI) using Cox proportional hazards regression. The significant variables ( $P < 0.05$ ) in the univariate model were included in the multivariate model. Kaplan-Meier curves were constructed to compare quality of life between well-nourished and malnourished patients, and for this purpose, we identified the time when patients were diagnosed with cirrhosis and the estimated time when patients noticed impairment of their quality of life. Statistical significance was considered as a  $P$ -value  $< 0.05$ .

## RESULTS

A total of 127 patients with cirrhosis were included, 70 of which were female (55.1%) and 57 were male (44.9%); the mean age was  $54.1 \pm 12.3$  years-old. Regarding the etiology of the cirrhosis, 68 patients (53.3%) had alcoholic cirrhosis, 23 (18.1%) had chronic hepatitis C, 21 (16.5%) had cryptogenic etiology, 11 (8.7%) had autoimmune hepatitis, 3 (2.4%) had non-alcoholic steatohepatitis, and 1 (0.8%) had chronic hepatitis B. According to Child-Pugh scoring, 25 patients (19.7%) were classified as A (compensated), 76 (59.8%) as B, and 26 (20.5%) as C (B/C = decompensated). As determined by the SGA, 58 patients (45.7%) were well-nourished and 69 (54.3%) had some degree of malnutrition, including 66 (52%) with mild to moderate malnutrition and 3 (2.3%) with severe malnutrition. A total of 51 patients (40.2%) assessed their HRQL as good quality of life or similar to other healthy subjects; on the other hand, 76 patients (59.8%) perceived impairment of their HRQL in comparison with other healthy subjects. Characteristics of patients according to their self-perception of HRQL are shown and compared in Table 1. In the univariate analysis, decompensated cirrhosis, presence of ascites, need for paracentesis, hospitalization for any cause, and malnutrition were factors significantly associated with poor HRQL.

Multivariate analysis to determine associations between predictor variables and self-perception of an impairment of HRQL is shown in Table 2. The most important factor related to poor HRQL was malnutrition ( $P < 0.0001$ ). Also, patients with malnutrition had poorer HRQL through the time course of their chronic liver disease, when compared with the well-nourished patients ( $P < 0.0001$ ) (Figure 1).

Finally, the comparison of characteristics evaluated through CLDQ between malnourished and well-nourished patients is shown in Table 3. The most important impaired characteristics in malnourished patients were: Presence of body pain, dyspnea on exertion with daily activities, decreased appetite, generalized weakness, trouble lifting or carrying heavy objects, and decreased

**Table 1** Comparison between the patient characteristics according to the self-perception of quality of life

Characteristic	Good quality of life ( <i>n</i> = 51)	Impairment of quality of life ( <i>n</i> = 76)	<i>P</i>
Male	24 (47.1)	33 (43.4)	0.69
Age (yr)	54.8 ± 10.3	53.7 ± 13.5	0.61
Decompensated or Child B/C	30 (58.8)	63 (82.9)	0.003
Etiology			
Alcohol	28 (55.0)	40 (52.7)	0.83
Viral	9 (17.6)	15 (19.7)	
NASH	2 (3.9)	1 (1.3)	
Cryptogenic	8 (15.7)	13 (17.1)	
Autoimmune	4 (7.8)	7 (9.2)	
Weight in kg	65.2 ± 14.9	63.7 ± 13.4	0.55
Body mass index (kg/m <sup>2</sup> )	26.6 ± 5.2	26.8 ± 4.0	0.32
Triceps skinfold thickness (cm)	1.4 ± 0.7	1.4 ± 0.8	0.79
Mid-arm circumference (cm)	26.4 ± 4.7	23.9 ± 3.7	0.001
Ideal mid-arm muscle circumference (cm)	22.1 ± 4.1	19.6 ± 2.8	< 0.0001
Malnourished according to SGA	14 (27.5)	55 (72.4)	< 0.0001
Presence of ascites	19 (37.3)	48 (63.2)	0.004
Need for paracentesis	7 (13.7)	25 (32.9)	0.02
Development of variceal bleeding	12 (23.5)	18 (23.7)	0.98
Development of hepatic encephalopathy	19 (37.3)	30 (39.5)	0.80
Bacterial infection requiring hospitalization	6 (11.8)	14 (18.4)	0.45
Any complication requiring hospitalization	32 (62.7)	62 (81.6)	0.02

Categorical variables are expressed as *n* (%), and compared by  $\chi^2$  or Fisher's exact test. Numeric variables are expressed as median and SD, and compared by Student's *t*-test. Statistical significance was considered as a *P*-value of < 0.05. NASH: Non-alcoholic steatohepatitis; SGA: Subjective global assessment.

**Table 2** Multivariate analysis to identify factors associated with self-perception of impairment of quality of life

Characteristic	HR (95%CI)	<i>P</i>
Malnourished according to SGA	2.8 (1.6-5.0)	< 0.0001
Need for paracentesis	1.8 (1.0-3.2)	0.05
Presence of ascites	1.4 (0.7-2.7)	0.38
Any complication requiring hospitalization	1.1 (0.5-2.6)	0.82
Decompensated or Child B/C	1.8 (0.0-4.0)	0.14

Cox regression, statistical significance was considered as a *P*-value of < 0.05. HR: Hazard ratio; SGA: Subjective global assessment.

level of energy (*P* < 0.0001).

## DISCUSSION

Cirrhosis represents the final stage of all chronic liver diseases. In its decompensated form, cirrhosis can result in portal hypertension and hepatic dysfunction. Cirrhosis is a leading cause of morbidity and mortality worldwide, and not only is related to decreased survival but also to poor HRQL<sup>[16]</sup>.

Quality of life is a concept that reflects the positive and negative aspects of an individual's life. The term "HRQL" specifically addresses the impact of health on patients' wellbeing<sup>[9]</sup>. There are many factors that influence outcome and HRQL in patients with cirrhosis, however liver function clearly plays a major role affecting the HRQL of patients with cirrhosis. Patients with decompensated cirrhosis have an important impairment on HRQL<sup>[17]</sup>. Also, many symptoms can negatively impact HRQL in patients with cirrhosis; these symptoms can include abdominal bloating, nausea, somnolence, weight

loss, weakness, fatigue and itching. All of these may interfere with patient's work, schooling, social activities, and sense of wellbeing<sup>[18]</sup>.

In our study, we found that decompensated cirrhosis (Child B/C) is a factor related to impairment of HRQL; this finding is similar to other studies. Marchesini *et al.*<sup>[19]</sup> also reported that the severity of liver disease or the development of complications were conditions clearly related to deterioration of perception of health. Similarly, we found that the presence of ascites and need for paracentesis were associated factors related to poor quality of life. Furthermore, hospitalization for any cause was a condition related to poor HRQL in patients with cirrhosis.

In our study, interestingly we found that patients with cirrhosis and malnutrition had a poorer HRQL when compared with well-nourished patients with cirrhosis. Furthermore, malnutrition was the main factor contributing to impairment of HRQL in these patients. Cirrhosis is also associated with malnutrition, which is a complication that negatively affects cirrhotic patients, particularly those decompensated<sup>[20-23]</sup>. In patients with cirrhosis, the prevalence of malnutrition has been reported between 20% to 60%<sup>[24-27]</sup>. In a previous study conducted by Pérez-Reyes *et al.*<sup>[4]</sup> in a Hispanic population, the prevalence of malnutrition was as high as 56.3%. In the present study, we also found a high frequency of malnutrition in patients with cirrhosis (54.3%). Malnutrition in cirrhosis is related to development of ascites, encephalopathy, spontaneous bacterial peritonitis, other bacterial infections and hepatorenal syndrome<sup>[4,28-32]</sup>. But also, malnutrition deteriorates the HRQL in patients with cirrhosis<sup>[33-35]</sup> and several other gastrointestinal and non-gastrointestinal diseases<sup>[36,37]</sup>. Our study confirms that malnutrition is

**Table 3** Chronic Liver Diseases Questionnaire items comparison according to nutritional status

CLDQ item	Well-nourished (n = 58)	Malnourished (n = 69)	P
1 How much of the time during the last 2 wk have you been troubled by a feeling of abdominal bloating?	5.72 ± 1.531	4.67 ± 2.056	0.001
2 How much of the time have you been tired or fatigued during the last 2 wk?	3.69 ± 1.366	2.94 ± 1.259	0.002
3 How much of the time during the last 2 wk have you experienced body pain?	4.14 ± 0.868	3.57 ± 0.848	0.0001
4 How often during the last 2 wk have you felt sleepy during the day?	5.05 ± 1.343	4.55 ± 1.105	0.02
5 How much of the time during the last 2 wk have you experienced abdominal pain?	5.45 ± 1.273	4.96 ± 1.529	0.05
6 How much of the time during the last 2 wk have you experienced dyspnea on exertion, being a problem for you in your daily activities?	6.16 ± 0.951	5.33 ± 1.431	0.0001
7 How much of the time during the last 2 wk have you not been able to eat as much as you would like?	6.12 ± 1.010	3.55 ± 1.549	0.0001
8 How much of the time in the last 2 wk have you been bothered by having decreased strength?	4.91 ± 1.218	2.90 ± 1.447	0.0001
9 How often during the last 2 wk have you had trouble lifting or carrying heavy objects?	5.62 ± 0.834	4.09 ± 1.391	0.0001
10 How often during the last 2 wk have you felt anxious?	5.52 ± 1.112	5.33 ± 1.379	0.41
11 How often during the last 2 wk have you felt a decreased level of energy?	5.19 ± 1.100	3.20 ± 1.491	0.0001
12 How much of the time during the last 2 wk have you felt unhappy?	5.12 ± 1.077	4.41 ± 1.527	0.003
13 How often during the last 2 wk have you felt drowsy?	4.97 ± 1.324	4.55 ± 1.051	0.05
14 How much of the time during the last 2 wk have you been bothered by a limitation of your diet?	4.14 ± 1.206	3.91 ± 1.160	0.29
15 How often during the last 2 wk have you been irritable?	5.52 ± 1.128	5.36 ± 1.175	0.45
16 How much of the time during the last 2 wk have you had difficulty sleeping at night?	5.02 ± 1.493	4.87 ± 1.444	0.57
17 How much of the time during the last 2 wk have you been troubled by a feeling of abdominal discomfort?	5.62 ± 1.437	4.77 ± 1.816	0.004
18 How much of the time during the last 2 wk have you been worried about the impact your liver disease has on your family?	5.84 ± 1.056	5.94 ± 1.371	0.66
19 How much of the time during the last 2 wk have you had mood swings?	5.50 ± 1.417	5.83 ± 1.283	0.18
20 How much of the time during the last 2 wk have you been unable to fall asleep at night?	5.10 ± 1.360	4.67 ± 1.569	0.99
21 How often during the last 2 wk have you had muscle cramps?	5.52 ± 1.047	5.39 ± 1.074	0.51
22 How much of the time during the last 2 wk have you been worried that your symptoms will develop into major problems?	4.19 ± 1.515	4.45 ± 1.586	0.35
23 How much of the time during the last 2 wk have you had a dry mouth?	5.40 ± 1.184	5.30 ± 1.192	0.66
24 How much of the time during the last 2 wk have you felt depressed?	5.33 ± 1.082	4.68 ± 1.745	0.01
25 How much of the time during the last 2 wk have you been worried about your condition getting worse?	4.05 ± 1.191	4.28 ± 1.454	0.34
26 How much of the time during the last 2 wk have you had problems concentrating?	5.34 ± 1.132	4.74 ± 1.569	0.01
27 How much of the time have you been troubled by itching during the last 2 wk?	5.71 ± 1.451	6.20 ± 1.065	0.03
28 How much of the time during the last 2 wk have you been worried about never feeling any better?	4.07 ± 1.153	4.36 ± 1.382	0.20
29 How much of the time during the last 2 wk have you been concerned about the availability of a liver if you need a liver transplant?	4.22 ± 1.312	4.23 ± 1.467	0.97

Data are expressed as median and SD, and compared with Student's *t*-test. Statistical significance was considered as a *P*-value of < 0.05. CLDQ: Chronic Liver Diseases Questionnaire.

a key factor related to impairment of HRQL in patients with cirrhosis, even when we adjusted for advanced liver disease or decompensation status, and for other major complications such as ascites, need for paracentesis and need for hospitalization for any cause.

In conclusion, cirrhosis is the end-stage of all chronic liver diseases; it contributes importantly to morbidity and mortality worldwide but also has a negative impact on HRQL that must be considered. Several factors contribute to a poor HRQL in patients with cirrhosis, however malnutrition, which is a highly prevalent comorbidity in patients with cirrhosis, represents a key factor related to poor HRQL in these patients. There is a need for developing strategies to evaluate more accurately patients with cirrhosis and to identify promptly those patients at risk of malnutrition.

## COMMENTS

### Background

Cirrhosis is a significant contributor to global mortality. Prevalence of malnutrition is high in patients with cirrhosis and is related to increased complications or even death. Despite increased knowledge of the pathogenesis of cirrhosis, there

remains a paucity of information related to health-related quality of life (HRQL) in these patients.

### Research frontiers

The emotional impact of cirrhosis on individual's lives is rarely considered in clinical practice. The Chronic Liver Disease Questionnaire assesses HRQL in patients with chronic liver disease across diagnoses, at all stages of disease and treatment.

### Innovations and breakthroughs

Cirrhosis is a leading cause of morbidity and mortality worldwide, and not only is related to decreased survival but also to poor quality of life. The term "HRQL" addresses the impact of health on a patient's wellbeing. Many factors influence HRQL in patients with cirrhosis, however the impact of comorbidities, such as malnutrition, are not well understood. The authors found that patients with cirrhosis and malnutrition had worse quality of life when compared with well-nourished patients with cirrhosis. In this study, malnutrition was the main factor contributing to impairment of quality of life in these patients.

### Applications

In this study, the authors found that several factors contribute to a poor health-related quality of life in patients with cirrhosis, however malnutrition, which is a highly prevalent comorbidity in these patients, represents a key factor related to poor quality of life in these patients. There is a need for developing strategies to evaluate more accurately patients with cirrhosis and to identify promptly those patients at risk of malnutrition.



# Terminology

Nutritional status was defined through the Subjective Global Assessment and patients were divided as follows: Well-nourished, or moderately or severely malnourished. The HRQL is defined as the impact on three health domains-physical, psychological, and social health-on patient perception of their wellbeing.

# Peer-review

Very nice and well written paper.

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Observational Study

# Addition of simvastatin to carvedilol non responders: A new pharmacological therapy for treatment of portal hypertension

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**Author contributions:** The work was carried out in collaboration of all authors; Wani ZA designed the study, performed the research and analyzed the data; Wani ZA and Mohapatra S wrote the first draft of the article; Khan AA and Yatoo GN made critical revisions related to important intellectual content of the manuscript; Mohapatra S and Mohapatra A edited and revised the manuscript for final submission.

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**Informed consent statement:** All included patients gave their informed consent (written or verbal) prior to study inclusion.

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## Abstract

### AIM

To determine whether addition of simvastatin could be an important pharmacological rescue therapy for carvedilol non-responders.

### METHODS

One hundred and two consecutive patients of cirrhosis of liver with significant portal hypertension were included. Hepatic venous pressure gradient (HVPG) was measured at the base line and after proper optimization of dose; chronic response was assessed at 3 mo. Carvedilol non-responders were given simvastatin 20 mg per day (increased to 40 mg per day at day 15). Carvedilol plus simvastatin was continued for 1 mo and hemodynamic response was again measured at 1 mo.

## RESULTS

A total of 102 patients with mean age of  $58.3 \pm 6.6$  years were included. Mean baseline HVPg was  $16.75 \pm 2.12$  mmHg and after optimization of dose and reassessment of HVPg at 3 mo, mean reduction of HVPg from baseline was  $5.5 \pm 1.7$  mmHg and  $2.8 \pm 1.6$  mmHg among responders and non-responders respectively ( $P < 0.001$ ). Addition of simvastatin to carvedilol non-responders resulted in significant response in 16 patients (42.1%) and thus overall response with carvedilol and carvedilol plus simvastatin was seen in 78 patients (80%). Two patients were removed in chronic protocol study with carvedilol and three patients were removed in carvedilol plus simvastatin study due to side effects.

## CONCLUSION

Addition of simvastatin to carvedilol non-responders may prove to be an excellent rescue therapy in patients with portal hypertension.

**Key words:** Simvastatin; Cirrhosis; Carvedilol; Liver cirrhosis; Portal hypertension; Hepatocellular carcinoma

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**Core tip:** There is no pharmacological option available for treatment of carvedilol nonresponders in patients with portal hypertension. Addition of simvastatin could be an important pharmacological rescue therapy for carvedilol nonresponders. This study showed that addition of simvastatin to carvedilol non responders can increase overall response to around 80%, which is one of the best possible pharmacologically produced chronic response and it opens a new strategy for portal hypertension treatment.

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## INTRODUCTION

The prevalence of esophageal varices in an asymptomatic compensated patient is around 40%<sup>[1]</sup>. While the incidence of variceal development is roughly 6% per year, it doubles if hepatic venous pressure gradient (HVPg) rises above 10 mmHg. Thus, cirrhotics with HVPg of > 10 mmHg represent higher risk group. HVPg > 10 mmHg also correlates with higher risk of decompensation and hepatocellular carcinoma (HCC)<sup>[2,3]</sup>. The result of a number of meta-analysis has shown that, prognosis of cirrhotic patients improve with significant decrease in portal pressure, *i.e.*, when target decrease in HVPg (> 20% from baseline or to < 12 mmHg) is achieved<sup>[4,5]</sup>.

In practice, cirrhotic patients complicated with varices should be treated except for Child-Pugh (C-P) class A patients with small varices without red color signs<sup>[6]</sup>.

The role of non-selective beta blockers (NSBBs) and endoscopic variceal ligation (EVL) in the prevention of first variceal bleeding is conflicting. Analysis of a recent meta-analysis did not show any differences on mortality or bleeding rates between the two groups in trials with adequate bias control<sup>[7]</sup>. In contrast, another meta-analysis showed that compared with BBs, EVL reduced the risk of a first variceal bleed, although, there was no significant difference in survival<sup>[8]</sup>. Hence, the author concluded that EVL should be offered to patients with moderate to large oesophageal varices who are unlikely to comply or intolerant or who bleed while taking BB.

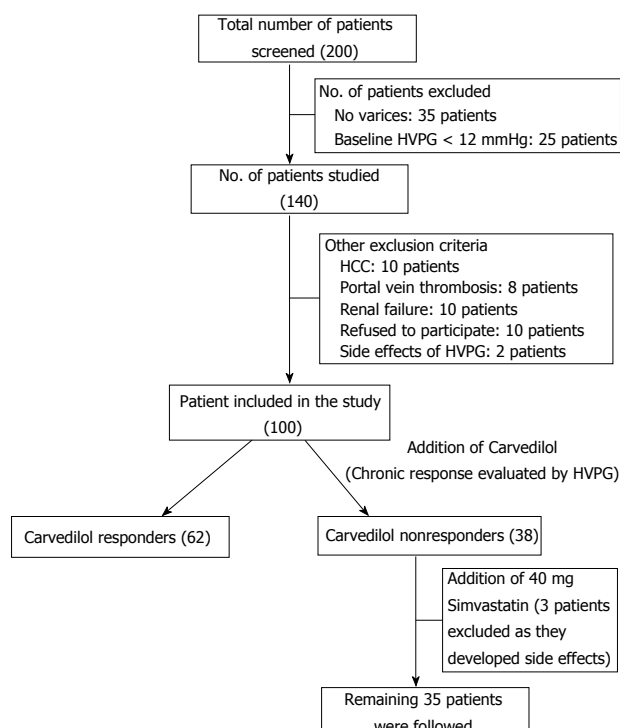
Still, the mainstream in pharmacological treatment of portal HTN (PHT) is NSBB like propranolol and nadolol which help in preventing first and recurrent variceal bleeding, gastropathy and spontaneous bacterial peritonitis (SBP)<sup>[9]</sup>. Drugs like isosorbide-5 mononitrate, prazosin or statins when added to NSBBs help in reducing the hepatic vascular tone and thus may turn many non-responders to responders<sup>[10,11]</sup>. Also, HVPg can be further decreased with these drugs. Recently our group has published a combined study on carvedilol in which 50% of the patients showed acute response and more than 60% of patients showed chronic response (please refer to definitions for details)<sup>[12]</sup>. We also showed in a separate study that, optimization of dose of carvedilol on chronic basis is an excellent policy for portal hypertension across different C-P class of liver disease<sup>[13]</sup>.

Simvastatin improves liver generation of nitric oxide (NO) and hepatic endothelial dysfunction in patients with cirrhosis. Hence, it could be an effective therapy for portal hypertension. Recently, ideal drug for portal hypertension was pictured as one that should reduce portal pressure by decreasing intrahepatic vascular resistance while maintaining or enhancing hepatic blood flow<sup>[14,15]</sup>. Other desirable action would be an antifibrotic effect and a capacity to improve liver function. The drug that would be able to increase NO bioavailability in liver would fulfill many of the requirements<sup>[15-18]</sup>. However in patients with advanced cirrhosis, non-selective NO donors such as organic nitrates which enhance peripheral vasodilatation further decrease arterial pressure and activate endogenous vasoactive system. Thus, selectivity for hepatic circulation is a further requirement for vasodilators used to treat portal hypertension<sup>[19]</sup>.

Recent experimental and human data<sup>[20,21]</sup> suggests that statins (3-hydroxy-3-methyl-COA reductase inhibitor) could decrease intra hepatic vascular resistance and improve flow mediated vasodilatation of liver vasculature in the cirrhotic liver. These effects are mediated by an up regulation of NO at the liver vasculature through an enhancement in endothelial NO synthetase activity<sup>[20]</sup>. Moreover, NO production in liver by statins is selective and could behave as true liver selective vasodilator.

Thus, the concept of our study was to assess the response of 3<sup>rd</sup> generation beta blocker carvedilol on





**Figure 1 Study design.** HVPG: Hepatic venous pressure gradient; HCC: Hepatocellular carcinoma.

chronic basis (after proper optimization of dose) and then to add simvastatin along with carvedilol, optimise dose in carvedilol non responders to have a new pharmacological approach and better rescue therapy.

## MATERIALS AND METHODS

### Patients and methods

We prospectively evaluated one hundred and two cirrhotic patients who were referred to our institution for hemodynamic evaluation from January, 2010 to December, 2014. The study was approved by the institutional review board (IRB) and all included patients gave informed consent for participation.

Diagnostic criteria for cirrhosis was based on clinical, biochemical, radiological and if needed on liver biopsy. The criteria for esophageal varices was based on quantitative grading used by Bavino consensus, *i.e.*, esophageal varices less than 5 mm are small varices and esophageal varices equal to or greater than 5 mm are considered large varices. Criteria used to diagnose ascites was according to international ascites club 2003, *i.e.*, grade I - mild (ultrasound based), Grade II - moderate, *i.e.*, (symmetrical abdominal distension) and Grade III - gross with marked abdominal distension.

The inclusion criteria of the study include evidence of esophageal varices on upper gastrointestinal (GI) endoscopy, without a previous history of hemorrhage and a baseline HVPG of greater than 12 mmHg. Exclusion criteria were age < 18 years; severe liver failure INR > 2.5, or PT < 40% of control, bilirubin > 5 mg/dL; active alcohol consumption; IV drug abuse; renal failure, *i.e.*,

creatinine > 1.5 mg/dL; HCC; contraindication to NSBB; pre or post hepatic cause of PHT; pregnancy; previous surgical shunt or TIPPS; treatment with calcium channel blockers; treatment with (3-hydroxy-3-methyl-COA reductase inhibitor) in past three months; a known hypersensitivity to simvastatin and refusal to participate in study.

### Dosing Of NSBB

Baseline HVPG was measured for all included patients after 8 h of fasting. They were started on carvedilol 6.25 mg/d from the next day and dose was titrated by steps of 6.25 mg/wk. Dose of carvedilol was increased weekly until arterial systolic blood pressure (BP) was not less than < 90 mmHg and heart rate (HR) not less than < 55 bpm. Compliance with therapy was monitored by recording HR and BP during clinical visit.

### Dosing of simvastatin

Carvedilol non-responders were added simvastatin 20 mg/d for 15 d (then increased to 40 mg). Complete clinical examination and blood tests were performed at day 15, patients were interrogated specifically for muscle weakness, if no safe end point was met, dose was increased to 40 mg/d and continuing with continuation of carvedilol. Treatment was maintained for 1 mo and then repeat hemodynamic response was measured.

### Definitions

**Acute response to carvedilol:** Acute response to carvedilol is defined as "a drop in HVPG greater than 20% and or less than 12 mmHg from baseline at 90 min after administration of a single dose (12.5 mg) of carvedilol".

**Chronic response to carvedilol:** Chronic response to carvedilol is defined as "a drop in HVPG greater than 20% and or less than 12 mmHg from baseline at 3 mo after proper optimization of dose of carvedilol".

### Response with addition of simvastatin

After 30 d of 40 mg simvastatin addition to carvedilol in carvedilol non responders, HVPG drop of greater than 20% from baseline and or less than 12 mmHg HVPG. The study design is illustrated in Figure 1. Dose optimization was done in all patients who were started with carvedilol. Once doses were optimized, weekly follow-up of each patient was done and HVPG was again measured at 3 mo of treatment. Patients were assessed for side effects; their BP and HR were measured on each follow-up visit. Carvedilol non responders were added with simvastatin 20 mg/d and after 15 d, all blood tests were taken for side effects of simvastatin and clinical history specifically muscle weakness was taken. With no clinical and biochemical evidence of adverse effects, patients were given 40 mg of simvastatin per day and continuing carvedilol for 1 mo, repeat hemodynamic assessment was done to see response in carvedilol non responders and thus overall response in the study group

**Table 1** Baseline characteristics of 102 patients

Parameters	Description
Age (mean $\pm$ SD)	58.35 $\pm$ 6.62
Gender (male:female)	63:39
Child-Pugh class (A:B:C)	43:32:27
Etiology (Alcohol:Viral:NASH or Cryptogenic:AIH)	31:37:29:5
Oesophageal Varices (small:large)	34:68
Ascites (No:Grade I :Grade II :Grade III)	63:6:25:8
Total bilirubin (mg/dL)	1.96 $\pm$ 0.81
Serum albumin (mg/dL)	3.20 $\pm$ 0.49
Prothrombin time	14.13 $\pm$ 1.91
International normalized ratio	1.29 $\pm$ 0.16

NASH: Non-alcoholic steatohepatitis; AIH: Autoimmune hepatitis.

was seen.

### Haemodynamic measurements

Under fluoroscopic guidance, hepatic vein catheterization was performed according to the standards described by Bosch *et al.*<sup>[22]</sup>. A 7F balloon tipped catheter was advanced to main right hepatic vein to measure wedged hepatic venous pressure gradient (WHPG). HVPG was measured as the difference between WHPG and free hepatic pressure gradient (FHPG). Swangaz catheter was advanced to pulmonary artery for measurement of cardio pulmonary pressures like pulmonary artery pressure (PAP), wedged pulmonary pressures (WPP), right arterial pressure (RAP), etc. All measurements were repeated three times and tracing were noted. Mean arterial pressure was measured non-invasively by automatic sphygmomanometer. HR was derived by continuous ECG monitoring and systemic vascular resistance (SVR) as (MAP - RAP/CO  $\times$  80).

### Statistical analysis

The statistical methods of this study were reviewed by Dr. Khan from Noora Multispeciality Hospital, Srinagar, India. Statistical analysis was performed by using statistical package for social sciences (SPSS) version 22.0. Descriptive statistics was presented as proportion, Mean  $\pm$  standard deviation (SD) and median with inter-quartile range. Comparative analysis was done by utilizing student's *t*-test and  $\chi^2$  test. The univariate and multivariate logistic regression was used for finding the predictors. A *P*-value less 0.05 was considered significant.

## RESULTS

A total of 68 patients (66.7%) had large varices and 34 patients (33.3%) had small varices on upper GI endoscopy and 63 (61.8%) patients had no ascites while others had ascites. The baseline parameters are shown in Table 1.

After optimization of dose and reassessment of HVPG after 3 mo, total number of chronic responders was 62. However two patients discontinued treatment because of side effects. Mean duration of dose optimization was

15  $\pm$  3 d. Mean reduction of HVPG from baseline and after 3 mo was 5.5  $\pm$  1.7 mmHg and 2.8  $\pm$  1.6 mmHg among responders and non-responders on chronic basis, respectively (*P* < 0.001). Mean dose of carvedilol was higher among non-responders (19.2  $\pm$  5.7 mg) as compared to responders (18.7  $\pm$  5.1 mg).

### Effect of simvastatin addition to carvedilol non responders with continuation of carvedilol on reduction of portal hypertension and hemodynamic parameters

After assessing the chronic response at 3 mo with carvedilol, there were 38 patients who did not respond significantly to carvedilol and were thus called as carvedilol non-responder. In these 38 patients, simvastatin 20 mg/d was added initially for 15 d and at 15 d, side effects like muscle weakness along with biochemical parameters like CPK and ALT was seen. If CPK > 5 times and ALT > 3 times was found in any patient, they were withdrawn from the study. One patient developed CPK > 5 times with normal ALT was withdrawn from study on 15<sup>th</sup> day. Second patient developed hepatic encephalopathy and 3<sup>rd</sup> patient developed severe dizziness and both of these were withdrawn from study. Four patients developed minor side effects with normal CPK and ALT and were continued with treatment.

Among 38 carvedilol non responders, therefore, 35 patients continued carvedilol and simvastatin for 1 mo and then a repeat hemodynamic assessment was done. There were 16 responders and 19 non-responders at one month after adding simvastatin. Thus, overall carvedilol response in the study was 79.56% (78 patients). The pre baseline mean HVPG of carvedilol non responders was 16.429 mmHg which dropped to 13.029 mmHg, i.e., 3.4 mmHg drop (> 20%) after adding simvastatin. The post carvedilol HVPG (post chronic) in carvedilol non responders was 14.457 mmHg which dropped to 13.029 mmHg, i.e., 1.428 mmHg drop (9.87%) by adding simvastatin. It means that, simvastatin is responsible for HVPG drop of 9.87% in isolation.

Baseline and hemodynamic parameters of patients in whom simvastatin was added are shown in the Tables 2 and 3.

Gender, etiology, C-P class, ascites and variceal size were not seen to be statistically significant between responders and non-responders in simvastatin protocol. Among baseline hemodynamic parameters, only pre WHPG was significantly higher in responders as compared to non-responders (*P* = 0.01). HVPG was higher, though not statistically significant predictor of response. All hemodynamic parameters significantly decreased from baseline after treatment with simvastatin except FHVP which was significantly raised. All hemodynamic parameter were significantly decreased after treatment with simvastatin except FHVP which was significantly raised with respect to their values after chronic treatment with carvedilol (chronic protocol). Pre (baseline), post chronic (chronic carvedilol at 3 mo) and post simvastatin haemodynamic parameters in carvedilol non responders

**Table 2** Baseline characteristics of 38 carvedilol non responders patients in whom Simvastatin was added

Parameters	Description
Age (mean $\pm$ SD)	58.45 $\pm$ 5.95
Gender (male:female)	21:17
Child-Pugh class (A:B:C)	14:13:11
Etiology (Alcohol:Viral:NASH or Cryptogenic)	12:15:11
Oesophageal Varices (small:large)	12:26
Ascites (No:Grade 1:Grade 2:Grade 3)	21:4:8:5
Total bilirubin (mg/dL)	2.042 $\pm$ 0.77
Serum albumin (mg/dL)	3.203 $\pm$ 0.54
Prothrombin time	14.105 $\pm$ 2.16
International normalized ratio	1.318 $\pm$ 0.15

NASH: Non-alcoholic steatohepatitis.

are shown as general linear model in Figure 2.

## DISCUSSION

The mechanism of portal hypertension primarily involves an increase in resistance to portal outflow circulation. It leads to the formation of portosystemic collateral veins, of which esophageal varices have the highest clinical impact and the most severe complications. Other manifestations of portal hypertension include portal hypertensive gastropathy and large spontaneous shunts which refer to presence of patent paraumbilical vein, spleno-renal shunt, ano-rectosigmoid varices<sup>[23]</sup>. Recently, it has been showed that identifying cirrhotic patients with high blood ammonia concentrations could be clinically useful, as high levels would lead to suspicion of being in presence of collaterals<sup>[24]</sup>. The first line pharmacological therapy in portal hypertension is NSBB therapy. It decreases portal pressure through a reduction in portal venous inflow as a result of a decrease in cardiac output ( $\beta_1$ -adrenergic blockade) and splanchnic blood flow ( $\beta_2$ -adrenergic blockade). However, a major drawback of NSBBs is that not all patients respond to beta-blockers with a reduction of the HVPg.

Clinicians and researchers have always been looking for a more powerful portal hypotensive agent than propranolol and nadolol either administered alone or combination with nitrovasodilators. Advantages of medical therapy include safety and correction of detrimental systemic effects of portal hypertension. Our study tries to use best portal hypotensive agent, *i.e.*, 3<sup>rd</sup> generation beta blocker (non-selective) with mild vasodilating properties, *i.e.*, carvedilol which has been proven to show excellent hemodynamic response on chronic basis to the tune of 50%-72% of patients<sup>[25]</sup>.

There are six studies which investigated chronic effects of carvedilol<sup>[26-28]</sup> with longest period of follow-up of 11 wk in one study. In another study by Stanley *et al.*<sup>[27]</sup>, seven of patients inclusively studied in acute protocol were unable to complete chronic administration of carvedilol as a result of side effects. This study suggests that, atleast for study group the administration of 25 mg without attempts to titrate according to response may

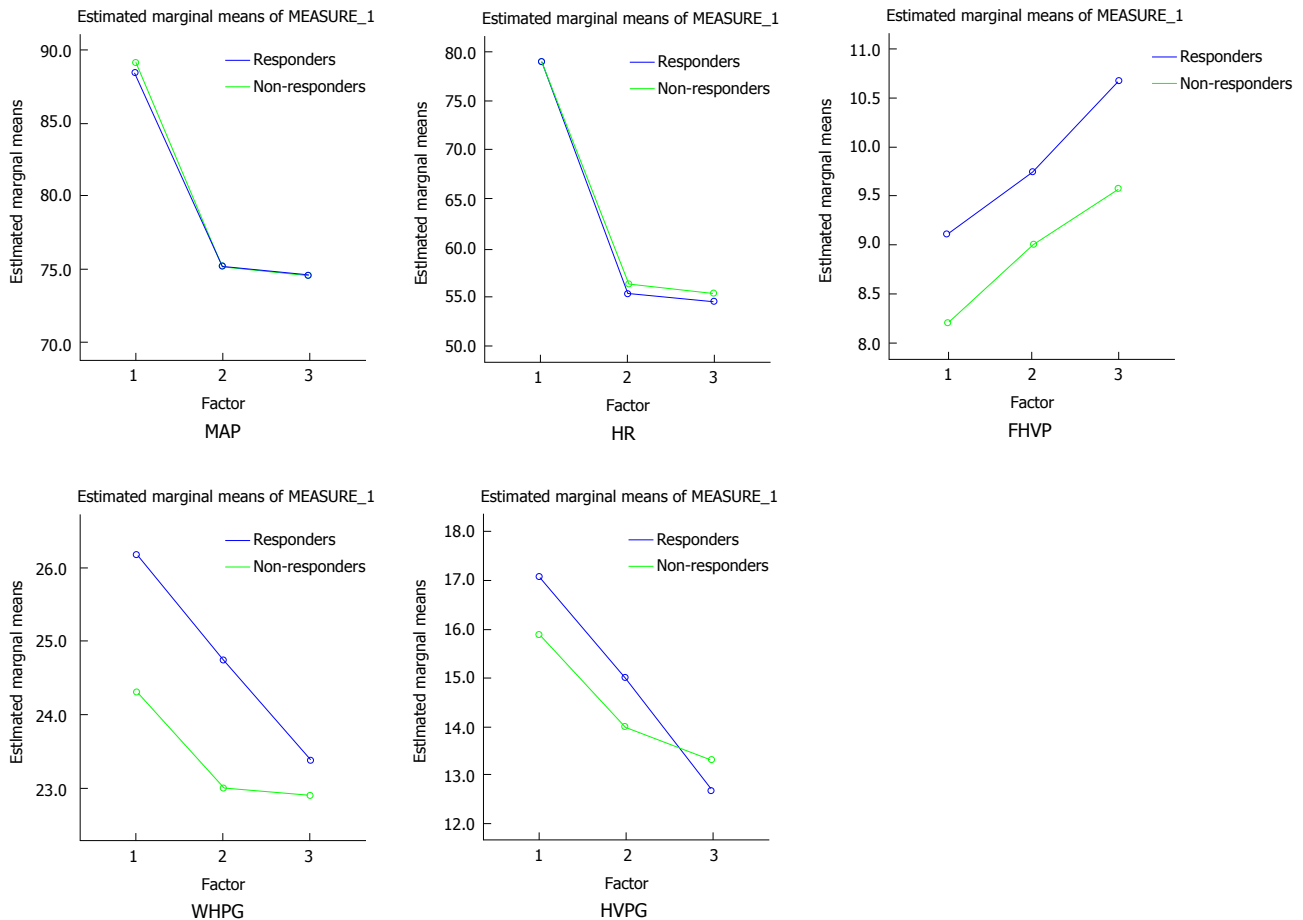
**Table 3** Hemodynamic parameters (mean) of studied population

Hemodynamic parameters	Baseline	Post chronic carvedilol (3 mo)	Post simvastatin
CO (L/min)	7.525 $\pm$ 0.19	6.38 $\pm$ 0.13	6.195 $\pm$ 0.17
HR (beats/min)	79.45 $\pm$ 2.50	57.45 $\pm$ 2.44	55.053 $\pm$ 1.67
MAP (mmHg)	89.53 $\pm$ 2.42	75.54 $\pm$ 1.97	74.500 $\pm$ 1.48
FHVP (mmHg)	8.28 $\pm$ 1.85	9.45 $\pm$ 1.90	10.086 $\pm$ 1.68
WHPG (mmHg)	25.08 $\pm$ 2.55	22.04 $\pm$ 2.56	23.114 $\pm$ 2.32
HVPG (mmHg)	16.75 $\pm$ 2.12	12.60 $\pm$ 2.24	13.029 $\pm$ 1.56

CO: Cardiac output; HR: Heart rate; MAP: Mean arterial pressure; FHVP: Free hepatic venous pressure; WHPG: Wedged hepatic venous pressure gradient; HVPG: Hepatic venous pressure gradient.

not be ideal. Keeping in view the results of the above study, we used a titration or dose optimization based strategy for assessing chronic carvedilol response. It also studies difference of response between early liver disease and advanced liver disease, *i.e.*, between C-P class A and B/C on chronic basis. Further this study looks into maximum dose tolerated by different C-P class of liver disease on chronic basis apart from looking into predictor of chronic response. Idea of our study was to further move to add an agent to carvedilol non responders which has no effects on MAP or peripheral vascular resistance and which behaves like a true liver selective vasodilator, *i.e.*, simvastatin. Thus, it is the first study which has used a new pharmacological agent simvastatin in carvedilol non responders. Additive effects of simvastatin may markedly increase the number of patients who are protected effectively from portal hypertensive related complication. Such an effect is in agreement with liver perfusion studies in experimental model of cirrhosis which showed statins exert their hepatic vasodilating effect by upregulating endothelial NO production<sup>[29,30]</sup>. Our study shows that, chronic carvedilol non-responders were 62 (60%) which increased to overall response of nearly 80% once simvastatin was added to it. Thus around 42% of carvedilol non responders became responders by adding simvastatin.

In titration protocol on chronic basis, mean dose of carvedilol was 18.7  $\pm$  5.1 mg and 19.7  $\pm$  5.4 mg in responders and non-responders respectively. It was difficult to further increase the carvedilol dose in non-responders because of apprehension of hypotension and bradycardia. On multivariate analysis, absence of adverse events (OR = 11.3, 95%CI: 1.9-67.8) were the only independent predictors of chronic response ( $P < 0.05$ ). Explanation for such results is that patients with less adverse events tolerated good dose to get good response. Major adverse events which resulted in drug discontinuation were hypotension in 2 patients and these patients could not be assessed further as shown in study design. Minor adverse events like fatigue, dyspnea, headache, temporary impotency, and dizziness were resolved without drug discontinuation. In addition, 2 patients had increase in ascites which resolved with escalation of diuretics. Further in our study, patients with



**Figure 2** General linear model comparing Pre (baseline), chronic (carvedilol at 3 mo) and post simvastatin haemodynamic parameters with respect to time in carvedilol responders and non-responders. HR: Heart rate; MAP: Mean arterial pressure; FHVP: Free hepatic venous pressure; WHPG: Wedged hepatic venous pressure gradient; HVPG: Hepatic venous pressure gradient.

C-P class A cirrhosis has shown better chronic response as compared to C-P class B and C but it was not statically significant.

Our studies showed that addition of simvastatin to carvedilol non responders can increase overall response to around 80%, which is one of the best possible pharmacologically produced chronic response and it opens a new strategy for portal hypertension treatment.

Etiology, C-P class, gender, ascites, adverse events, variceal size was not seen statically significant predictors of response for simvastatin protocol. Pre WHPG (baseline WHPG) was seen significantly higher among responders than non-responders and all hemodynamic parameters significantly decreased from baseline after treatment with simvastatin except FHVP which significantly raised. Similar results were observed after chronic treatment with carvedilol. In our study, HVPG after adding simvastatin decreased mainly because of increase in FHVP. Previous studies have shown that patients with cirrhosis have blood pooling in splanchnic region that correlates with degree of portal hypertension<sup>[20]</sup>. This might suggest that decreases in hepatic resistance by simvastatin could reduce splanchnic congestion and improving central blood volume<sup>[21]</sup> and alternatively simvastatin may have normalized venous compliance and by this mechanism

can inverse venacaval and right arterial pressure and thus increase FHVP.

It is well known that simvastatin improves hepatic clearance, intrinsic clearance, and hepatic extraction of indocyanine green, parameters that reflect effective liver perfusion. Thus, an increase in intrahepatic bioavailability of NO might result in improvement in amount of blood that has functional contact with hepatocytes that explains the improvement in quantitative tests of liver function after simvastatin. We have not done these tests of liver function in our study as it is already a proven fact<sup>[11,12]</sup>.

An important concern with the use of statins in patients with cirrhosis is potential for inducing liver toxicity. A number of studies have shown the safety of statins in patients with liver disease<sup>[31-33]</sup>. Our study particularly evaluated these issues in cirrhotic patients and our safety evaluation included Bil, ALP, GGT, ALT, AST, CPK and questionnaire for muscle weakness at 15<sup>th</sup> and 30<sup>th</sup> day of treatment. There was no major safety concern seen in our study. Some minor adverse events which were observed after addition of simvastatin are: (1) muscle weakness with CPK > 5 times in one patient and was withdrawn; (2) pruritis in one patient which settled and treatment continued; (3) diarrhea in one patient, self-settled and treatment continued; (4) severe dizziness and treatment



withdrawn; and (5) hepatic encephalopathy in one patient and withdrawn from the study, not related to simvastatin likely part of disease.

However, whether safety profile is maintained after long term administration needs further long term studies especially with larger doses in advanced liver disease. Newer drugs like rovastatin have been shown to be safe in chronic liver disease also.

Overall, 7 patients had adverse events, 4 (57.1%) among responders, and 3 (42.9%) among non-responders with no statistical significance. Three patients were withdrawn due to side effects, first one because of increase in CPK > 5 times with muscle weakness, second one developed dizziness and 3<sup>rd</sup> patients developed hepatic encephalopathy not related to simvastatin. Liver function test after 30 d and CPK did not change and remained static and no further side effects were observed after 30 d.

Thus in conclusion, our study is first study which clearly shows that a sequential treatment strategy is an excellent policy in the pharmacological management of portal hypertension by which around 80% of response can be achieved. Further long term safety profile of statins with large doses particularly in advanced disease needs further studies and safe drugs like pravastatin needs to be evaluated in future that can be used for adjuvant treatment along with carvedilol.

## COMMENTS

### Background

Carvedilol, a potent 3<sup>rd</sup> generation non-selective beta blocker (NSBB) has shown to be a promising therapy for reduction of portal hypertension. Although up to 60% of patients respond to carvedilol, options for carvedilol non responders in patients with portal hypertension is limited. Simvastatin improves liver generation of NO and hepatic endothelial dysfunction in patients with cirrhosis without affecting the hemodynamics such as heart rate and blood pressure. Hence, it could be used as an effective adjuvant therapy with carvedilol without causing any major side effects in patients with portal hypertension.

### Research frontiers

Current guidelines recommend using NSBB, such as propranolol or nadolol, with or without isosorbide-5-mononitrate to prevent variceal bleeding. Carvedilol, which blocks both  $\alpha$  and  $\beta$  receptors, was shown to have better results than NSBBs by further reducing intrahepatic resistance and thus, could be used for propranolol non-responders. However, treatment option for carvedilol non-responders has not been studied yet.

### Innovations and breakthroughs

Addition of simvastatin could be an important pharmacological rescue therapy for carvedilol nonresponders. This study showed that addition of simvastatin to carvedilol non responders can increase overall response to around 80%, which is one of the best possible pharmacologically produced chronic response and may open a new strategy for the treatment of portal hypertension.

### Applications

Addition of simvastatin to carvedilol non-responders may prove to be an excellent therapy in patients with portal hypertension.

### Terminology

NSBB are very useful drugs in preventing first variceal bleeding and re-bleeding in patients with cirrhosis.

## Peer-review

The observational study of Wani *et al* seems to be the first which demonstrate that a sequential treatment (carvedilol + simvastatin) strategy is an excellent policy in the pharmacological management of portal hypertension. The study is well designed and well presented.

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# Influence of vitamin D on liver fibrosis in chronic hepatitis C: A systematic review and meta-analysis of the pooled clinical trials data

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## Abstract

### AIM

To investigate the relationship between vitamin D and liver fibrosis in hepatitis C-monoinfected or hepatitis C virus (HCV)-human immunodeficiency virus (HIV) co-infected patients.

### METHODS

Pertinent studies were located by a library literature search in PubMed/Embase/Cochrane/Scopus/LILACS by two individual reviewers. Inclusion criteria: (1) studies with patients with HCV or co-infected HCV/HIV; (2) studies with patients  $\geq 18$  years old; (3) studies that evaluated liver fibrosis stage, only based on liver biopsy; and (4) studies that reported serum or plasma 25(OH)D levels. Studies that included pediatric patients, other etiologies of liver disease, or did not use liver biopsy for fibrosis evaluation, or studies with inadequate data were excluded. Estimated measures of association reported in the literature, as well as corresponding measures of uncertainty, were recorded and corresponding odds ratios with 95%CI were included in a meta-analysis.

### RESULTS

The pooled data of this systematic review showed that 9 of the 12 studies correlated advanced liver disease defined as a Metavir value of F3/4 with 25(OH) D level insufficiency. The meta-analysis indicated a significant association across studies.

## CONCLUSION

Low vitamin D status is common in chronic Hepatitis C patients and is associated with advanced liver fibrosis.

**Key words:** Vitamin D; Liver fibrosis; Hepatitis C virus; Chronic hepatitis C

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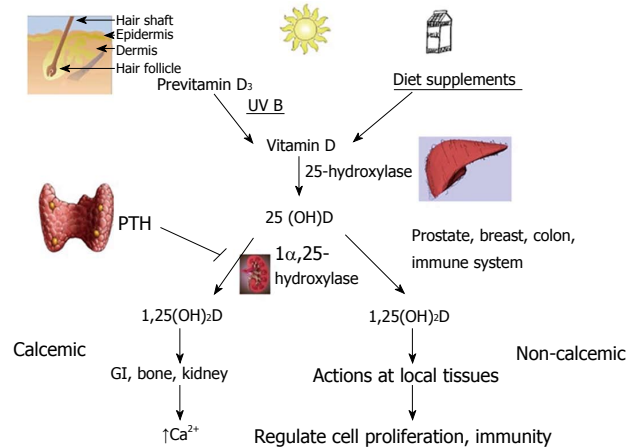
**Core tip:** Vitamin D levels are associated with more advanced fibrosis in chronic hepatitis C.

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(5): 278-287 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i5/278.htm> DOI: <http://dx.doi.org/10.4254/wjgh.v9.i5.278>

## INTRODUCTION

Hepatitis C virus (HCV) infection remains one of the most common etiologies of liver disease worldwide. A number of epidemiological papers have addressed the global prevalence of Hepatitis C. Lanini *et al.*<sup>[1]</sup> reported that 100 million people globally have serological evidence of current or past HCV infection causing 700000 deaths annually while others suggest that the actual occurrence is double<sup>[2]</sup>. HCV remains the most common indication for liver transplantation in the United States<sup>[3]</sup>. Chronic infection with HCV can lead to liver inflammation, liver fibrosis, cirrhosis, and hepatocellular carcinoma. Liver fibrosis is a result of excessive accumulation of extracellular matrix proteins, as part of the wound healing response to chronic injury and chronic inflammation<sup>[4]</sup>. Various factors have been associated with progression of fibrosis including duration of infection, age, male sex, diabetes, alcohol consumption and human immunodeficiency virus (HIV) co-infection<sup>[5]</sup>.

Vitamin D is a hormone that has numerous biological properties that influence host physiology by regulating epigenetic regulation of more than 2000 genes throughout the body. Vitamin D is best known for its role in maintaining bone mineralization but has diverse and profound influences which can determine disease development and outcome. Although referred to as a vitamin, this steroid hormone is synthesized in the body by a series of hydroxylation reactions that occur in skin (7-hydroxylation), the liver (25-hydroxylation) and the kidney (1-hydroxylation)<sup>[6]</sup> (Figure 1). Reduction of the enzymatic conversion of 7-dehydrocholesterol to 1,25 hydroxy vitamin D at any of the three conversion steps can result in suboptimal vitamin D status<sup>[7]</sup>. Vitamin D has a number of influences on innate and adaptive immunity which are pertinent to study in conditions that are driven by



**Figure 1 Vitamin D metabolism.** Vitamin D has diverse influences throughout the body as vitamin D receptors present on virtually every cell. The actions of vitamin D can be subdivided into two larger categories: Calcemic and non-calcemic actions. The non-calcemic actions of vitamin D are legion and have been reviewed elsewhere<sup>[6,54-56]</sup>. Reproduced with permission<sup>[6]</sup>.

chronic inflammation and maladaptive tissue injury<sup>[8,9]</sup>. Given the ubiquitous distribution of vitamin D receptors in virtually every cell in the body-suboptimal vitamin D status has been studied for its relationship to numerous diseases<sup>[10]</sup>. For example, there is substantial evidence that vitamin D benefits rheumatoid arthritis, due to its immunomodulatory effect<sup>[11]</sup>. The role of vitamin D in various cancers has been established linked to its anti-proliferative action mediated through vitamin D nuclear receptor<sup>[12]</sup>. There have been numerous reports on lower serum vitamin D levels in patients with chronic liver disease from various etiologies<sup>[13]</sup>. In chronic HCV, Low vitamin D levels have been reported in 46% to 92% of patients<sup>[10]</sup> raising suspicion of its potential contribution to disease pathogenesis. There is growing evidence from various groups, that vitamin D levels are inversely correlated with liver inflammation and stage of liver fibrosis in patients with HCV; however, the studies are heterogeneous with occasionally the results are conflicting. Additionally, the methods of reporting liver fibrosis were variable.

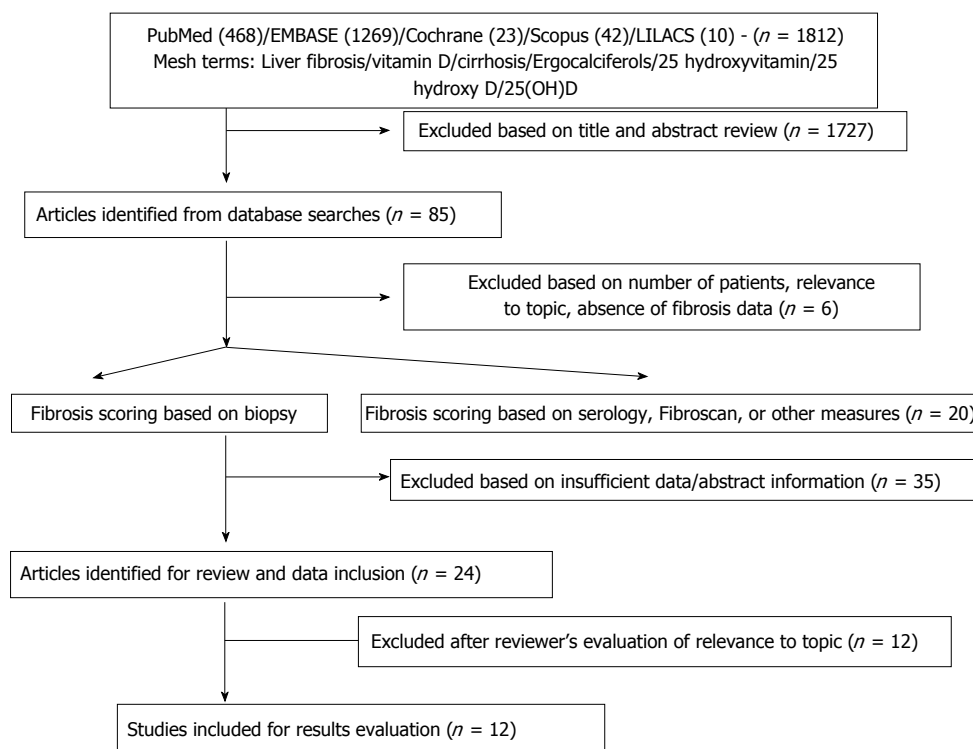
The aim of this study was to evaluate the relationship between vitamin D status and hepatic fibrosis based on histopathological staging in patients with chronic HCV mono-infection or co-infected HIV-HCV infection, by performing a systematic review of the scientific literature followed by a meta-analysis.

## MATERIALS AND METHODS

### Search method

Applicable studies were identified by a library literature search in Pubmed/Embase/Cochrane/Scopus/LILACS utilizing the PRISMA checklist<sup>[14]</sup> "Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated" and the Cochrane review reporting guidelines (6.6.2.2)<sup>[15]</sup>.





**Figure 2 Flowchart of study selection process.** Eighteen hundred and twelve articles were identified using PubMed ( $n = 468$ )/EMBASE ( $n = 1269$ )/Cochrane ( $n = 23$ )/Scopus ( $n = 42$ )/LILACS ( $n = 10$ ) search engines. Detailed evaluation of the articles by at least two independent reviewers (total of three) narrowed the studies to twelve ( $n = 2521$ ) based upon inclusion and exclusion criteria as listed in Table 1.

The search terms were as follows: ["Liver cirrhosis" or "liver" and ("cirrhosis" or "fibrosis")] and ["vitamin D" or "Ergocalciferols" or "25 hydroxyvitamin D" or "25 hydroxy vitamin D" or "25 hydroxy D" or "25(OH)D"]. Also, the studies cited by the selected articles were searched for further pertinent studies. The search was performed before July 6, 2016.

### Selection criteria

The title and abstract of the studies were carefully reviewed by two individual reviewers, based on the inclusion and exclusion criteria. If there was an agreement between two reviewers, then the study was selected for further analysis. When there was a disagreement, a third reviewer determined if the study qualified for inclusion. Once the articles met the criteria, then the text was reviewed, and data extraction was completed.

Inclusion criteria: (1) studies with patients with HCV or co-infected HCV/HIV; (2) studies with patients  $\geq 18$  years old; (3) studies that evaluated liver fibrosis stage, only based on liver biopsy; and (4) studies that reported serum or plasma 25(OH)D levels. Exclusion criteria: (1) liver diseases other than hepatitis C; (2) studies with inadequate data; (3) studies that used non-invasive methods in evaluating liver fibrosis; and (4) age  $< 18$  years.

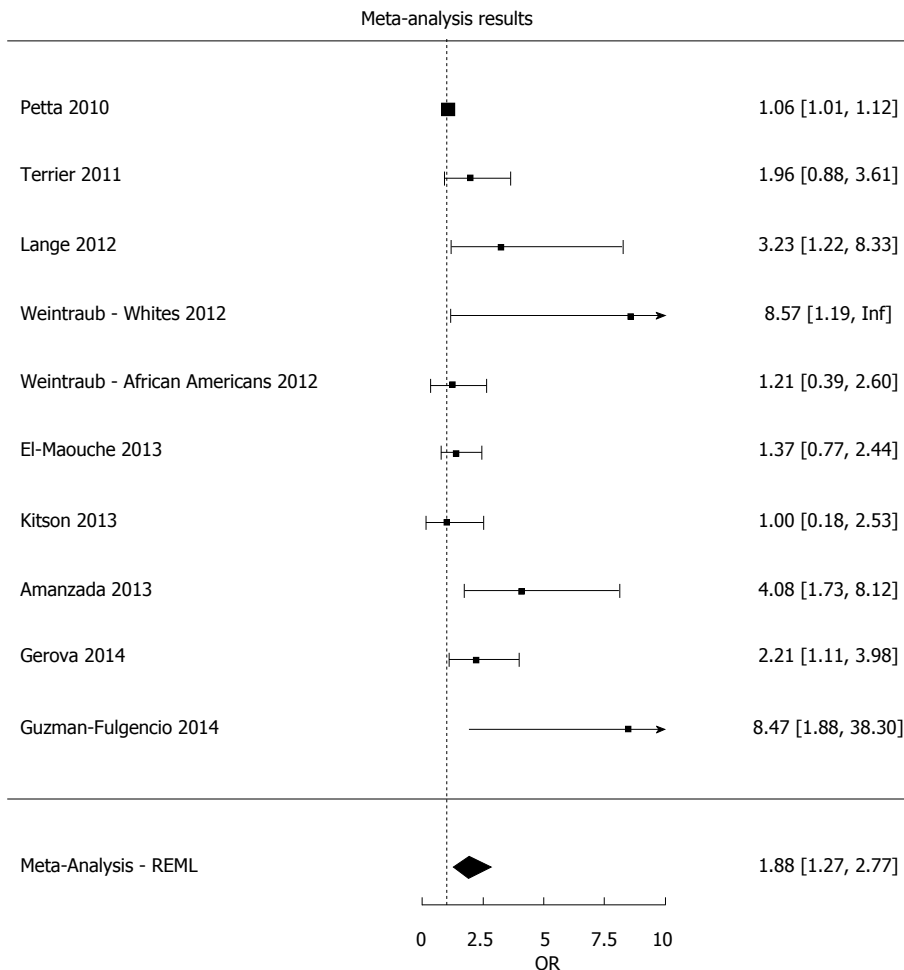
### Data extraction

A total of 12 studies were included for extraction which was performed by two independent reviewers based on

data quality, sufficiency, and relevance. Disagreements were resolved by a third reviewer to reach a consensus. The following data were extracted: Last name of the first author, demographic information of patients, publication year, sample size, HCV genotype, presence or absence of HIV co-infection, pathological fibrosis stage using Metavir score, vitamin D levels, and association of serum vitamin D level and fibrosis stage (Figure 2). The quality of evidence was ascertained by two independent reviewers using The Grading of Recommendations Assessment, Development and Evaluation (GRADE) analysis whereby very low = 1, low = 2, moderate = 3, high = 4<sup>[16]</sup>. The strength of recommendations were 1 (strong) or 2 (weak)<sup>[17]</sup>. When there was a disagreement, a third reviewer determined GRADE assessment and strength of recommendations.

### Statistical analysis

All statistical computations were conducted in R (Version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria, 2016)<sup>[18]</sup>. Estimated odds ratios (OR) reported in the literature, as well as 95%CI, were inverted when necessary and included in a meta-analysis. In several studies, the odds ratio for severe fibrosis corresponding to vitamin D deficiency was not reported, but the distribution (mean and standard deviation or inter-quartile range) of vitamin D levels were reported for subjects with and without severe fibrosis separately. To estimate the odds ratio from these studies, a Monte Carlo simulation approach was adopted: For each such study, 1000



**Figure 3** Meta-analysis of the pooled data from the 12 included studies. The odds ratio for severe fibrosis comparing low vitamin D levels was estimated by meta-analyzing studies including a total of 2521 patients. Details concerning the analytic strategy are provided in the Materials and Methods section.

simulated studies were created assuming that vitamin D levels were normally distributed with the reported parameters and the observed number of subjects in each group. The odds ratio for severe fibrosis comparing vitamin D levels with a cutoff of 15 ng/mL was estimated for each simulated dataset. A sensitivity analysis was also conducted by using thresholds of 20 ng/mL and 30 ng/mL. The average odds ratio across simulated datasets were then estimated, and quantile-based confidence intervals were also recorded and included into the meta-analysis. A random-effects meta-analysis fit using restricted maximum likelihood was then fit using the Metafor package in R<sup>[19]</sup>.  $P < 0.05$  was considered statistically significant.

## RESULTS

The initial protocol established a series of mesh terms used to identify articles that would evaluate the severity of liver fibrosis in chronic hepatitis C patients with vitamin D levels. Eighteen hundred and twelve articles were found using PubMed ( $n = 468$ )/EMBASE ( $n = 1269$ )/Cochrane ( $n = 23$ )/Scopus ( $n = 42$ )/LILACS ( $n = 10$ ) search engines. Mesh terms used were liver fibrosis/

vitamin d/cirrhosis/Ergocalciferols/25 hydroxyvitamin/25 hydroxy d/25(OH) D. Detailed evaluation of the articles by at least two independent reviewers (total of three) assessed the sufficiency of data, method of fibrosis qualification, relevance to the topic to narrow the studies to twelve. The data extraction algorithm is summarized in Figure 3. Table 1 reflects the characteristics of the studies relating fibrosis to chronic hepatitis C and vitamin D level. When patients were stratified according to vitamin D status, we found substantial differences between the levels of severity of liver fibrosis. The sensitivity analysis with different cutoffs for the Monte Carlo simulations showed robustness of the result to the choice of cutoff, with significant effects for all thresholds employed.

### Definition of vitamin D levels

Vitamin D insufficiency was defined in most studies as below  $< 30$  ng/mL, and deficiency ranged from  $< 20$  ng/mL to 10 ng/mL. While there was some variability in these definitions, there was consistency in the lower limit of normal being  $< 30$  ng/mL. Two of the studies used nmol/L to express 25(OH)D, but were consistent with vitamin D insufficiency below the lower limit of normal  $< 80$  nmol/L.

**Table 1** Pooled data of vitamin D levels and liver fibrosis from the 12 included studies

Year	Author	Country	Design	n	HCV GT	HIV	Definition of vitamin D insufficiency (I)/deficiency (D)	Outcome (serum vitamin D and liver fibrosis)	P value/OR 95%CI	GRADE quality of evidence very low = 1, low = 2, moderate = 3, high = 4 and strength of recommendation: 2 = strong 1 = weak
2010	Petta	Italy	Prospective	197	1	No	< 30 ng/mL for low vitamin D level	Low 25(OH)D associated with severe fibrosis (F3/F4)	0.942 [0.893, 0.994] P = 0.009	GRADE 3 Strong
2011	Terrier	France	Prospective	189	1,-4 other	Yes	< 10 ng/mL D, 10-30 ng/mL (I)	Low 25(OH)D correlate with severe fibrosis (F3/F4)	P = 0.04	GRADE 3 Strong
2012	Lange	Sweden	Retrospective	496	1, 4	No	< 10 ng/mL D, < 20 ng/mL (I)	Advanced fibrosis stage F2-F4 vs F0-F1 associated with low 25(OH)D	0.31 [0.12, 0.82] P = 0.018	GRADE 2 Weak
2012	Weintraub	United States	Cross- sectional	171	1	No	< 20 ng/mL or < 30 ng/mL (I)	Higher 25(OH)D predictive of milder fibrosis (F0-F2) in white patients but not in African Americans	P = 0.007	GRADE 2 Weak
2012	Baur	Switzerland	Cohort	251	1, 3	No	< 20 ng/mL (I)	(1) 25(OH)D lower in more advanced fibrosis (F2 vs F0-1); (2) low 25-OH vitamin D associated with rapid fibrosis progression rate.	P = 0.005, P = 0.013	GRADE 3 Strong
2013	El- Maouche	United States	Prospective	116	-	Yes	< 15 ng/mL (D)	(1) The prevalence of significant fibrosis (F2 ≥ 2) was similar among those with and without low Vitamin D; (2) low 25(OH)D not associated with significant fibrosis after adjusting for other confounders	P = 0.43  1.37 [0.77, 2.44]	GRADE 3
2013	Mandorfer	Austria	Prospective	65	1, 4	Yes	< 10 ng/mL D, 10-30 ng/mL (I)	Patients with D-DEF displayed a higher prevalence of advanced liver fibrosis than patients with D-NORM	P = 0.009	Strong GRADE 3
2013	Kitson	Australia and New Zealand	Prospective	274	1	No	< 50 nmol/L D < 75 nmol/L (I)	Baseline 25(OH)D level did not vary with fibrosis stage (F3/4 vs F0-2)	P = 0.18	Strong GRADE 3
2013	Amanzada	Germany	Prospective	191	1	Yes	< 30 ng/mL (I)	Low 25(OH)D associated with advance fibrosis (F0-2 vs F3/4)	P = 0.02	Strong GRADE 3
2014	Gerova	Bulgaria	Retrospective	296	1, 4	No	< 25 nmol/L (D), 25-50 nmol/L for profound (I), 50 -80 nmol/L for mild (I)	Lower 25OHD levels were registered in cases with advanced fibrosis compared to those with mild or absent fibrosis	P > 0.05	Strong GRADE 2
2014	Guzman- Fulgencio	Spain	Retrospective	174	1, 4	Yes	< 10 ng/mL (D), 10-30 ng/mL (I)	Low 25(OH)D deficiency associated with advanced fibrosis (F3/4 vs F0-2)	P = 0.005	Weak GRADE 2
2015	Esmat	Egypt	Prospective	101	4	No	< 20 ng/mL (D), 20-30 (I)	No correlation found between vitamin D levels and stage of liver fibrosis	P = 0.26	Weak GRADE 3

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; GRADE: Grading of Recommendations Assessment, Development and Evaluation.

**Table 2 Selection criteria for inclusion and exclusion**

Inclusion criteria
Age $\geq$ 18 yr
Studies including mono-infected HCV or co-infected HCV/HIV
Studies that evaluated liver fibrosis stage, only based on liver histology
Studies that reported serum or plasma 25(OH)D levels
Exclusion criteria
Age < 18 yr
Other etiologies of liver disease, other than hepatitis C
Studies that used non-invasive methods in evaluating liver fibrosis
Inadequate data

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus.

### Association between vitamin D deficiency and severity of liver disease

Among the articles used for data extraction, there were seven prospective studies, three retrospective studies, one cross-sectional analysis, and one cohort study (Table 1). In a review of the results, nine studies demonstrated a significant association between plasma levels of vitamin D and degree of HCV-related hepatic fibrosis. Three studies showed no correlation was found between vitamin D levels and stage of liver fibrosis. Patient characteristics between these studies were all similar and could not account for the variability of the findings between the three negative studies and the nine positive studies. Only one of the three negative studies was conducted in the northern hemisphere. Overall, hepatitis C genotypes were not different among the negative studies, although El-Maouche *et al*<sup>[20]</sup> did not identify which genotype(s) were included. The forest plot of the data used in this systematic review showed that advanced liver disease defined as a Metavir value of F3/4 was associated with severe 25(OH)D insufficiency as follows; OR (95%CI): 1.88 (1.27, 2.77), and  $I^2$  (total heterogeneity/total variability): 66.94% indicated substantial heterogeneity between studies.

### Plasma vitamin D levels and seasonal variation

Notably there were several latitudes identified in the studies which can affect Vitamin D levels, however, the scope of this difference in this analysis's outcome was not assessed. In the article by Guzmán-Fulgencio *et al*<sup>[21]</sup> significant seasonal variation of plasma 25(OH)D levels was observed with the subjects in the first semester (winter/spring) having lower plasma 25(OH)D levels than patients evaluated in the second semester (summer/autumn) ( $P < 0.001$ ). A higher percentage of patients with vitamin D deficiency (25(OH)D < 25 nmol/L) was found in the first semester (winter/spring) ( $P < 0.001$ ). Since not all the studies identified the time frame of vitamin D levels and biopsy procurement, we were unable to qualify the significance of this on the study results.

## DISCUSSION

The results of our systematic analysis of the literature

demonstrated an association between advanced liver fibrosis (defined as Metavir F3/F4) in chronic hepatitis C (CHC) with vitamin D status as reflected by 25-hydroxyvitamin D [25(OH)D] serum levels. In nine<sup>[21-29]</sup> of twelve studies (75%) that qualified for data extraction (Tables 1 and 2) the final analysis demonstrated an overall association between low vitamin D status as defined as serum 25(OH)D < 15 ng/mL with advanced liver fibrosis (F3/F4 stage disease) in CHC as proven by biopsy analysis for fibrosis stage. These data are highly consistent with prior reports, and the expected pathophysiological interference of 25-hydroxylation of vitamin D as liver fibrosis increases and functional hepatic capacity decreases over the course of hepatitis C disease progression<sup>[6]</sup>.

A recent systematic review of the literature by Abbasi *et al*<sup>[30]</sup> studied the relationship between low vitamin D status [ $< 20$  ng/mL 25 OH(D)] and the severity of the CLD. A comparatively abridged search strategy yielded 641 articles for consideration and ultimately 19 articles and 4895 study patients with CLD for data extraction showing that almost 80% of patients with chronic liver disease had severe vitamin D deficiency. García-Álvarez *et al*<sup>[31]</sup> conducted a systematic review evaluating the relationship of vitamin D status to advanced liver fibrosis in CHC-naïve patients and sustained virological response (SVR) to therapy using pegylated interferon/ribavirin (Peg-IFN/RBV). Seven of fourteen papers utilized for their extraction evaluated advanced liver fibrosis (1083 patients) and eleven for SVR (2672 patients). Approximately 70% of CHC patients had low 25(OH)D whereby the definition of insufficiency varied (20 or 30 ng/mL), and 50% of the HCV-infected patients had 25(OH)D levels < 10 or 20 ng/mL. Overall, low vitamin D status was related to a diagnosis of advanced stage of liver disease. Luo *et al* utilized a search methodology restricted to PubMed and Embase databases before October 2013 included studies that analyzed the association between serum vitamin D status and the severity of liver fibrosis in 8231 CHC patients without other restrictions yielding six global studies for data extraction<sup>[13]</sup>. One study recruited 6567 participants as part of the Swiss Hepatitis C Cohort Study<sup>[23]</sup> raising concerns for skewing of the extracted data. The mean data from extracted studies suggested that lower serum vitamin D is a risk factor for progressive liver fibrosis in CHC patients. However, there was a high heterogeneity and inconsistencies depending upon data set utilized (OR data studies vs mean data extracted). Our search methodology instead included 2521 patients which incorporated the 2012 study by Lange *et al*<sup>[32]</sup> which evaluated 468 HCV patients treated with alpha interferon-based regimens for vitamin D status and advanced disease demonstrating that fibrosis stages F2-F4 vs F0-F1 associated with low 25(OH)D.

The nine studies showing a positive association between low vitamin D with an advanced stage of fibrosis had variations in their definition of vitamin D status which challenged our ability to Meta-analyze the data. Low vitamin D was stratified according to by either



insufficient (I) or deficient (D) (Table 1) in eight<sup>[21-27,29]</sup> of the nine studies. Gerova *et al.*<sup>[28]</sup> used three categories; mild insufficiency, profound insufficiency, and deficiency. Overall, of the twelve papers in our final analysis, two<sup>[28,33]</sup> utilized nmol/L to measure serum 25(OH) vitamin D status. Insufficiency was defined as < 30 ng/mL in seven with another two using equivalent levels in nmol/L<sup>[28,34]</sup>, < 20 ng/mL in two<sup>[23,25]</sup> while El-Maouche studied only deficient patients (< 15 ng/mL)<sup>[20]</sup>. The definition of "deficiency" was utilized by all but two<sup>[20,34]</sup> of the studies as < 10 ng/mL 25(OH) vitamin D. The prevalence of vitamin D deficiency in a population depends on upon the definition used [ $< 20$  or  $< 30$  ng/mL (50 or 75 nmol/L)]. In the National Health and Nutrition Examination Survey (NHANES), 41.6 percent of United States adults had (25[OH]D) levels < 20 ng/mL (50 nmol/L)<sup>[35]</sup>. The Institute of Medicine recommends the attainment of the serum 25(OH)D levels of  $> 20 < 40$  ng/mL (50 to 100 nmol/L), however, many define sufficient vitamin D status as 25(OH)D  $> 30$  and  $< 50$  ng/mL (75 to 125 nmol/L)<sup>[36,37]</sup>.

Hepatitis C genotype (1-6) did not change the outcome of analyses between advanced fibrosis in CHC with vitamin D status<sup>[20,33,34]</sup>. The geographical latitudes of study site and variable seasonal fluctuations have provided challenges to vitamin D status, but did not appear to influence the outcome of the negative outcome studies<sup>[20,33,34]</sup>. Esmat *et al.*<sup>[34]</sup> conducted a open-labelled RCT of 101 HVC4 Egyptian patients undergoing standard of care (SOC) Peg-IFN/RBV plus/minus 15000 IU vitamin D<sub>3</sub> (cholecalciferol). The fibrosis stage (F1-F3) at baseline was not different according to 25(OH) vitamin D levels. El-Maouche *et al.*<sup>[20]</sup> evaluated HIV-HCV co-infected patients for histological fibrosis using the Metavir system [0 (no fibrosis) to 4 (cirrhosis)] and used banked serum as a source for vitamin D determination. Similar to Esmat *et al.*<sup>[34]</sup>, the prevalence of significant fibrosis (F2  $\geq$  2) was similar among those with and without low vitamin D while low 25(OH)D status was not associated with significant fibrosis after adjusting for other confounders. Finally, Kitson *et al.*<sup>[38]</sup> from Australia evaluated pre-treatment 25-hydroxyvitamin D [25(OH)D] level in a cohort of 274 treatment-naïve patients with HCV-1 to evaluate the association between vitamin D status, virological response, and liver histology after 48 wk of pegylated interferon alfa-2a plus ribavirin therapy. Baseline 25(OH)D level did not vary with fibrosis stage (F3/4 vs F0-2).

The manner by which vitamin D may influence the course of CHC may be due to effects on viral clearance, immune modulation, cell differentiation and proliferation and inflammation regulation. Vitamin D is not only involved in calcium homeostasis but has also has been associated with the mechanism of cellular proliferation, and immunomodulation<sup>[39]</sup>. Several studies have shown that vitamin D levels are inversely correlated with stage of liver fibrosis in patients with CHC. Nine<sup>[21-29]</sup> of the twelve studies that we included for data extraction reported the inverse correlation of vitamin D levels with the stage of liver fibrosis in patients with CHC. Vitamin D has anti-

inflammatory, anti-proliferative and anti-fibrotic effects that dampen inflammatory cell recruitment to the liver and mitigate hepatic fibrosis progression<sup>[40]</sup>. HCV may also have its own direct actions that impair vitamin D activity and status. It has been hypothesized that HCV affects 25-hydroxylation of vitamin D through cytokine induction or oxidative stress or through disruption in lipid metabolism where HCV suppress 25(OH)D levels due to a decrease in the production of vitamin D precursor, 7-dehydrocholesterol<sup>[10]</sup>.

The profound relationship of vitamin D to immunity and inflammation, and our findings raise questions about how vitamin D status may impact the outcome of the many non-HCV chronic liver diseases. Individuals with chronic liver disease have significant global prevalence, morbidity, poor quality of life and mortality. Prior works have demonstrated adverse survival outcomes in patients with lowered vitamin D status<sup>[41,42]</sup>. In our analyses, we excluded papers reporting the analysis of vitamin D in chronic liver diseases other than HCV including chronic hepatitis B (CHB) which has a higher global prevalence of approximately 300 million infected individuals. Yu *et al.*<sup>[43]</sup> evaluated the potential association between serum vitamin D level and liver histology or virological parameters in treatment-naïve patients with chronic hepatitis B infection in Southern China. They reported that patients infected with genotype B had a higher prevalence of vitamin D insufficiency than individuals with CHC. Furthermore, in chronic hepatitis B patients, serum 25(OH) D was not correlated with viral load or fibrosis. Mi *et al.*<sup>[44]</sup> reported that vitamin D status was not different among Asians with non-cirrhotic CHB and CHC.

Low vitamin D status is associated with the risk of progression and the severity of hepatic inflammation in patients with non-alcoholic fatty liver disease<sup>[45,46]</sup>. Primary biliary cirrhosis has been extensively analyzed for correlations of vitamin D status predicting the outcome to ursodeoxycholic acid (UCDA) therapy and the influence of vitamin D supplementation to UCDA intervention<sup>[47-49]</sup>. Autoimmune hepatitis (AIH) has also been studied for the potential influence of vitamin D given the epidemiological association of this hormone with a number of diseases with autoimmunity<sup>[50,51]</sup>. However, there are not sufficient studies to draw meaningful conclusions of serum 25(OH)D and AIH at this time.

Altered vitamin D physiology *via* resistance from genetic polymorphisms of the vitamin D receptor (VDR) could also influence the outcome of CHC. Baur *et al.*<sup>[25]</sup> demonstrated that low 25(OH)D plasma levels and VDR bAt[CCA]haplotype were associated with rapid fibrosis progression in CHC, separately and synergistic when co-present. Petta *et al.*<sup>[52]</sup> reported that low hepatic VDR expression was inversely related to the severity of advanced liver fibrosis in patients with genotype 1 cCHC patients. Grunhage reported that a single nucleotide polymorphism (SNP) linked to the *DHCR7* gene coding vitamin D precursor dehydrocholesterol was related to altered serum 25(OH)D in chronic liver disease patients

with no or mild fibrosis<sup>[53]</sup>.

CHC with severely low vitamin D status is accompanied by advanced liver fibrosis. Interventional trials aimed to normalize vitamin D status in early stages of CHC may shed light on whether correction of vitamin D status in this patient population should become the standard of care.

## COMMENTS

### Background

Hepatitis C remains a global health burden affecting over 100 million people worldwide. There is growing evidence that vitamin D is inversely associated with liver inflammation and fibrosis in patients with chronic hepatitis C.

### Research frontiers

Currently hepatitis C is being dramatically eradicated with DAA therapy. Possible augmentation of DAA therapy by vitamin D in those patients who already have fibrosis may decrease long term damage in the liver parenchyma.

### Innovations and breakthroughs

The pooled data of this systematic review showed that 9 of the 12 studies correlated advanced liver disease defined as a Metavir value of F3/4 with 25(OH) D level insufficiency. The meta-analysis indicated a significant association across studies. Low vitamin D status is common in chronic Hepatitis C patients and is associated with advanced liver fibrosis.

### Applications

Augmentation of standard hepatitis C therapy of direct acting antiviral meds with vitamin D may assist with long term decrease in liver fibrosis.

### Peer-review

This is a very interesting and informative paper, and it deserves publication.

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## Is it time to rethink combined liver-kidney transplant in hepatitis C patients with advanced fibrosis?

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### Abstract

#### AIM

To reduce hepatic and extrahepatic complications of chronic hepatitis C in kidney transplant recipients.

#### METHODS

We conducted a systematic review of kidney only transplant in patients with hepatitis C and advanced fibrosis.

#### RESULTS

The 5 year patient survival of kidney transplant recipients with and without hepatitis C cirrhosis ranged from 31% to 90% and 85% to 92%, respectively. Hepatitis C kidney transplant recipients had lower 10-year survival when compared to hepatitis B patients, 40% and 90% respectively. There were no studies that included patients with virologic cure prior to kidney transplant that reported post-kidney transplant outcomes. There were no studies of direct acting antiviral therapy and effect on patient or graft survival after kidney transplantation.

#### CONCLUSION

Data on kidney transplant only in hepatitis C patients that reported inferior outcomes were prior to the development of potent direct acting antiviral. With the development of potent direct acting antiviral therapy for hepatitis C with high cure rates studies are needed to determine if patients with hepatitis C, including those with advanced fibrosis, can undergo kidney transplant alone with acceptable long term outcomes.

**Key words:** Cirrhosis/cirrhotics; Renal transplantation; Kidney transplantation; Mortality; Systematic review; Graft outcomes; Meta-analysis

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**Core tip:** Individuals with chronic hepatitis C with advanced fibrosis and kidney failure who undergo kidney transplant alone are believed to have lower long-term survival. Surprisingly, we have only a few studies with inconsistent results. The concern about isolated-kidney-transplant alone is that the liver disease would progress to decompensated cirrhosis and liver failure in the setting of immunosuppression after kidney transplant. Earlier, interferon was associated with low virologic cure and high adverse events including graft rejection. However, with development of newer directly acting anti-virals we wish to invite our readers to reconsider the need for a combined liver-kidney transplant in hepatitis C patients with advanced fibrosis or compensated cirrhosis.

Shah NJ, Russo MW. Is it time to rethink combined liver-kidney transplant in hepatitis C patients with advanced fibrosis? *World J Hepatol* 2017; 9(5): 288-292 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i5/288.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i5.288>

## INTRODUCTION

Patients with hepatitis C virus (HCV) cirrhosis undergoing kidney transplantation only have lower post-transplant survival rates compared to recipients without hepatitis C or cirrhosis<sup>[1]</sup>. After the implementation of the model for end-stage liver disease (MELD) scoring system for allocating liver transplants, the number of simultaneous liver-kidney transplantation has increased by 300%<sup>[2]</sup>. Some of these patients may have relatively well compensated cirrhosis and patients with well compensated cirrhosis but kidney failure may receive a MELD score of 20 based upon a creatinine of 4 mg/dL. These patients may have compensated cirrhosis without complications of portal hypertension. Thus, kidney failure, not liver failure may be the driving factor for priority for liver transplant in this subgroup. This is particularly relevant in areas of the country where patients may receive liver transplants at relatively low MELD scores compared to areas with higher demand.

The reason for dual listing patients with hepatitis C cirrhosis and kidney failure who may be well compensated is the concern of decompensation after liver-kidney transplant. Immunosuppressive therapy to prevent rejection increases the titers of HCV RNA and immunosuppression has been associated with accelerated hepatitis injury such as fibrosing cholestatic hepatitis C<sup>[3]</sup>. However, the impact on treating and curing candidates before or after kidney transplant has not been well studied. The high virologic cure rates may have important implications for patients in kidney failure with hepatitis C and advanced liver fibrosis.

The guidelines for liver kidney transplant are conflicting or without detailed recommendations. The AASLD

and KDIGO guidelines do not directly address the issue of isolated kidney transplant in the setting of cirrhosis or advanced liver fibrosis. The EASL guidelines state that patients with established cirrhosis and portal hypertension who fail (or are unsuitable for) HCV antiviral treatment, isolated renal transplantation may be contra-indicated and consideration should be given to combined liver and kidney transplantation<sup>[4]</sup>. Patients with symptomatic or presence of portal hypertension are considered candidates for kidney-liver transplantation<sup>[2]</sup>. There is no consensus for patients with hepatitis C and periportal fibrosis or bridging fibrosis who are kidney transplant candidates.

The aim of this systematic review was to assess the outcome of hepatitis C cirrhotics undergoing kidney only transplant and suggest areas for further study in patients with hepatitis C and advanced fibrosis who are kidney transplant candidates.

## MATERIALS AND METHODS

### Literature search

We conducted online electronic searches (published human clinic trials in English) of the National Library of Medicine's (Bethesda, MD, United States) MEDLINE database, Cochrane Library and manual searches of selected specialty journals to identify any pertinent literature. Three MEDLINE database engines (Ovid, PubMed and EMBASE) were searched using the key words "cirrhosis", "cirrhotics", "chronic hepatitis C", "renal transplantation", "kidney transplantation", "mortality", "graft outcomes". The references of articles were reviewed for additional articles.

### Inclusion criteria

Clinical studies (prospective and retrospective) from the last 20 years on kidney transplant recipients with HCV cirrhosis (both compensated and decompensated) were included. The studies required a minimum of a 1 year post transplant follow-up with information regarding graft and patient survival outcomes.

### Exclusion criteria

Studies not published in English or published only in the abstract form were excluded.

### Primary end point

To compare post kidney transplant survival in hepatitis C cirrhotics undergoing kidney transplant alone to recipients without hepatitis C and without cirrhosis.

### Source of support

This systematic review was not supported by any pharmaceutical company, governmental agency or other grants.

## RESULTS

Figure 1 shows studies<sup>[5-9]</sup> in patients with hepatitis C

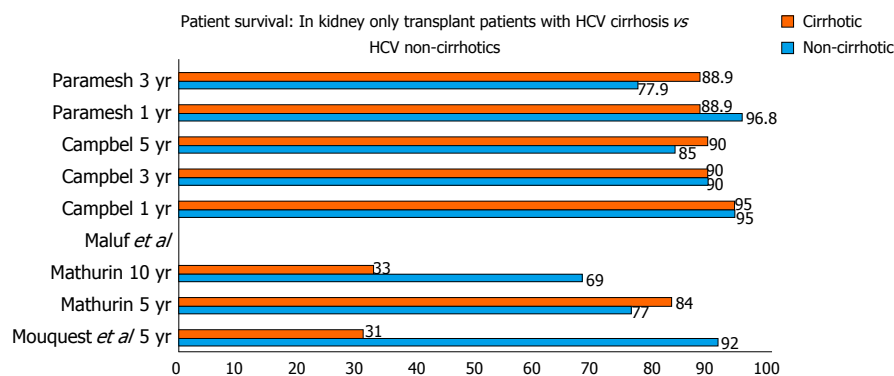


Figure 1 Studies in patients with hepatitis C who underwent kidney transplant only. HCV: Hepatitis C virus.

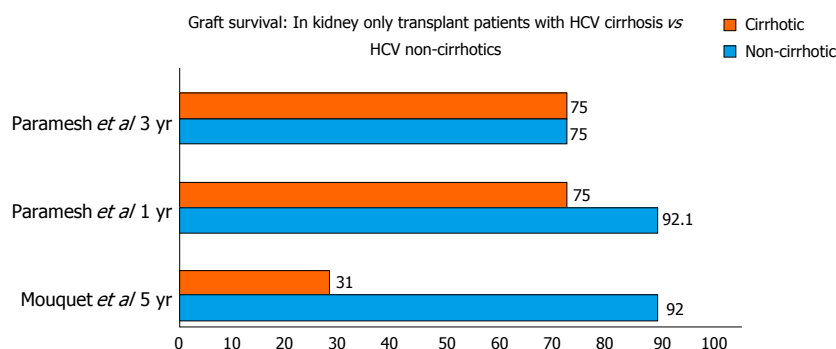


Figure 2 Graft survival in kidney only transplant patients. HCV: Hepatitis C virus.

who underwent kidney transplant only. Five studies were identified that included 2511 patients. Of these 2511 patients, 458 had hepatitis C while 69 were confirmed to have cirrhosis based on a liver biopsy. The mean age ranged from 35 to 57 years with a male to female ratio of 1.73:1. The study by Mathurin *et al*<sup>[6]</sup> consisted of 66% Europeans and 31% Africans, while in most of the other studies 66%-79% of the study population was African-American. The most common etiology of kidney disease was diabetes mellitus. Only one study provided the mean MELD score (20.6)<sup>[9]</sup>. Data on hepatitis C genotyping was not reported in any study. In all the studies the donors were deceased donors. One patient in the Mouquet *et al*<sup>[5]</sup> study was coinfectd with hepatitis B. Two studies reported the specific immunosuppressive regimen with either cyclosporine or tacrolimus.

### Outcomes of studies

The studies reported either 1, 3, 5 or 10 year survival of HCV cirrhotics vs non-cirrhotics. One year and three year survival were available for 3 studies. The 1-year and 3-year patient survival was 88.9% to 95% and 37% to 90% in cirrhotics vs 95% to 96.3% and 76% to 90% in non-cirrhotics. The 5-year and 10-year graft survival was 31%-90% and 33%  $\pm$  11% in cirrhotics when compared to 85%-92% and 69%  $\pm$  7% in non-cirrhotics.

Mathurin *et al*<sup>[6]</sup> reported that the presence of cirrhosis ( $P = 0.02$ ) and HbsAg positive status ( $P < 0.0001$ ) were associated with poor 5 and 10-year survival, 84%

$\pm$  7% and 33%  $\pm$  11%, respectively. Maluf *et al*<sup>[7]</sup> demonstrated the Knodell histology score was associated with mortality in hepatitis C kidney transplant patients ( $P = 0.012$ ).

The study by Campbell *et al*<sup>[8]</sup> reported that survival after kidney transplant only in recipients with hepatitis C was similar between patients with minimal liver fibrosis compared to patients with advanced fibrosis. Paramesh *et al*<sup>[9]</sup> reported kidney transplant alone to be safe in compensated hepatitis C cirrhosis; HR = 1.4,  $P = 0.7817$  compared to graft survival in non-cirrhotics: HR = 0.81,  $P = 0.758$ ) (Figure 2).

## DISCUSSION

Individuals with chronic hepatitis C with advanced fibrosis and kidney failure who undergo kidney transplant alone are believed to have lower long term survival although there are surprisingly few studies on this patient population. Furthermore, there has not been consistent results among studies reporting outcomes of isolated kidney transplant in hepatitis C infected recipients. The concern about isolated kidney transplant alone in a patients with hepatitis C and advanced liver fibrosis is that the liver disease will progress to decompensated cirrhosis and liver failure in the setting of immunosuppression after kidney transplant. The progression of liver disease from hepatitis C after kidney transplant was of particular concern during the interferon era because of limited

therapy for hepatitis C. Interferon is associated with low virologic cure and high adverse events including graft rejection. However, with the development of interferon free regimens and direct acting antiviral agents the need of combined liver-kidney transplant in hepatitis C patients who have hepatitis C and advanced fibrosis or compensated cirrhosis needs to be readdressed.

Patients with cirrhosis after kidney transplant may be at a greater risk of immune dysfunction and developing lethal infections because patients with cirrhosis have multiple immunological defects. Cirrhotic patients have reduced cell-mediated immunity<sup>[10,11]</sup> reduced neutrophil phagocytic ability<sup>[12]</sup> and impaired macrophage Fc receptor function<sup>[13]</sup>. In the setting of immunosuppression the risk of infection in patients with cirrhosis is likely higher than without immunosuppression. However, if liver fibrosis regresses then the risk of infection may be reduced. In a 10-year study following 51 kidney transplant recipients with hepatitis C who underwent serial liver biopsies, Kamar *et al*<sup>[14]</sup> showed that HCV infection was not associated with worsening liver histology in 50% of patients. Furthermore, there may be regression of liver fibrosis in some patients after kidney transplantation<sup>[15]</sup>. In fact, Paramesh *et al*<sup>[9]</sup> concluded that the presence of cirrhosis in HCV-positive patients is not a significant variable affecting either graft or patient survival.

One strategy is to treat all chronic hepatitis C patients with direct acting antiviral therapy while waiting for kidney transplant. The regimens that are currently available include sofosbuvir/ledipasvir, sofosbuvir/daclatasvir, and paritaprevir/ritonavir/ombitasvir/dasabuvir. Each of these regimens may require the addition of ribavirin depending on patient characteristics such as genotype or presence of cirrhosis. Sofosbuvir is renally cleared and not indicated in patients with glomerular filtration rates less than 30 mL/min. Ribavirin is renally cleared and although there is renal dosing for ribavirin it may be associated with a 2-4 g/dL drop in hemoglobin which may not be tolerated in some patients with kidney failure. Thus, given these limitations many patients with kidney failure may not be candidates for therapy with the currently available direct acting antiviral agents. Paritaprevir/ritonavir/ombitasvir/dasabuvir has been studied in patients with hepatitis C and kidney failure with virologic cure rates exceeding 85%<sup>[16]</sup>. There are other direct acting antiviral agents in development for hepatitis C patients with kidney failure that will provide additional treatment options for this patient population.

During the era of interferon based regimens for hepatitis C high rates of rejection in kidney transplant recipients was reported. Rejection rates of 40%-60% were reported with interferon based regimens with rare cases of graft loss<sup>[17-22]</sup>. The mechanism of rejection is believed to be the immune mediated injury from interferon. The direct-acting antiviral agents regimens are interferon free and due not stimulate the T cell response and should not be associated with rejection. The direct acting agents have been studied in liver transplant recipients with virologic cure exceeding 90% and

acceptable safety profile with little or no rejection<sup>[23-27]</sup>. Although there is no theoretical reason to believe the direct acting antiviral agents would be associated with increased risk of kidney rejection this would be studied in clinical trials. Additional important findings from this review include the lack of reporting of relevant data related to hepatitis C including genotype, liver fibrosis, viral load and prior treatment history. Studies of hepatitis C in patients with kidney disease should systematically report these data in a standardized fashion. Furthermore, the number of subjects with hepatitis C and advanced fibrosis was small and it is likely a multicenter study will best demonstrate if there is any difference in outcomes between kidney transplant recipients without hepatitis, with hepatitis C and mild liver fibrosis, and hepatitis C and advanced fibrosis.

We suggest we should treat all chronic hepatitis C patients irrespective of the fibrotic staging; especially those that we anticipate may be on the waiting list for a longer time.

In conclusion, data are lacking or outdated on post renal transplant outcomes in recipients with chronic hepatitis C. There is no substantiated evidence on which to base a decision to perform kidney transplant alone or a kidney-liver transplantation in a patient with chronic hepatitis C and advanced fibrosis or well compensated cirrhosis. Given limited resources of organs data are sorely needed so evidence based decisions can be made on how best to allocate kidneys in patients with liver disease. The time has come to conduct a large multicenter trial in kidney transplant candidates and recipients with hepatitis C to determine how organs should best be allocated.

## COMMENTS

### Background

Individuals with chronic hepatitis C with advanced fibrosis and kidney failure who undergo kidney transplant alone are believed to have lower long-term survival. Surprisingly, the authors have only a few studies with inconsistent results. The concern about isolated-kidney-transplant alone is that the liver disease would progress to decompensated cirrhosis and liver failure in the setting of immunosuppression after kidney transplant.

### Research frontiers

With further research on the use of direct-acting antiviral agents's (DAA's) in this subgroup of patients with hepatitis C virus (HCV) listed for renal transplant; the authors could come to a consensus to draft acceptable guidelines for better management of this subgroup of patients.

### Innovations and breakthroughs

Earlier, interferon was associated with low virologic cure and high adverse events including graft rejection. This has been replaced by newer DAA's that are safe and potent with fewer side events.

### Applications

The main objective is to invite hepatologist, transplant hepatologist and transplant nephrologist to consider DAA's in all HCV patients on the renal transplant list.

### Terminology

DAA's: Directly acting anti-virals.



# Peer-review

This is a correct, well-written review on the different autoimmune forms of liver disease, clinical manifestations and evolution, and treatment.

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## Hepatorenal syndrome: Update on diagnosis and therapy

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### Abstract

Hepatorenal syndrome (HRS) is a manifestation of extreme circulatory dysfunction and entails high morbidity and mortality. A new definition has been recently

recommended by the International Club of Ascites, according to which HRS diagnosis relies in serum creatinine changes instead that on a fixed high value. Moreover, new data on urinary biomarkers has been recently published. In this sense, the use of urinary neutrophil gelatinase-associated lipocalin seems useful to identify patients with acute tubular necrosis and should be employed in the diagnostic algorithm. Treatment with terlipressin and albumin is the current standard of care. Recent data show that terlipressin in intravenous continuous infusion is better tolerated than intravenous boluses and has the same efficacy. Terlipressin is effective in reversing HRS in only 40%-50% of patients. Serum bilirubin and creatinine levels along with the increase in blood pressure and the presence of systemic inflammatory response syndrome have been identified as predictors of response. Clearly, there is a need for further research in novel treatments. Other treatments have been assessed such as noradrenaline, dopamine, transjugular intrahepatic portosystemic shunt, renal and liver replacement therapy, *etc.* Among all of them, liver transplant is the only curative option and should be considered in all patients. HRS can be prevented with volume expansion with albumin during spontaneous bacterial peritonitis and after post large volume paracentesis, and with antibiotic prophylaxis in patients with advanced cirrhosis and low proteins in the ascitic fluid. This manuscript reviews the recent advances in the diagnosis and management of this life-threatening condition.

**Key words:** Hepatorenal syndrome; Acute-on-chronic liver failure; Liver cirrhosis; Terlipressin; Acute kidney injury

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**Core tip:** Hepatorenal syndrome (HRS) is a life-threatening complication present in very advanced liver cirrhosis. This manuscript addresses many recent advances in this field, including the recent change in the definition of HRS according to acute kidney injury

criteria, the potential consequences of the adoption of this new definition, and the use of biomarkers to help in the diagnostic algorithm. Moreover, it reviews the recent advances in treatment of HRS such as the use of continuous infusion of terlipressin instead of bolus and the low efficacy of midodrine plus octreotide. Potential areas of research are identified as well.

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## INTRODUCTION

Hepatorenal syndrome (HRS) is a manifestation of extreme circulatory dysfunction. It develops in the setting of advance stage in cirrhosis and carries an ominous prognosis.

HRS is diagnosed clinically. Its definition has been updated recently in accordance with the acute kidney injury (AKI) criteria.

Current standard of care involves the use of vasoconstrictor therapy (*i.e.*, terlipressin) and volume expansion with albumin. Treatment is effective in only 40%-50% of cases and it recurs in up to 50% of those cases responding to treatment. Liver transplant (LT) should be considered in all patients without contraindications for it.

Areas of research would be aimed at improving the accuracy of diagnosis of HRS, identifying predictors of non-response, and testing novel treatments.

## PATHOPHYSIOLOGY

HRS is caused by extreme circulatory dysfunction. Hepatocytes and stellate cells in a cirrhotic liver produce numerous local acting vasodilators such as nitric oxide, cannabinoids, *etc.* These vasodilators act locally on the splanchnic circulation producing splanchnic arterial vasodilation. Splanchnic circulation represents an important part of the circulation of the body. Thus, splanchnic vasodilation produces a decrease in mean arterial pressure (MAP), which in turn triggers the activation of the sympathetic nervous system, leading to high levels of circulating noradrenaline, which along with an increase in cardiac output are the early mechanisms compensating circulatory dysfunction during this early stage and keep MAP stable<sup>[1]</sup>.

As the disease progresses and splanchnic vasodilation gets worse other vasoconstrictor systems get activated such as the renin-angiotensin-aldosterone system and vasopressin release<sup>[1]</sup>.

Aldosterone enhances retention of sodium and water by the kidneys leading to development of ascites. Vasopressin enhances retention of free water conducting to hyponatremia. The splanchnic vascular bed is refractory to the action of all these vasoconstrictor systems which

on the contrary act effectively on other vascular beds such as the femoral and brachial vessels (producing cramps), in vessels in the brain (potentially playing a role in encephalopathy) and in the renal arteries (leading to HRS)<sup>[1,2]</sup>. In this sense, mean renal artery resistive index increases gradually from patients with cirrhosis but no ascites, in those with ascites, refractory ascites and HRS<sup>[3,4]</sup>.

Therefore, HRS is a functional disease characterised by marked vasoconstriction of the renal arteries secondary to the effect of hyper-activation of different vasoconstrictor systems aimed at compensating the systemic vasodilation caused by the initial splanchnic vasodilation. HRS always develops in the setting of advance circulatory dysfunction and it is always accompanied by ascites and usually by hyponatremia<sup>[1]</sup>.

HRS can develop in the setting of infection, mainly after spontaneous bacterial peritonitis (SBP), as a consequence of a worsening degree of circulatory dysfunction caused by sepsis. Volume expansion with albumin prevents effectively development of HRS in patients with SBP<sup>[5]</sup>.

HRS can also develop in the setting of circulatory dysfunction after large volume paracentesis (LVP). This complication is prevented by replacing albumin after LVP<sup>[6]</sup>.

## DIAGNOSIS OF HRS ACCORDING TO THE NEW DEFINITION OF AKI

Classically, acute renal failure in cirrhosis was defined as an increase in serum creatinine (sCr) levels of  $\geq 50\%$  from baseline to a final level above 1.5 mg/dL (133  $\mu\text{mol/L}$ ), and classical definition of HRS type-1 was doubling sCr levels over 2.5 mg/dL or 220  $\mu\text{mol/L}$  within 2 wk. Serum creatinine overestimates renal function in cirrhotic patients due to a number of factors: Creatinine production in patients with cirrhosis is reduced due to muscle wasting, there is an increased secretion of creatinine in the renal tubules, sCr may be diluted due to an increased volume of distribution, and finally, high bilirubin levels may interfere with the assays to measure accurately its level. Recently, the International Club of Ascites (ICA) has adopted the concept of AKI which was developed originally to be used in general critically-ill patients. AKI is defined as the increase of at least 0.3 mg/dL (26  $\mu\text{mol/L}$ ) and/or  $\geq 50\%$  from baseline, within 48 h<sup>[7]</sup>.

Diagnostic criteria of HRS according to ICA-AKI criteria are the following<sup>[7]</sup>: (1) diagnosis of cirrhosis and ascites; (2) diagnosis of AKI according to ICA-AKI criteria; (3) no response after 2 consecutive days of diuretic withdrawal and plasma volume expansion with albumin (1 g/kg of body weight); (4) absence of shock; (5) no current or recent use of nephrotoxic drugs (non-steroidal anti-inflammatory drugs, aminoglycosides, iodinated contrast media, *etc.*); and (6) no macroscopic signs of structural kidney injury, defined as absence of proteinuria ( $> 500$  mg/d), absence of microhematuria ( $> 50$  red blood

cells per high power field) and normal findings on renal ultrasound.

The main change produced by adopting the new definition of HRS is the removal of a rigid very high cut-off value of sCr (2.5 mg/dL or 220  $\mu$ mol/L) to start pharmacologic treatment. In this way, treatment can be administered early and potentially better efficacy could be achieved.

However, these clinical criteria do not allow differentiation between HRS and parenchymal renal disease, which is extremely important because vasoconstrictors will not be effective and could even worsen the renal dysfunction. Thus, there is a wide interest in developing urinary biomarkers to help in the differential diagnosis of HRS.

## URINARY BIOMARKERS IN AKI

Currently, numerous biomarkers have been assessed in the setting of AKI and liver cirrhosis including neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18, liver-type fatty acid binding protein (L-FABP), kidney injury molecule-1, toll-like receptor 4,  $\pi$ -glutathione S-transferase and  $\alpha$ -glutathione S-transferase<sup>[8]</sup>. Among all of them, current data show that NGAL is the most useful marker. NGAL detects patients with acute tubular necrosis (ATN). On the contrary, NGAL is not helpful to differentiate between pre-renal azotemia and HRS. NGAL urinary levels are much higher in patients with ATN compared to patients with other causes of AKI. Urinary levels of NGAL in ATN were 417  $\mu$ g/L, compared with levels at 30  $\mu$ g/L in pre-renal azotemia, 82  $\mu$ g/L in chronic kidney disease and 76  $\mu$ g/L in HRS,  $P < 0.001$ <sup>[9,10]</sup>. Thus, incorporating NGAL into the clinical decision algorithm would be of benefit to rule out structural kidney injury and detecting a group of patients in whom treatment with vasoconstrictors wouldn't be effective and only would produce potentially serious side effects<sup>[11]</sup>.

## CURRENT TREATMENT (STANDARD OF CARE)

Once patients with AKI have received volume expansion with albumin (1 g per kilogram) with no response achieved in the following 48 h, and criteria of HRS are fulfilled, then treatment with terlipressin is recommended. Expansion with albumin should be continued at the dose of 20–40 g daily.

Response to treatment should be assessed regularly and terlipressin should be titrated gradually up to a maximum dose of 12 mg per day. Terlipressin should be used for a maximum of 14 d and stopped in case of lack of response<sup>[7]</sup>.

Response is defined as a reduction of at least 25% from baseline sCr level, that is from sCr level before treatment with terlipressin was started<sup>[7]</sup>.

Response is achieved in around 40%–50% of patients. The rate of recurrence of HRS is 30%. A definitive treatment of the circulatory dysfunction and the underlying liver

cirrhosis with liver transplantation should be considered in all cases with no contraindications. Otherwise, the persistent advanced circulatory dysfunction makes HRS recur frequently and predispose the patient to other major decompensations<sup>[12]</sup>. This is the rationale supporting prioritization of patients with HRS on the waiting list for LT in some centres. Terlipressin and albumin is not a definitive treatment but should be considered as a bridge to a definitive treatment, *i.e.*, LT.

Two randomized studies showed that HRS reversal rate when terlipressin plus albumin was employed was higher compared to the reversal achieved employing albumin alone. Martín-Llahí *et al.*<sup>[13]</sup> reported a much higher rate of improvement in renal function in patients treated with terlipressin and albumin compared to those patients treated only with albumin (43.5% vs 8.7%,  $P = 0.017$ ). This result may be influenced by the fact that patients who did not tolerate terlipressin were excluded from the analysis. Sanyal *et al.*<sup>[14]</sup> also showed that HRS reversal was achieved more frequently in those patients treated with terlipressin and albumin compared with those treated only with albumin (33.9% vs 12.5%,  $P = 0.008$ ). Any of these studies showed difference in survival at 3-mo and 6-mo. A large randomized trial has been published recently and it showed a higher rate of HRS reversal in those patients receiving terlipressin (23.7% vs 15.2%,  $P = 0.13$ ). This difference did not reach statistical significance, probably due to the fact that one third of patients received fewer than three days of treatment, which could affect the effectiveness of the treatment. When the analyses were done stratifying patients by the degree of reduction in serum creatinine level, data showed that a decrease in sCr level, even if not reaching a complete reversal, has a positive impact on survival<sup>[15]</sup>.

Traditionally, terlipressin has been used in bolus 0.5–1.0 mg every 4–6 h. Recent data show that continuous infusion of terlipressin has the same efficacy compared with bolus administration and it is better tolerated presenting fewer side effects (35.29% vs 62.16%,  $P < 0.025$ ). Probably, side effects were lower because the total effective daily dose required was lower in the infusion groups compared to the bolus group ( $2.23 \pm 0.65$  mg/d vs  $3.51 \pm 1.77$  mg/d,  $P < 0.05$ )<sup>[16]</sup>.

Therefore, we recommend employing terlipressin at 2 mg per day in continuous infusion (diluted in 250 mL of Dextrose 5%) along with albumin (20–40 g per day). Response should be assessed every 48 h. If response is not achieved in 48 h, then terlipressin dose should be increased in a stepwise manner (increase in 2 mg per day).

These patients need careful observation, including review of ischaemic side effects on acral parts, ischaemic heart events, bowel ischaemia (diarrhoea). They can also develop hyponatremia and arrhythmias.

## PREDICTORS OF RESPONSE TO TERLIPRESSIN AND ALBUMIN

There are only few published studies assessing pre-



dictors of response to treatment in HRS. These studies show there is a close relationship between effectiveness of treatment and capacity to improve systemic hemodynamics. Patients in whom terlipressin did not increase the MAP in at least 5 mmHg at day 3 of treatment had a lower rate of response. Effectiveness of treatment is also related with degree of liver dysfunction. Those patients who did not increase MAP at day 3 and who also had high baseline bilirubin levels  $\geq 171 \mu\text{mol/L}$  (10 mg/dL) had a poor response rate, of only 9%<sup>[17]</sup>. Another study showed that baseline creatinine levels predicted HRS reversal, suggesting that early intervention would be more effective<sup>[18]</sup>. A recent retrospective study showed that those patients with systemic inflammatory response syndrome (SIRS) had a much higher response rate to terlipressin (42.9% vs 6.7%,  $P = 0.018$ ), while terlipressin did not show more efficacy than placebo when employed in patients without SIRS (15.9% vs 18.8%,  $P = \text{NS}$ )<sup>[19]</sup>.

A recent abstract showed that no response to treatment was associated with higher urinary NGAL levels (728.8  $\mu\text{g/L}$  vs 182.9  $\mu\text{g/L}$ ,  $P = 0.02$ ), probably related to the presence of acute tubular necrosis in those patients<sup>[20]</sup>.

In summary, the following markers to predict response to treatment (terlipressin) have been identified: Low baseline creatinine and bilirubin levels, increase in blood pressure, presence of SIRS and low urinary NGAL.

## OTHER TREATMENTS

### LT

Patients with HRS type-1 with no contraindications for a LT should be invariably worked up and place in the LT waiting list because LT is the only definitive treatment for HRS. LT reverses liver dysfunction and portal hypertension. Patients with HRS have worse survival expectancy than other patients with cirrhosis for any given value of MELD score, which suggests HRS is a factor of poor prognosis independently from MELD score<sup>[21,22]</sup>. Furthermore, there is evidence that structural injury to the renal tubules occur early in the course of HRS-1 and the longer the patient is awaiting the transplant and suffering from HRS the higher the risk of not recovering their renal function or even requiring a renal transplant after LT<sup>[23]</sup>. In this sense, experts recommend to prioritize these patients by using pre-treatment levels of creatinine or considering the pharmacological treatment of HRS as haemodialysis when calculating MELD score<sup>[24]</sup>. Currently, there is no general consensus about prioritization of patients with HRS awaiting a LT. Some centres prioritize these patients and some others don't. The major challenge LT programmes face is the shortage of donors and consequently optimization in the allocation of the few organs available becomes extremely necessary. Thus, we suggest that those patients with recurrent episodes of HRS-1, hence at high risk of developing refractory HRS, are at high risk of dropping out of the LT waiting list or at risk of not recovering their renal function after LT, and therefore will

get most benefit from early transplantation.

### Midodrine and octreotide

Combination of midodrine and octreotide (MID/OCT) plus albumin is widely used in countries where terlipressin is not available. A recent randomized trial showed a much lower response rate in patients treated with MID/OCT compared to patients treated with terlipressin (4.8% vs 55.6%,  $P < 0.01$ ). Three-month survival rate, after exclusion of patients who received rescue treatment, was also lower in the MID/OCT group (29% vs 56%,  $P = 0.06$ )<sup>[25]</sup>. These data show midodrine in combination with octreotide is not an effective treatment for HRS.

### Noradrenaline

A recent randomized study comparing noradrenaline with terlipressin showed HRS reversal is achieved in 43.4%, similar to the reversal rate achieved with terlipressin (39.1%). Survival at 15 d of therapy was similar in the noradrenaline and terlipressin group (39.1% vs 47.8%,  $P = 0.461$ )<sup>[26]</sup>. A recent meta-analysis analysed 4 studies including 152 patients and suggested that treatment with noradrenaline is as effective as terlipressin in reversing HRS when used along with albumin<sup>[27]</sup>. Therefore, noradrenaline is an effective therapy for HRS. Noradrenaline main drawback is that its use generally requires an intensive care unit setting.

### Dopamine

Low-dose dopamine increases renal blood flow but shows no effect on glomerular filtration rate or on the outcome in HRS. In a recent study, dopamine didn't show reduction of creatinine levels after 5 d of treatment<sup>[28,29]</sup>. It is not considered an appropriate treatment for HRS.

### Transjugular intrahepatic portosystemic shunt

HRS type-1 usually occurs in the setting of advanced liver dysfunction and transjugular intrahepatic portosystemic shunt (TIPS) is usually contraindicated on this basis. There are few small trials showing improvement on renal function and deactivation of vasoconstrictor system, *i.e.*, reduction in levels of renin, aldosterone and noradrenaline after TIPS insertion<sup>[30,31]</sup>. However, data is very limited to recommend its use in clinical practice.

### Renal and liver replacement therapy

Haemodialysis is employed in those patients awaiting LT whose renal function failed to respond to medical treatment and at the same time bring the extra points required for prioritization.

Liver support with molecular adsorbent recirculating system (MARS) has been tested in small cohorts of patients who did not respond to vasoconstrictors and had advanced liver dysfunction, which usually precludes TIPS insertion. One trial showed the reduction in creatinine and bilirubin levels was higher in the MARS group compared with the continuous haemodialysis group<sup>[32]</sup>. Another study showed no significant changes in systemic

haemodynamics and glomerular filtration rates following MARS treatment<sup>[33]</sup>. Treatments employed at this stage should be restricted to patients awaiting a definitive treatment (*i.e.*, LT). It would be controversial to employ such invasive treatments in patients with contraindication for LT, and thus with no option for a definitive treatment.

### Serelaxin

Serelaxin is a recombinant form of the human peptide hormone relaxin-2, increases renal perfusion in healthy human volunteers. Its properties have been explored in a pilot study on compensated cirrhotic patients and it showed increase renal blood flow by 65.4% from baseline with no effect on systemic blood pressure<sup>[34]</sup>. Data on this hormone is still scarce.

## PREVENTION

HRS can be prevented in different clinical scenarios. The first one is in the setting of SBP. The deleterious effect on circulatory dysfunction produced by SBP can be prevented by volume expansion with albumin. The pioneer study of the Barcelona group showed that those patients receiving albumin prevented development of renal failure (10% vs 33%,  $P = 0.002$ ) and reduced short-term mortality (mortality at 3-mo, 22% vs 41%,  $P = 0.03$ )<sup>[9]</sup>. There are still no convincing data to recommend plasmatic expansion with albumin in patients with other types of infections different from SBP. One trial showed a tendency to develop renal failure less frequently in those patients without renal failure at baseline and receiving expansion with albumin (3% vs 10%,  $P = \text{NS}$ )<sup>[35]</sup>.

HRS can be prevented after LVP, albumin at a dose of 6-8 g per litre of ascites removed is the dose most commonly used to prevent worsening of circulatory dysfunction, and thus minimize the impact on electrolytes, creatinine and renin levels. Volume expansion with albumin also improves survival after LVP and it is recommended by international societies<sup>[36,37]</sup>.

HRS can also be prevented with primary antibiotic prophylaxis of SBP. Fernández *et al.*<sup>[38]</sup> showed in a cohort of patients with advanced cirrhosis that SBP primary prophylaxis reduced development of HRS (28% vs 41%,  $P = 0.02$ ) and mortality at 3 mo (94% vs 62%,  $P = 0.003$ ), this effect is probably related to the effect of Norfloxacin in reducing the levels of bacterial products within the gut and hence reducing bacterial translocation.

## AREAS FOR FUTURE RESEARCH

Definition of HRS is continuously changing and it is based on clinical grounds, relying on serum creatinine levels, which has many limitations as marker of renal function. Research focused on new biomarkers, such as urinary NGAL, to make the diagnostic algorithm of HRS more accurate is clearly needed and fortunately, interest in this field is increasing.

Moreover, identifying patients with low probability of

responding to treatment is of major importance in order to start early alternative treatments and potentially prioritize these patients on the LT waiting list.

Finally, research looking for novel treatments besides intravenous terlipressin and expansion with albumin is also needed.

## CONCLUSION

HRS is a major decompensation in advanced liver cirrhosis. It entails a high short-term mortality rate. Current definition is based on clinical grounds and has been recently modified adopting AKI definition. Recent data on urinary NGAL show it is useful to differentiate acute tubular necrosis and should be incorporated in the diagnostic algorithm of HRS. Terlipressin and noradrenaline are the only effective treatment currently available and reversal rate is only 40%-50% of cases. Data on predictors of response to treatment suggest that treatment should be started as early as possible. In this sense, ICA new definition of HRS allows an early diagnosis. New treatments should be tested for this life-threatening condition. Finally, LT is the only curative treatment and should be always considered.

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## Clinical features, histology, and histogenesis of combined hepatocellular-cholangiocarcinoma

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### Abstract

Combined hepatocellular-cholangiocarcinoma (CHC) is a rare tumor with poor prognosis, with incidence ranging from 1.0%-4.7% of all primary hepatic tumors. This entity will be soon renamed as hepato-cholangiocarcinoma. The known risk factors for hepatocellular carcinoma (HCC) have been implicated for CHC including viral hepatitis and cirrhosis. It is difficult to diagnose this tumor pre-operatively. The predominant histologic component within the tumor largely determines the predominant radiographic features making it a difficult distinction. Heterogeneous and overlapping imaging features of HCC and cholangiocarcinoma should raise the suspicion for CHC and multiple core biopsies (from different areas of tumor) are recommended before administering treatment. Serum tumor markers CA19-9 and alpha-fetoprotein can aid in the diagnosis, but it remains a challenging diagnosis prior to resection. There is sufficient data to support bipotent hepatic progenitor cells as the cell of origin for CHC. The current World Health Organization classification categorizes two main types of CHC based on histo-morphological features: Classical type and CHC with stem cell features. Liver transplant is one of the available treatment modalities with other management options including transarterial chemoembolization, radiofrequency ablation, and percutaneous ethanol injection. We present a review paper on CHC highlighting the risk factors, origin, histological classification and therapeutic modalities.

**Key words:** Combined hepatocellular-cholangiocellular carcinoma; Hepatocellular carcinoma; Cholangiocellular carcinoma; Hepatic progenitor cell(s); Histogenesis; Classification

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**Core tip:** Combined hepatocellular-cholangiocarcinoma is a rare tumor with ambiguous data in literature in

relation to its clinical features, histogenesis, pathological classification and prognosis. The goal of our study was to review the literature and highlight the new updates on this entity.

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## INTRODUCTION

Combined hepatocellular-cholangiocarcinoma (CHC) is a rare tumor, with variation reported from 1.0%-4.7% of all primary hepatic tumors in series of patients undergoing hepatic resection<sup>[1-6]</sup>, although accurate incidence is not known. CHC has been known with several nomenclatures in the literature including mixed hepatocellular carcinoma-cholangiocarcinoma (HCC-CC), hybrid HCC-CC or combined liver and bile duct carcinoma<sup>[7]</sup>. Some of the more common risk factors for CHC mentioned in the literature are hepatitis B virus (HBV) infection, male predominance, cirrhosis and hepatitis C virus (HCV) infection<sup>[8-12]</sup>. Molecular evidence supports that the hepatic progenitor cell (HPC) is the cell of origin for CHC. On histology, it is divided into two main subtypes-classical type and subtypes with stem cell features (further discussed in histology section)<sup>[13]</sup>. Separate HCC and CC in the same liver does not classify as CHC. We present a review of current understanding of clinical features, histogenesis and histology of the combined hepatocellular-cholangiocarcinoma that will be soon renamed as hepato-cholangiocarcinoma.

## CLINICAL FEATURES AND RISK FACTORS

Owing to the rarity of CHC, the majority of the series describing clinical features and prognostic factors consist of retrospective studies from single institutions encompassing small patient populations with little statistical power. This is further compounded by inconsistent histologic inclusion criteria and definition of CHC across the studies, with many series including collision tumors and separate nodules of HCC and CC as CHC. Thus, it is not surprising that identification of clinical features and prognostic factors are not consistent or reproducible among the different studies. A clear profile of the patient demographic afflicted by this rare primary hepatic malignancy has remained vague and is highly dependent on geographic region.

Etiology and risk factors for these tumors may be common or differ in different regions in eastern and western series. This reflects variability in the prevalence of infectious agents such as hepatitis viruses and liver flukes, as well as the lifestyle and nutritional differences.

Multiple studies have highlighted risk factors such as male gender, cirrhosis, hepatitis infection, family history of liver cancer, heavy alcohol consumption and diabetes mellitus<sup>[4,8-11,14-16]</sup>. The high male: Female ratio and prevalence of HBV in CHC patients in Asian countries are generally more similar to HCC compared to CC<sup>[15,16]</sup>. On the other hand, western studies have shown a less pronounced male predominance of CHC, paralleling with relatively low prevalence of HBV (15%-16.6%) and high prevalence of HCV<sup>[1,4,12,17]</sup>. Taken together, these findings suggest that geographical characteristics heavily influence clinical profiles of patients with CHC. This demonstrates that CHC is associated with overlapping clinical features of both HCC and CC. Some studies have reported that CHC has poor prognosis and more aggressive behavior in comparison to HCC and CC<sup>[18-20]</sup>, which some authors attribute to increased lymph node involvement<sup>[8]</sup>.

## IMAGING CHARACTERISTICS AND PRE-OPERATIVE DIAGNOSIS

Historically, CHC has been an elusive and difficult pre-operative diagnosis. This is due to its heterogeneous imaging characteristics with overlapping features of both HCC and CC. The predominant histologic component within the tumor largely determines the predominant radiographic features. This is also true in tissue specimens, as sampling error (e.g., sampling only the area of HCC or CC within a CHC) may also lead to an erroneous pre-operative diagnosis. Thus, the majority of CHC cases in the literature were initially misdiagnosed as either HCC or CC and the proper diagnosis was only reached in the surgical resection specimens. Correct pre-operative diagnosis is important, especially distinction from HCC, as it may determine different management strategy. In the United States the vast majority of HCCs are diagnosed based on characteristic radiological features alone without pathologic confirmation. HCC in selected patients is an indication for liver transplant, with excellent outcomes equivalent to non-neoplastic entities and 5-year survival > 70%<sup>[21]</sup>. Taking into account the scarcity of grafts available for transplantation and the poor prognosis associated with CHC, differentiation from HCC becomes paramount.

The characteristic features of HCC on contrast enhanced CT and MRI are arterial phase diffuse enhancement, portal venous washout, and an enhanced pseudocapsule on delayed imaging. The hallmark radiological findings of CC are arterial peripheral rim enhancement with progressive fibrous stroma central enhancement, dilation of the biliary system, and retraction of the capsule<sup>[22]</sup>. CHC may show all of these radiographic characteristics to varying degrees, making distinction from HCC and particularly from CC very challenging. Some authors have suggested that the presence of heterogeneous or overlapping imaging features should prompt an extended tissue biopsy from different appearing tumor areas to aid in this diagnostic

conundrum and mitigate sampling bias<sup>[22]</sup>. Another clue that should raise suspicion for CHC pre-operatively is discordant tumor markers. Generally, elevated alpha-fetoprotein (AFP) levels are associated with HCC, while elevated CA 19-9 levels are associated with CC. If a tumor shows characteristic imaging features of HCC, but is associated with elevated CA 19-9 levels, or if a tumor has characteristic CC imaging features and is associated with elevated AFP levels, or if both serum markers are elevated, biopsy for pathologic confirmation should be strongly considered.

## HISTOGENESIS

The concept of cancer stem cells may explain the origin and progression of different kinds of cancers, and CHC is no exception. Although histogenesis of the CHC has been a topic of debate, three types of tumor origins have been hypothesized: (1) collision tumors; (2) de-differentiation or re-differentiation of a primary HCC into a biliary phenotype or vice versa; (3) derivation from bipotent HPC<sup>[5,23]</sup>. The first theory, collision tumor consisting of separate populations of HCC and CC occurring in the same liver without intimate relationship, does not qualify as CHC. De-differentiation or redifferentiation of HCC or CC into the other component is controversial, as some studies have shown differences in the clinical features, histology and molecular genetics of CHC and HCC/CC while others have supported this theory owing to existing similarities between CHC and HCC as well as CHC and CC.

The bipotent HPC is a stem cell that differentiates into both hepatocytes and bile duct epithelial cells, and is suspected to be the cancer stem cell responsible for CHC growth<sup>[5,23-26]</sup>. Theise *et al.*<sup>[23]</sup> described four primary liver cancers with three different components including hepatocellular, cholangiocellular and a third component of small or undifferentiated cells (oval-like cells) with high N/C ratio, scant basophilic cytoplasm and nuclear pleomorphism. Oval cells (described in animal models) or intermediate cells (in humans) or stem cells can be found in regenerative nodules. These cells are located in canals of Hering<sup>[23,27]</sup> and can differentiate bidirectionally into hepatocytes and cholangiocytes, also called as bipotent progenitor cells<sup>[5,23,24]</sup>. There is morphological and immunohistochemical similarity between these oval-cell like progenitors and hepatoblasts (both positive for CK19 and Hep-Par1).

HPC and stem cell markers which have been studied in relation to CHC include CD133, CD90, CD44, epithelial cell adhesion molecule (EpCAM), nuclear cell adhesion molecule (NCAM/CD56), OV6, CD13, c-kit, YAP1, SALL4 and Delta-like 1 homolog (DLK-1)<sup>[5,28-33]</sup>. Kim *et al.*<sup>[24]</sup> demonstrated that c-kit-positive HPCs have a potential to differentiate into both hepatocytes and cholangiocytes and are neoplastic counterparts of HPCs.

Evidence including identification of HPC-like cells merging with HCC and CC components, as well as shared expression of HPC markers in the different components,

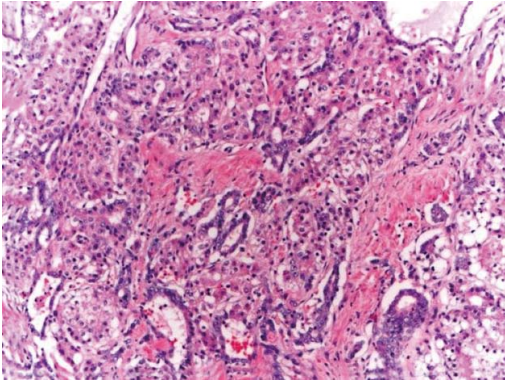
supports these cells as the origin for CHC<sup>[23,25]</sup>. Furthermore, inoculation of cells from a CHC cell line positive for the HPC marker EpCAM has been associated with development of CHC in mice<sup>[26]</sup>. HPC activation in non-tumor liver in CHC cases has been linked with recurrence and poor prognosis in CHC<sup>[34]</sup>. Microdissection has shown that both components share a single clonal background, which is consistent with the shared origin of both components deriving from HPCs<sup>[35]</sup>.

The role of the HPC is reflected in the current World Health Organization (WHO) classification, which is subdivided into CHC, classical type and three subtypes of CHC with stem cell features<sup>[13]</sup>. In making this classification, the authors noted that it was uncertain whether biological differences existed between these subtypes, and determination of subtype relied mainly on histological and immunohistochemical features. HPC marker expression has been shown to varying degree in all stem cell subtypes and to a lesser degree in the classical subtype, while prominent in transitional areas of CHC<sup>[24,29-31]</sup>. Ikeda *et al.*<sup>[30]</sup> showed that the stem cell markers DLK-1 and NCAM/CD56 were expressed most frequently in CHC with stem cell features, and were most frequently expressed in typical and cholangiocellular subtypes. Akiba *et al.*<sup>[29]</sup> showed that stem cell markers CD133 and EpCAM were more often expressed in CHC with stem cell features compared to those with classical features. They further showed that among CHC subtypes with stem cell features, cholangiocellular subtype more often expressed CD133 and EpCAM in comparison to intermediate subtype (their study did not include sufficient cases of typical subtype for statistical analysis)<sup>[29]</sup>. Komuta *et al.*<sup>[36]</sup> supported origin of cholangiocellular carcinoma from HPCs that was initially a subtype of cholangiocarcinoma.

Molecular studies have shown that CHC shares some traits with HCC and others with CC, confirming its status as a distinct entity. Gene profiling of CC, HCC and CHC by microarray shows increased differential expression in CC vs HCC as compared to CC vs CHC, reflecting this concept<sup>[37]</sup>. Analysis of copy number changes in CC and HCC components of CHC showed concordance in the overall trend of gain or loss for several target genes although magnitude of copy number change differed. The copy number gains in the CC component were likely to be paired with a similar but not identical copy number gain in the HCC component of the tumor, with the same holding true for copy number losses. The specific genes most often amplified in this study were MYC, ADAMTSL4, TM4SF1 and CUL4A, which are each associated with HCC although CUL4A has also been associated with CC<sup>[38]</sup>. Similarly, comparative genomic hybridization showed specific chromosomal gains and losses similar to those of HCC<sup>[39]</sup>. This study also showed high prevalence of chromosomal imbalances similar to those seen in CC. Similarly, a high level of chromosomal instability, in addition to recurrent loss of heterozygosity at 3p and 14q is also noted<sup>[40]</sup>.

Genome-wide transcriptional analysis of 20 CHC cases





**Figure 1** Representative picture classic type combined hepatocellular-cholangiocarcinoma, hematoxylin and eosin, 10 × - intermediate areas with both hepatocytic and cholangiocytic components.

showed that CHC clustered with CC and separately from HCC, with upregulated signaling pathways of TGF $\beta$  and Wnt similar to those seen in CC. The TGF $\beta$  pathway upregulated in CHC recalled the key role of fibrosis and extracellular matrix remodeling in CC, and the Wnt pathway signature was similar to that seen in biliary ductal morphogenesis. However, CHC also clustered with a subset of poorly differentiated HCC with progenitor cell features, as would be expected given the postulated HPC origin of CHC, while CHC showed repression of the transcription factor HNF4A associated with mature hepatocyte differentiation<sup>[41]</sup>. Likewise it is also shown that CHCs were clustered with CC by gene expression profiling<sup>[42]</sup>. A recent whole genome sequencing analysis showed that genome-wide substitution patterns in liver cancers of biliary phenotype (both CC and CHC) overlapped with those of HCC in cases associated with chronic viral hepatitis, while biliary cancers (mostly CC in this study) unrelated to chronic hepatitis differed from HCC<sup>[43]</sup>. TERT promoter mutations, for example, were common in CHC and in other hepatitis-related cancers. Carcinogenesis arising from HBV acts largely through the HBV X protein that promotes HPC tumorigenesis so it is possible that these tumors may share a similar pathogenesis<sup>[44]</sup>.

## HISTOLOGY

### Classification

There are multiple classifications for CHC in the literature. Allen and Lisa<sup>[7]</sup> made the first histological classification for CHC in 1949. They described three subtypes. Type 1 consisted of discrete foci of HCC and CC. Type 2 had contiguous masses with features of both HCC and CC. Type 3 was described as a solitary mass comprising of both components. Goodman *et al.*<sup>[45]</sup> in 1985 proposed another classification, also encompassing three subtypes: Type 1 or collision tumor with separate and colliding areas of HCC and CC in the same liver; type 2 or transitional tumor with transitional areas with intimate intermingling of two components with actual transition of HCC elements to CC elements in the same tumor; and type 3

or mucin producing fibrolamellar tumor. Allen and Lisa<sup>[7]</sup>'s type 3 and Goodman *et al.*<sup>[45]</sup>'s type 2 has similar features to the current WHO criteria for CHC. The current edition of the WHO classification describes two main types of CHC: Classical type and CHC with stem cell features. The stem-cell features type is further divided into 3 subtypes: Typical subtype, intermediate cell subtype and cholangiolocellular subtype<sup>[13]</sup>. Representative images of each subtype highlighting histological features and immunohistochemical profile are shown in Figures 1-4.

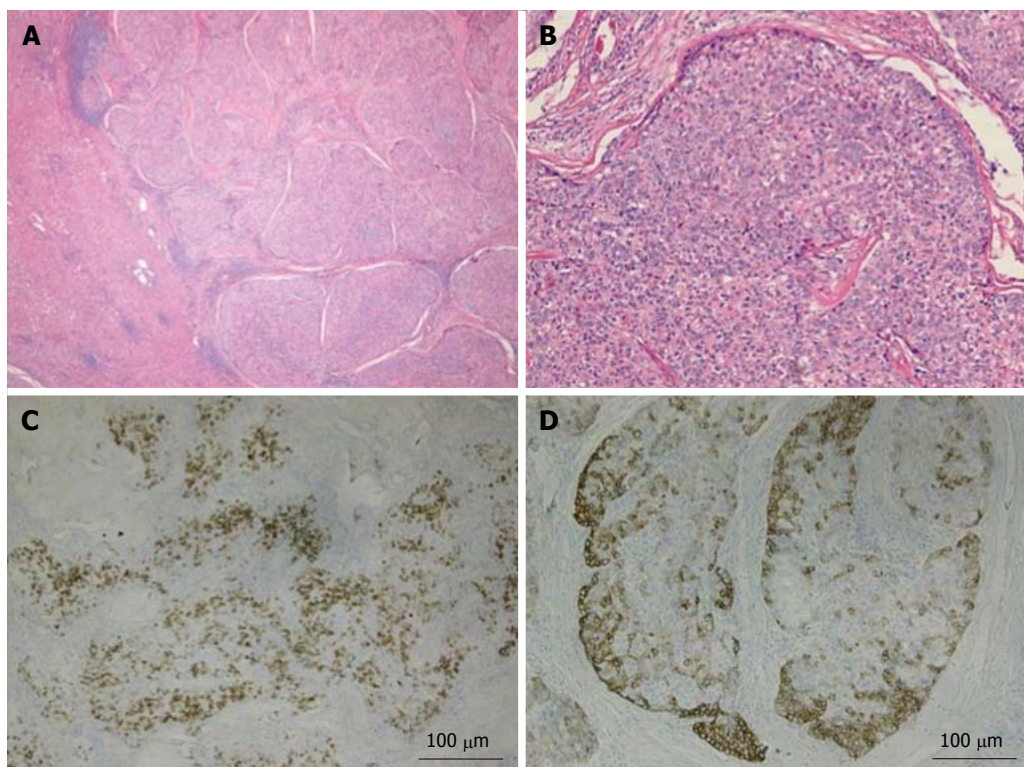
### Histopathology

WHO 2010 classification defines these tumors as histological demonstration of unequivocally differentiated hepatocellular and biliary components in the tumor with intermingling of the two components<sup>[13]</sup>. Collision tumor, which is a separate entity, consists of HCC and CC occurring in the same liver without intimate relationship and is not categorized as CHC. The definite diagnosis of CHC can be made by histology only along with use of IHC and special stains<sup>[5]</sup>. The diagnosis of CHC is a challenging diagnosis on core biopsy as it depends on the area sampled<sup>[46]</sup>. Hepatocytic differentiation is defined by bile production, Mallory-Denk bodies, alpha-1 antitrypsin globules and trabecular arrangement of tumor cells. The cholangiocarcinoma component is appreciated by mucin production, prominent desmoplastic stroma and glandular structures. Combined fibrolamellar HCC in combination with cholangiocellular component has also been described in the literature<sup>[45,47]</sup>.

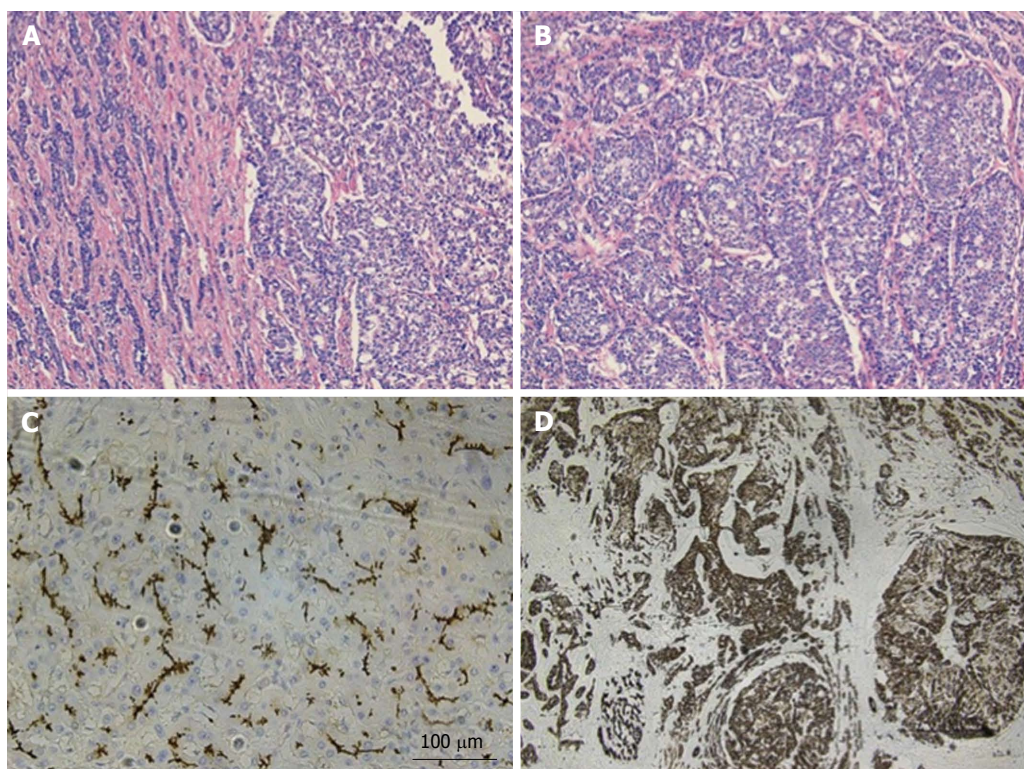
In the CHC classic type (Figure 1), both the hepatocytic and cholangiocarcinoma components are present and can vary from well to poorly differentiated<sup>[13]</sup> with intermediate areas demonstrating features of both the components. Hepatocytic component is usually represented by thickened trabeculae composed of polygonal cells with abundant granular eosinophilic cytoplasm and scant stroma while cholangiocarcinoma component has gland formation with low cuboidal/columnar cells and dense fibrotic stroma. HCC with pseudoglandular pattern and expression of CK7/CK19 is not classified as CHC<sup>[5]</sup>.

The first subtype of CHC with stem cell features, the typical subtype (Figure 2A and B), is characterized by peripheral small cells with hyperchromatic nuclei and a high nuclear to cytoplasmic ratio with nests of mature appearing hepatocytes in the center. The intermediate cell subtype (Figure 3A and B) consists of tumor cells with intermediate features between hepatocytes and cholangiocytes. Kim *et al.*<sup>[24]</sup> described these tumor cells as small and oval shaped with hyperchromatic nuclei and scant cytoplasm arranged in either trabeculae, solid nests or strands, present within a desmoplastic stroma. Well-formed glands are not seen but ill-formed gland like structures may be present. These tumors were initially termed as intermediate carcinomas (hepatocyte-cholangiocyte) as these had features that were intermediate between HCC and CC<sup>[13,24]</sup>. The cholangiolocellular subtype (Figure 4A and B) is characterized



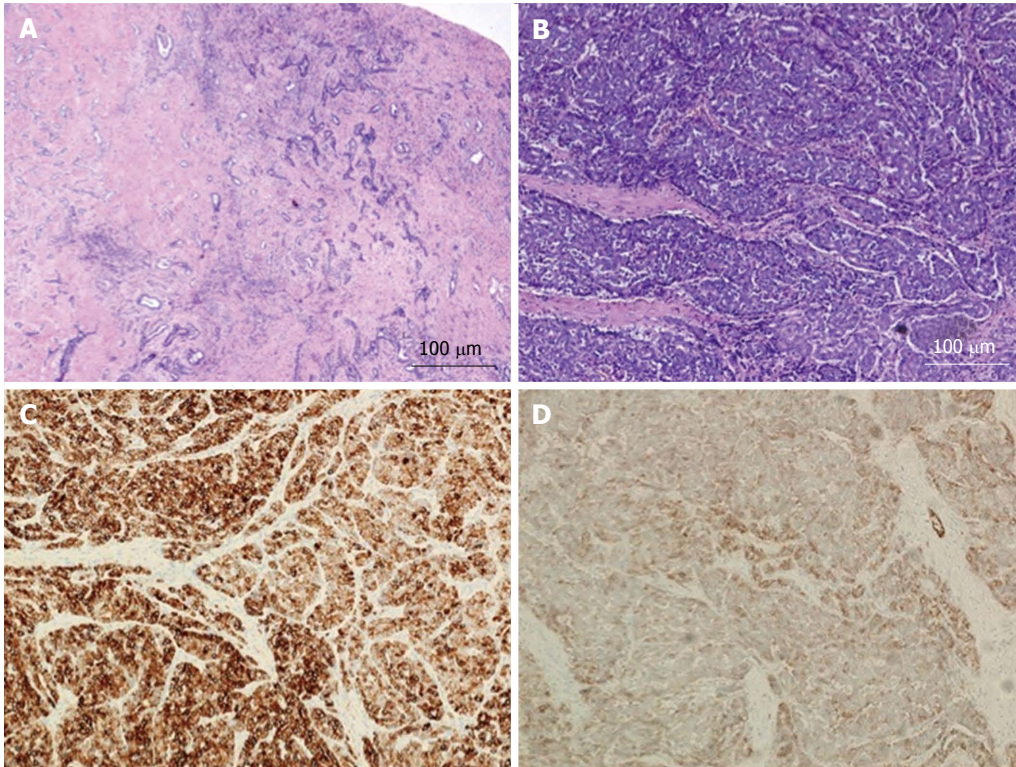


**Figure 2 Combined hepatocellular-cholangiocarcinoma with stem cell features, typical subtype.** A: H and E, 4 × - tumor nests present on the right side with non-neoplastic liver on the left side; B: H and E, 10 × - peripheral small cells with hyperchromatic nuclei with mature appearing hepatocytes in the center; C: CK7, 4 × - scattered expression of CK7 by tumor cells; D: CK19, 4 × - patchy staining of the tumor and highlighting small tumor cells located at the periphery. Tumor was also positive for Hep-Par1 (not shown). H and E: Hematoxylin and eosin.



**Figure 3 Combined hepatocellular-cholangiocarcinoma with stem cell features, intermediate subtype.** A: H and E, 4 × - tumor is present in trabecular/nested pattern on the right side with ill-formed gland like structures seen on the left side; B: H and E, 10 × - tumor cells with intermediate features between hepatocytes and cholangiocytes; C: CD10, 10 × - tumor showing canalicular staining pattern for CD10 (hepatocytic marker); D: CK19, 4 × - tumor cells strongly and diffusely expressing CK19 (cholangiocytic marker). Focal tumor cells were positive for Hep-Par1 and CD56 (not shown). H and E: Hematoxylin and eosin.





**Figure 4 Combined hepatocellular-cholangiocarcinoma with stem cell features, cholangiocellular subtype.** A: H and E, 4 × - tumor cells present in tubular, anastomosing (antler-like) pattern; B: H and E, 10 × - small hyperchromatic tumor cells with high nuclear to cytoplasmic ratio present within dense fibrous stroma; C: CK7, 10 × - tumor is diffusely positive for CK7; D: CD56, 10 × - CD56 staining the cholangiolocellular component as well as the tumor cells at the periphery of the trabeculae. The tumor was diffusely positive for CK19 while negative for HepPar-1 and AFP (not shown). H and E: Hematoxylin and eosin.

by small cells with a high nuclear to cytoplasmic ratio and hyperchromatic oval shaped nuclei arranged in a tubular, cord like, anastomosing pattern (also referred to as an “antler-like” pattern) within a dense fibrous stroma. No significant cellular atypia or evidence of mucin production is seen in both intermediate and cholangiolocellular subtypes<sup>[13]</sup>. Although there are distinct histological features described by the WHO to classify all these types/subtypes, there is no mention of percentage of stem cell area required to categorize CHC with stem cell features. If the stem cells predominate, the tumor is classified as one of the subtype of CHC with stem cell features depending on the histologic appearance. It appears that CHCs with less than 5% stem cell area have better prognosis than those with stem cell areas greater than 5%<sup>[30]</sup>. Sasaki *et al.*<sup>[48]</sup> proposed certain clinico-pathological findings for CHC with stem cell features. The intermediate subtype was more commonly associated with female patients, larger tumor size, higher histological grade of HCC component, and less fibrosis while cholangiocellular subtype had smaller tumor size and lower histological grade of HCC. The typical subtype has less inflammation in comparison with the cholangiocellular subtype<sup>[48]</sup>.

On cytology specimens, diagnosis of CHCs can be challenging<sup>[49,50]</sup>. Cell blocks and immunohistochemical stains can prove helpful in reaching a correct diagnosis of CHC. With CHC being an uncommon tumor, arriving at a diagnosis and classification of CHC can be difficult with histology alone.

### Special and immunohistochemical stains

Immunohistochemical stains are required for demonstrating hepatic and biliary phenotypes. The hepatocellular component is positive for HepPar1, pCEA, CD10 and glypican<sup>[5]</sup>. The CC component shows expression of CK7, CK19 and mucin/mucicarmine although HCC components can also express CK7 and CK19<sup>[5]</sup>. Mucin is essential to demonstrate the biliary component<sup>[51]</sup>. CAM 5.2 and AE1 can also be useful to differentiate between HCC and CC component; HCC will be positive for CAM5.2 while AE1 will be positive in CC component<sup>[49]</sup>. CHC with stem cell features expresses stem cell markers including c-kit, NCAM, and EpCAM. Kim *et al.*<sup>[32]</sup> demonstrated similar expression of these stem cell markers in CHC and HCC. Oval cell-like progenitors or small cells (or HPCs) described originally by Theise *et al.*<sup>[23]</sup> are focally positive for AFP and alpha-antitrypsin while negative for Hep-Par1, c-Kit, vimentin and CHR-A. CD44, which is one of the cancer stem cell markers, is associated with poor prognosis and early recurrence in patients with CHC<sup>[32]</sup>. DLK1 is another marker of HPCs in adult liver<sup>[30]</sup>. Survival of patients with high expression of DLK1 is worse<sup>[30]</sup> suggesting that patients with CHC with stem cell features do worse in comparison to classical type CHC.

CHC with stem cell features, typical subtype stains positively with CK7, CK19 (Figure 2C and D), NCAM1/CD56, cKIT and/or EpCAM.

The intermediate subtype that is characterized by

intermediate cells (between hepatocytes and cholangiocytes), shows simultaneous expression of hepatocyte and biliary markers (Figure 3C and D). Akiba *et al.*<sup>[52]</sup> demonstrated that intermediate cells stain better with Arginase-1 and CK8. Biliary phenotypes (CK7 and CK19) are more commonly positive in intermediate subtype than Hep-Par1<sup>[29]</sup>.

The cholangiolocellular subtype is positive for CK19, and stem cell markers- cKIT, NCAM1/CD56 and EpCAM (Figure 4C and D).

## TREATMENT OPTIONS

Currently, minor or major hepatic resection, with or without lymph node dissection, is the consensus recommended treatment for CHC. However, the absence of randomized prospective studies precludes the determination of optimal management strategies in these patients.

The role of liver transplant in the treatment of CHC remains to be defined. Most of the data on transplanted CHC patients comes from patients that were initially misdiagnosed with HCC or from incidentally discovered tumors in the explanted livers. The outcome data of this cohort is mixed, although outcomes are consistently worse when compared with HCC due to associated higher recurrence rates after transplant in CHC<sup>[53,54]</sup>. A recent study found no survival benefit of transplant over resection in CHC, with 3-year overall survival of 48% and 46% ( $P = 0.56$ ), respectively<sup>[55]</sup>. Interestingly, a few studies have reported favorable outcomes in CHC patients undergoing transplantation. Chan *et al.*<sup>[56]</sup> reported three cases, with two of them alive without recurrence 25 and 35 mo post-transplant. The other patient died of metastatic disease 16.5 mo after transplant. Another more recent publication found that transplanted CHC patients have better 5-year overall survival than those treated with major resection (41.1% vs 28.1%,  $P = 0.039$ )<sup>[57]</sup>. However, the 5-year overall survival rate of transplanted CHC patients was still much worse than transplanted HCC patients in both studies (41.1% vs 67%,  $P < 0.001$  and 48% vs 78%,  $P = 0.01$ )<sup>[55,57]</sup>. These findings were further supported by Vilchez *et al.*<sup>[12]</sup> in their UNOS database analysis. They found an overall 5-year survival of 40% in transplanted CHC patients, which was similar to CC (47%) but much worse than HCC (62%,  $P = 0.002$ ). The authors concluded that currently, liver transplant is not a viable option for these patients. It should be emphasized that these results may be influenced by the fact that majority of CHC patients are misdiagnosed pre-operatively and managed as HCC. Improved initial diagnostic accuracy may allow for optimization and more aggressive neo-adjuvant therapies for CHC patients. This may in turn improve outcomes in this group and make transplant a viable option in the future.

Other treatment modalities that have been reported in CHC include transarterial chemoembolization, radio-frequency ablation, and percutaneous ethanol injection, but the data regarding the benefits of these interventions

are inconclusive. The role of chemotherapy and radiotherapy remains to be defined.

## PROGNOSIS

Despite the ambiguity and discordance of CHC clinical features reported in the literature, the studies consistently supported that CHC is associated with a more aggressive course and a worse prognosis than HCC. With regards to CC, the studies are varied with some showing CHC to have a worse<sup>[9]</sup> or similar prognosis while others show an improved outcome. Overall most studies showed that the prognosis of CHC is grim. Reported 3-year and 5-year overall survivals range from (37.3%, 47%, 46%, 12.4%, 10.5%, 34.6%) and (9.2%, 40%, 32%, 23.1%, 33%), respectively<sup>[8-12,55,57,58]</sup>.

Adverse clinicopathologic prognostic factors associated with increased tumor recurrence and worse survival in various studies include large tumor size ( $> 5$  cm), presence of satellite nodules, lymph node involvement, multifocality, vascular invasion, portal vein invasion, high tumor stage, high levels of CA 19-9, decreased capsule formation, free surgical resection margins  $< 2$  cm, and GGT levels  $> 60$  U/L<sup>[11,58,59]</sup>. However, many of these factors did not reach statistical significance on multivariate analysis. This may be due to the retrospective nature and low number of patients within each study due to the low incidence of CHC.

A recent population level study analyzed the SEER database for patients diagnosed with CHC between 1988-2009 in the United States. Of the 465 cases studied, they founded that a majority of CHC patients were male (66%) and Caucasian (74.9%), and a plurality were 66 years or older (44.3%)<sup>[57]</sup>. Clinical features of CHC patients fell in between those for HCC and CC, suggestive of the mixed characteristics associated with this tumor and in concordance with its bi-phenotypic differentiation. The authors found that CHC had a worse overall survival when compared to HCC, but better when compared with CC. The reported 5-year overall survival and disease specific survival for HCC, CHC, and CC were 11.7%, 10.5%, 5.7% and 21%, 17.8% and 11.9%, respectively ( $P < 0.001$ ). The authors of the study concluded that CHC patients have intermediate clinical characteristics, demographics, and prognosis when compared with HCC and CC patients. Another study compared the post-resection outcomes of CHC, HCC and CC and found no significant differences in tumor recurrence rates, but did find worse survival rates when compared to HCC. However, results of this study are not representative of true CHCs as the authors used the Allen and Lisa<sup>[7]</sup> classification. The majority of patients in this study were classified as "combined type" or type 2 of the Allen and Lisa classification, which is not currently considered a true CHC according to the WHO classification<sup>[6]</sup>.

The intermediate biological behavior of CHC has been further supported by other studies. Multiple series have found clinico-pathologic features in CHC that are commonly associated with either HCC or CC. These



include a high rate of lymph node metastasis, commonly associated with CC, and vascular invasion and portal vein invasion, commonly associated with HCC. Reported rates of lymph node metastasis in CHC cases are as high as 42%<sup>[8]</sup>. This may be explained by the biphenotypic nature of these malignancies<sup>[9]</sup>.

In summary, CHC is a rare tumor with bad prognosis and overlapping clinical and radiological features with HCC and CC. More studies are required to adequately define its histogenesis including molecular genetics of this tumor.

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Retrospective Study

## Fibrosis assessment using FibroMeter combined to first generation tests in hepatitis C

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## Abstract

### AIM

To evaluate the performance of FibroMeter<sup>Virus3G</sup> combined to the first generation tests aspartate amino-transferase-to-platelet ratio index (APRI) or Forns index

to assess significant fibrosis in chronic hepatitis C (CHC).

## METHODS

First generation tests APRI or Forns were initially applied in a derivation population from Rio de Janeiro in Brazil considering cut-offs previously reported in the literature to evaluate significant fibrosis. FibroMeter<sup>Virus3G</sup> was sequentially applied to unclassified cases from APRI or Forns. Accuracy of non-invasive combination of tests, APRI plus FibroMeter<sup>Virus3G</sup> and Forns plus FibroMeter<sup>Virus3G</sup> was evaluated in the Brazilian derivation population. APRI plus FibroMeter<sup>Virus3G</sup> combination was validated in a population of CHC patients from Angers in France. All patients were submitted to liver biopsy staged according to METAVIR score by experienced hepatopathologists. Significant fibrosis was considered as METAVIR  $F \geq 2$ . The fibrosis stage classification was used as the reference for accuracy evaluation of non-invasive combination of tests. Blood samples for the calculation of serum tests were collected on the same day of biopsy procedure or within a maximum 3 mo interval and stored at  $-70^{\circ}\text{C}$ .

## RESULTS

Seven hundred and sixty CHC patients were included (222 in the derivation population and 538 in the validation group). In the derivation population, the FibroMeter<sup>Virus3G</sup> AUROC was similar to APRI AUROC (0.855 *vs* 0.815,  $P = 0.06$ ) but higher than Forns AUROC (0.769,  $P < 0.001$ ). The best FibroMeter<sup>Virus3G</sup> cut-off to discriminate significant fibrosis was 0.61 (80% diagnostic accuracy; 75% in the validation population,  $P = 0.134$ ). The sequential combination of APRI or Forns with FibroMeter<sup>Virus3G</sup> in derivation population presented similar performance compared to FibroMeter<sup>Virus3G</sup> used alone (79% *vs* 78% *vs* 80%, respectively,  $P = 0.791$ ). Unclassified cases of significant fibrosis after applying APRI and Forns corresponded to 49% and 54%, respectively, of the total sample. However, the combination of APRI or Forns with FibroMeter<sup>Virus3G</sup> allowed 73% and 77%, respectively, of these unclassified cases to be correctly evaluated. Moreover, this combination resulted in a reduction of FibroMeter<sup>Virus3G</sup> requirement in approximately 50% of the entire sample. The stepwise combination of APRI and FibroMeter<sup>Virus3G</sup> applied to the validation population correctly identified 74% of patients with severe fibrosis ( $F \geq 3$ ).

## CONCLUSION

The stepwise combination of APRI or Forns with FibroMeter<sup>Virus3G</sup> may represent an accurate lower cost alternative when evaluating significant fibrosis, with no need for liver biopsy.

**Key words:** Chronic hepatitis C; Fibrosis; Liver biopsy; Non-invasive methods; FibroMeter<sup>Virus3G</sup>; Combination algorithms

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**Core tip:** Liver fibrosis assessment still poses a challenge

when prioritizing hepatitis C treatment due to logistical and financial barriers in the use of direct acting antiviral drugs. We introduced a new stepwise combination of first generation fibrosis tests - aminotransferase-to-platelet ratio index (APRI) and Forns-followed by FibroMeter<sup>Virus3G</sup> whenever results remained unclassified after first generation tests to identify significant fibrosis. This combination presented similar accuracy to FibroMeter<sup>Virus3G</sup> used as the only test, reduced APRI and Forns grey zone, and spared FibroMeter<sup>Virus3G</sup> requirement in 50% of cases. This approach represents a lower-cost alternative to assess fibrosis, with no need for liver biopsy.

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## INTRODUCTION

Fibrosis staging in chronic hepatitis C (CHC) has evolved in recent years with the introduction of blood tests for liver fibrosis as well as physical methods such as elastometry. Although liver biopsy has been classically considered the standard tool to evaluate fibrosis, it presents well-known inconveniences<sup>[1-3]</sup> and limitations<sup>[4-7]</sup> which make its use to assess fibrosis staging controversial amongst various authors<sup>[8-11]</sup>. However, even when considering the recent advances in CHC therapy, the diagnosis of significant fibrosis still represents a challenge to define which patients should have priority in treatment, mainly in resource limited countries. Thus, the development and improvement of alternative methods to identify candidates for an early treatment or intensive fibrosis monitoring is still recommended<sup>[11]</sup>. Most of the commonly used first generation non-invasive tests such as aspartate aminotransferase-to-platelet ratio index (APRI)<sup>[12]</sup>, FIB-4<sup>[13]</sup> and Forns index<sup>[14]</sup> have been constructed and evaluated as binary diagnosis tools aiming to predict or exclude significant fibrosis, advanced fibrosis or cirrhosis at specific cut-offs. Although they are all free of charge, easily accessible and well validated for CHC, these non-invasive tests are limited to classify all patients<sup>[12-14]</sup>.

The interest in detailed fibrosis class classification for non-invasive tests of fibrosis has recently grown<sup>[15-18]</sup>, representing a more comprehensive and sophisticated approach to assess liver fibrosis. In this line, FibroMeters are a group of blood tests providing classifications intended to evaluate liver fibrosis in chronic viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease<sup>[19-21]</sup>. FibroMeter dedicated for viral aetiology has recently evolved from FibroMeter<sup>Virus2G</sup><sup>[20]</sup> to a less costly



hyaluronic acid free test FibroMeter<sup>Virus3G[21]</sup>, which discriminates seven different fibrosis classes. FibroMeters provide scores ranging from 0 to 1 which are correlated with METAVIR staging system<sup>[22]</sup>. Although this new non-invasive test represents a better strategy to evaluate fibrosis in CHC, it may signify an economic burden hindering easy access mainly in developing countries. Thus, in order to identify patients with significant fibrosis and optimize costs, we evaluated the performance of a stepwise combination using APRI and Forns followed by FibroMeter<sup>Virus3G</sup> in cases whose results remained unclassified after use of these first generation tests, always considering liver biopsy as reference.

## MATERIALS AND METHODS

### Patients

A cross-sectional study with prospective inclusion of compensated CHC patients submitted to percutaneous liver biopsy was performed at the Federal University of Rio de Janeiro, Brazil, as part of a pre-treatment routine evaluation. This group represented the derivation population of the study. Patients with concomitant human immunodeficiency virus infection, hepatitis B virus, alcohol abuse, metabolic, autoimmune or biliary diseases, liver transplantation or those who had previously undergone antiviral treatment were excluded. The validation population was composed by an independent cohort of CHC patients from Angers in France, who fulfilled the same inclusion and exclusion criteria. All patients signed an informed consent form and the study was approved by the Ethics Committee of both Institutions.

### Liver biopsy

In the derivation population, all consecutive biopsies were guided by ultrasonography using a 14 or 16 G disposable Tru Cut needle (Surecutw, TSK Laboratory, Akasaka, Japan) obtaining a maximum length of 20 mm for each pass. In validation population, Menghini needle was used. Samples were considered inappropriate when length presented < 10 mm or contained < 6 portal tracts. Serial sections 5 µm thick were cut from each paraffin block and routinely stained with hematoxylin and eosin, periodic acid-Schiff diastase, reticulin, Masson Trichrome and Picrosirius red. Liver fibrosis was staged from F0 to F4 according to METAVIR staging system<sup>[22]</sup> by an experienced hepatopathologist in each centre, blinded for biological and clinical results. METAVIR F ≥ 2 was considered significant fibrosis. This fibrosis stage classification was used as the reference for accuracy calculation of non-invasive tests.

### Blood fibrosis tests

Serum tests of fibrosis were performed with blood samples collected from fasting patients on the same day of biopsy procedure or within a maximum 3 mo interval and stored at -70 °C. APRI and Forns were selected due to their free accessibility and their good

accuracy to discriminate significant fibrosis. The values of APRI<sup>[12]</sup>, Forns<sup>[14]</sup> and FibroMeter<sup>Virus3G[21]</sup> tests were calculated according to the original studies: (1) APRI = AST level/ULN/platelet counts (10<sup>9</sup>/L) × 100; (2) Forns index = 7.811 - 3.131 × ln(platelet count) + 0.781 × ln(GGT) + 3.467 × ln(age) - 0.014 × cholesterol; and (3) FibroMeter<sup>Virus3G</sup> = patented formula including the biologic parameters prothrombin index, AST, ALT, Urea, GGT, alpha-2-macroglobulin and platelets. The calculations were performed by Echosens (Paris, France) laboratory.

### Statistical analysis

Quantitative variables were expressed as mean ± SD values or proportions. Student's *t* test or ANOVA were used to compare continuous variables, and McNemar  $\chi^2$  test to compare paired proportions. The performance of APRI, Forns and FibroMeter<sup>Virus3G</sup> to predict significant fibrosis was expressed by the AUROC. In order to evaluate the applicability of FibroMeter<sup>Virus3G</sup>, considering an economic approach, we determined the best cut-off point of FibroMeter<sup>Virus3G</sup> to discriminate significant fibrosis using the Youden index that maximizes sensitivity and specificity. The performance of APRI and Forns were separately assessed to exclude or predict significant fibrosis respectively, at cut-offs already established in literature as follows: APRI: cut-off of 0.5 and 1.5<sup>[12]</sup>; Forns: cut-off of 4.2 and 6.9<sup>[14]</sup>. FibroMeter<sup>Virus3G</sup> was then sequentially tested in a stepwise use, considering the results allocated in unclassified APRI values (between 0.5 and 1.5) and Forns values (between 4.2 and 6.9), using histology as reference. The overall accuracy of the aforementioned approaches was calculated considering the sum of true positive and negative cases as a proportion of the total. Sensitivity, specificity, predictive positive (PPV) and negative values (NPV) of first generation tests and sequential use of APRI + FibroMeter<sup>Virus3G</sup> and Forns + FibroMeter<sup>Virus3G</sup> were evaluated.

Data were analyzed using the statistics software programs SPSS version 20 for Windows and MedCalc version 14.8.1. A *P* value < 0.05 was considered statistically significant.

## RESULTS

### Patients' characteristics

The initial series of liver specimens in Rio population consisted of 231 biopsies, of which four (1.7%) were excluded, due to evidence of other hepatic diseases associated with hepatitis C, and five (2.0%) were considered inadequate for analysis. Thus, the final Rio population included 222 biopsies of CHC patients. The validation cohort was represented by 538 French CHC patients. Excepted for gender, demographic characteristics, laboratory data and histological features of derivation and validation cohort were similar and described in Table 1.

The mean length of liver fragments was 24 ± 5 mm in derivation population vs 22 ± 10 mm in validation cohort (*P* = 0.315). The prevalence of significant fibrosis

**Table 1** Demographic, laboratory and histological features of patients with chronic hepatitis C of both populations

Variables	All ( <i>n</i> = 760)	Rio population ( <i>n</i> = 222)	Angers population ( <i>n</i> = 538)
Females, <i>n</i> (%)	401 (54)	134 (60)	179 (35)
Age (yr, mean ± SD)	46 ± 11	51 ± 11	46 ± 11
AST, IU/L (mean ± SD)	67 ± 58	68 ± 52	66 ± 60
ALT, IU/L (mean ± SD)	101 ± 84	100 ± 67	101 ± 90
Platelet count, 10 <sup>6</sup> /mm <sup>3</sup> (mean ± SD)	208 ± 68	203 ± 63	210 ± 70
GGT, IU/L (mean ± SD)	144 ± 171	124 ± 135	110 ± 184
APRI	1.0 ± 1.2	0.9 ± 1.2	1.1 ± 1.3
Forns		6.0 ± 1.9	
FibroMeter <sup>Virus3G</sup>	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.3
Biopsy length (mm, mean ± SD)	22 ± 9	24 ± 5	22 ± 10
METAVIR stage, <i>n</i> (%)			
F0	22 (3)	5 (2)	17 (3)
F1	283 (37)	91 (41)	192 (36)
F2	215 (28)	55 (25)	160 (30)
F3	145 (19)	54 (24)	91 (17)
F4	95 (13)	17 (8)	78 (14)

AST: Aspartate transaminase; ALT: Alanine aminotransferase; GGT: Glutamyl transpeptidase; APRI: Aspartate transaminase to platelet ratio index.

**Table 2** Rates of correct FibroMeter<sup>Virus3G</sup> stage classification in comparison to liver biopsy in the Rio population

FibroMeter stage	METAVIR fibrosis classification						Correct fibrosis class classification according to liver biopsy (%)
	0	1	2	3	4	<i>n</i>	
F0/F1	0	10	0	0	0	10	10/10 = 100
F1	0	6	1	0	0	7	6/7 = 86
F1[F1-F2]	3	21	4	2	0	30	25/30 = 83
F2[F1-F2]	1	26	10	4	1	42	36/42 = 86
F2[F1-F3]	1	15	12	10	0	38	37/38 = 97
F2/F3	0	9	17	12	3	41	29/41 = 71
F3[F2-F4]	0	4	8	22	6	40	36/40 = 90
F4[F3-F4]	0	0	3	4	7	14	11/14 = 79
Total	5	91	55	54	17	222	190/222 = 86

was 57% vs 61% ( $P = 0.399$ ) comparing derivation population to validation cohort, respectively, considering liver biopsy as reference.

### Rio (derivation) population

FibroMeter<sup>Virus3G</sup> applied as a class classification test presented an overall rate of correct diagnosis of 86% considering any of the results reported in FibroMeter<sup>Virus3G</sup> stage class in accordance with fibrosis scored by METAVIR (Table 2). The AUROCs of both tests comparing METAVIR F0-F1 vs F2-F4 were similar between FibroMeter<sup>Virus3G</sup> and APRI [0.855 (0.801-0.898) vs 0.815 (0.757-0.864)] but the difference was at the limit of significance ( $P = 0.06$ ). The FibroMeter<sup>Virus3G</sup> AUROC was higher in comparison to Forns AUROC [0.855 (0.801-0.898) vs 0.769 (0.708-0.823);  $P < 0.001$ ]. The best cut-off that predicted significant fibrosis was 0.61, demonstrating an accuracy of 80% compared to liver biopsy. The PPV, NPV and accuracy of all tests were shown in Table 3. The stepwise combination of APRI or Forns followed by FibroMeter<sup>Virus3G</sup> provided an overall accuracy of 79% (Figure 1) and 78% (Figure 2), respectively ( $P = 0.791$ ), when identifying significant fibrosis. It also enabled 49% ( $n = 109$ ) and 54% ( $n = 120$ ) of the total sample, representing the grey zone of APRI and Forns for significant fibrosis, to be correctly classified in 73% and 77% of cases, respectively.

Thus, diagnostic accuracy did not differ comparing the use of FibroMeter<sup>Virus3G</sup> test alone or combined with APRI or Forns (80% vs 79% vs 78%, respectively,  $P = 0.79$ ), but represented a lower cost alternative since this procedure led to a 51% reduction of FibroMeter<sup>Virus3G</sup> test requirement using APRI + FibroMeter<sup>Virus3G</sup> and 46% reduction using Forns + FibroMeter<sup>Virus3G</sup> ( $P = 0.25$ ). Rates of well classified patients applying the algorithm APRI + FibroMeter<sup>Virus3G</sup>, using METAVIR score as reference, were as follows: 100% for F0, 81% for F1, 67% for F2, 80% for F3 and 94% for F4.

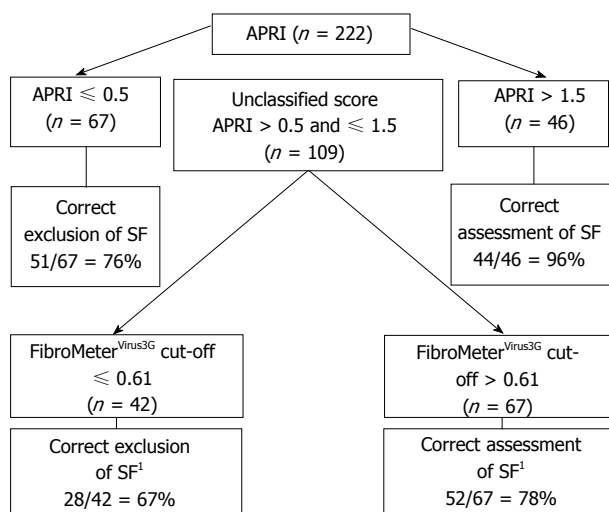
### Angers (validation) population

The cut-off of 0.61 found in derivation population presented an overall accuracy of 75% when discriminating significant fibrosis in the validation cohort in comparison to 80% in derivation population ( $P = 0.13$ ), considering histology as reference. The diagnostic accuracy of APRI + FibroMeter<sup>Virus3G</sup> combination in validation population to detect significant fibrosis and advanced fibrosis was respectively 69% and 74%. Rates of correct classification of this algorithm according to METAVIR score were as follows: 100% for F0, 88% for F1, 39% for F2, 64% for F3 and 85% for F4. When analysing the subgroup of false negative patients in this population we observed that 69% are represented by F2, 23% are F3, and only

**Table 3** Performance of aspartate transaminase to platelet ratio index, Forns, FibroMeter<sup>Virus3G</sup> and combination algorithm to discriminate significant fibrosis (FO-F1 vs F2-F4) in derivation and validation population

Serum fibrosis tests	AUROC (95%CI)	Cut-off value	Se (%)	Sp (%)	PPV (%)	NPV (%)	OA (%)
Derivation population (n = 222)							
FM score	0.855 (0.801-0.898)	0.61	79	81	85	74	80
APRI	0.815 (0.757-0.864)	0.5 <sup>1</sup>	87	53	71	76	72
		1.5 <sup>2</sup>	35	98	96	53	62
Forns	0.769 (0.708-0.823)	4.2 <sup>3</sup>	94	31	64	79	66
		6.9 <sup>4</sup>	41	87	81	53	61
Apri + FM			76	82	85	72	79
Forns + FM			81	75	81	75	78
Validation population (n = 538)							
FM score	0.854 (0.821-0.888)	0.61	67	87	89	63	75
Apri + FM			57	88	87	57	69

<sup>1</sup>APRI ≤ 0.5 exclude significant fibrosis; <sup>2</sup>APRI > 1.5 predict significant fibrosis; <sup>3</sup>Forns ≤ 4.2 exclude significant fibrosis; <sup>4</sup>Forns > 6.9 predict significant fibrosis. FM: FibroMeterVirus3G; AUROC: Area under ROC curve; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; OA: Overall accuracy; APRI: Aspartate transaminase to platelet ratio index.

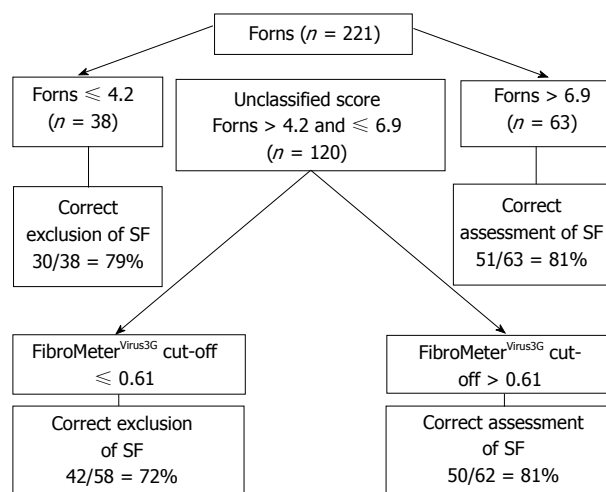


**Figure 1** Sequential algorithm of aminotransferase-to-platelet ratio index + FibroMeter<sup>Virus3G</sup> to predict significant fibrosis, in the Rio population. Accuracy of sequential use of aminotransferase-to-platelet ratio index (APRI) + FibroMeter<sup>Virus3G</sup> was determined considering: Number of correct assessments of SF (96) + number of correct exclusions of SF (79)/total of liver biopsies (222) = 175/222 = 79%. <sup>1</sup>Considering liver biopsy as reference.

8% are cirrhotic. The PPV, NPV and accuracy of APRI + FibroMeter<sup>Virus3G</sup> combination on validation population are shown in Table 3.

## DISCUSSION

Although new treatment regimens with very high rates of sustained virologic response are now available to treat hepatitis C patients, the logistical and financial barriers to treat all infected patients represent an important limitation, even in resource-replete countries<sup>[23]</sup>. Thus, it is necessary to determine an optimal and practical approach to prioritize these highly efficacious, but extremely costly therapies, for a selected population at risk of disease progression or for those who require immediate therapy. There is a consensus that non-invasive evaluation of liver



**Figure 2** Sequential algorithm of Forns + FibroMeter<sup>Virus3G</sup> to predict significant fibrosis, in the Rio population. Accuracy of sequential use of Forns + FibroMeter<sup>Virus3G</sup> was determined considering: Number of correct assessments of SF [(101) + number of correct exclusions of SF (72)]/total of liver biopsies (221) = 173/221 = 78%.

fibrosis may be useful to complement or even replace liver biopsy in CHC owing to its low risk of complications and good accuracy. However, non-invasive tests also present some limitations related to cost, fibrosis discrimination and accuracy.

The present study originally evaluated the combination of a more robust patented fibrosis test, FibroMeter<sup>Virus3G</sup>, with low cost first generation tests to enhance its applicability in the clinical practice. Although APRI and Forns are well established non-invasive tests to assess fibrosis in CHC, their main limitation is that when used alone, almost half of the patients could not be classified according to the possibility of presenting or not significant fibrosis. Thus, using a second test to improve discrimination would enhance the accuracy of these results in order to diagnose significant fibrosis. In the present study, the use of first generation tests combined with FibroMeter<sup>Virus3G</sup> demonstrated to be a lower cost strategy since it reduced

FibroMeter<sup>Virus3G</sup> requirement, without loss of accuracy, eliminating the requirement for liver biopsy procedure.

When analyzed as a class classification test, FibroMeter<sup>Virus3G</sup> presented an overall accuracy of 86% similar to the rate of 87% described in FibroMeter<sup>Virus3G</sup> original report<sup>[21]</sup>. The AUROC for significant fibrosis was 0.85, comparable to previous reports ranging from 0.84 to 0.86<sup>[16,17,21]</sup>. Analysing under a practical point of view, and considering significant fibrosis as the criteria to treat or not the patient, we presented important results that may help hepatologists on clinical decision-making. We found a cut-off of 0.61 as the best numeric value to discriminate significant fibrosis for FibroMeter<sup>Virus3G</sup>, which is close to the value displayed in the manufacturer's bar graph of FibroMeter<sup>Virus3G</sup> report of 0.63, representing the transition from F1 to F2 METAVIR stage<sup>[24]</sup>. The cut-off of 0.61 presented similar performance in comparison to manufacturer's cut-off of 0.63 in the French validation cohort.

FibroMeter<sup>Virus3G</sup> applied as a numeric score also enables its association to other scores. Sebastiani *et al.*<sup>[25]</sup> provided a sequential algorithm for fibrosis evaluation (SAFE) biopsy combining APRI and Fibrotest, another biomarker based on fibrosis class classification, resulting in a 46% reduction of liver biopsy requirement to identify significant fibrosis. In a more recent study performed with 1785 CHC patients, Boursier *et al.*<sup>[26]</sup> reported that the diagnosis of significant fibrosis using SAFE still required liver biopsy in 64% of the cases. To our knowledge, to date the stepwise combination of APRI or Forns with FibroMeter<sup>Virus3G</sup> has never been evaluated. The sequential algorithm of either APRI or Forns combined with FibroMeter<sup>Virus3G</sup> represents a lower cost method with similar accuracy when compared to the isolated use of FibroMeter<sup>Virus3G</sup> test. This represents an advantage when reducing the number of unclassified APRI and Forns patients in the grey zone, without the need for liver biopsy. This is a useful and alternative approach in countries with less financial resources, considering the easy applicability and low cost of APRI and Forns for significant fibrosis. This procedure may represent a more comprehensive proposal to apply these non-invasive tests in the clinical setting.

Some limitations may be discussed in this study. The prevalence of significant fibrosis in our population was higher than the prevalence reported in a meta-analysis including more than 30000 CHC patients which showed a rate of 48% of significant fibrosis histologically assessed<sup>[27]</sup>. Our prevalence is greatly influenced by the fact that this study was carried out in a tertiary-care setting. Another limitation is that derivation and validation populations came from different racial ethnic backgrounds. Nevertheless, most patients included in the derivation population were Caucasians and both populations shared similar characteristics regarding laboratorial results and distribution of significant fibrosis.

The diagnostic accuracy of the APRI and FibroMeter<sup>Virus3G</sup> combination in validation population was 69%. A decrease in diagnostic accuracy is usually expected in the validation population when compared to the derivation

population; however some points need to be emphasized. The PPV of the algorithm APRI and FibroMeter<sup>Virus3G</sup> in the validation population was high (87%). Consequently the algorithm enabled the selection of a subset of patients where very few false positive results were found. In other words, this algorithm allowed treatment to be given to those patients who really required antiviral drugs. The low sensitivity (57%) remains a limitation, since a considerable number of patients who need to be treated will not be correctly identified by the algorithm. Nevertheless, when analysing the subgroup of false negative patients in the validation population, we observed that the majority (69%) were represented by F2 and only 8% were cirrhotic. In the whole validation population, most of the F0 patients (100%), the F1 patients (88%) and the F4 (85%) were well classified by the algorithm, as well as two thirds of the F3. Therefore, the algorithm identifies the more severe patients (F3 and F4) while most of the misclassification concerns the F2 stage. In the derivation population, even though the accuracy of the algorithm was found to be better, the worst result was also observed in F2 stage. And lastly, even when considering a gold standard such as liver biopsy, there is a considerable misclassification of F2 patients<sup>[28]</sup>. In low income countries, where therapy is offered only to patients with advanced fibrosis, a close follow-up is required until these untreated patients fulfil the criteria for direct antiviral therapy. We may consider following the "missed" patients by reapplying the algorithm to better identify when patients change their fibrosis stage and require treatment<sup>[29]</sup>. Since most misclassified patients present F2 fibrosis stage, there is sufficient time before they become cirrhotic.

Our findings demonstrated that the association of a more robust non-invasive marker of fibrosis such as FibroMeter<sup>Virus3G</sup> and first generation tests may represent a useful alternative for fibrosis staging in CHC without loss of accuracy and without the need for liver biopsy. This might be an attractive approach mainly in limited resource countries.

## ACKNOWLEDGMENTS

Fibrometer tests results were granted by Echosens, Paris, Fr.

## COMMENTS

### Background

Despite recent advances in chronic hepatitis C therapy, diagnosis of significant fibrosis still represents a challenge when defining which patients should have priority in treatment, mainly in resource limited countries. Although liver biopsy has been classically considered the standard tool to evaluate fibrosis, it presents well-known inconveniences and limitations which make its use controversial amongst various authors. Thus, the development and improvement of alternative methods to identify candidates for an early treatment or intensive fibrosis monitoring is still recommended. First generation non-invasive tests such as aminotransferase-to-platelet ratio index (APRI), FIB4 and Forns are free of charge, easily accessible and well validated for chronic hepatitis C, however are limited when classifying all patients. Therefore, in order to increase assessment availability for patients



with significant fibrosis, the authors evaluated the performance of a stepwise combination of first generation tests of fibrosis - APRI and Forns - followed by FibroMeter<sup>Virus3G</sup>, a more robust test, whenever results remained unclassified after first generation tests, always using liver biopsy as reference. This proposed combination allows costs to be optimized with no loss of accuracy and no need of liver biopsy, thus representing a favorable economic approach in resource limited areas.

### Research frontiers

This study introduces alternative approaches to evaluate significant fibrosis in chronic hepatitis C using a stepwise algorithm with first generation tests and FibroMeter<sup>Virus3G</sup> both to improve clinical decision-making and reduce costs. The authors considered this topic of great interest for clinicians and hepatologists in the daily practice management of chronic hepatitis C.

### Innovations and breakthroughs

The present study demonstrated that the association of a more robust non-invasive marker of fibrosis such as FibroMeter<sup>Virus3G</sup> and first generation tests such as APRI and Forns may represent a useful alternative for fibrosis staging in chronic hepatitis C. This might be an attractive non-invasive approach to evaluate liver fibrosis and to optimize the access to potent but expensive direct-acting antiviral agents. To our knowledge, to date the stepwise combination of APRI or Forns with FibroMeter<sup>Virus3G</sup> has never been evaluated.

### Applications

The sequential algorithm of either APRI or Forns combined with FibroMeter<sup>Virus3G</sup> represents an alternative approach to recognize and prioritize patients with chronic hepatitis C to antiviral therapy. It reduces the number of unclassified APRI and Forns patients allocated in the grey zone and reduces the total FibroMeter<sup>Virus3G</sup> requirement in 50%, representing an alternative approach in countries with less financial resources, without loss of accuracy, eliminating the requirement for liver biopsy procedure. This procedure may represent a more comprehensive proposal to apply these non-invasive tests in the clinical setting.

### Terminology

FibroMeter<sup>Virus3G</sup> is a non-invasive test to evaluate liver fibrosis in chronic hepatitis C represented by a patented formula including the biologic parameters prothrombin index, AST, ALT, Urea, GGT, alpha-2-macroglobulin and platelets.

### Peer-review

It is a carefully planned study, it takes in to consideration the Liver biopsy and the Fibrometer Virus2 and Virus3 and makes a head to head comparison of the three, as to discover the safety profile and the accuracy when it comes to clinical use especially for the group patients with cirrhosis and to evaluate the change in fibrosis stage in pts with cirrhosis that are unable to undergo liver biopsy with a non-invasive procedure. The Fibre meter requires more wide use in the clinical setting as to prove its self as a reliable and non-invasive method of estimating the fibrosis stage.

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## Observational Study

# Thrombocytopenia in cirrhosis: Impact of fibrinogen on bleeding risk

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**Author contributions:** Thakrar SV and Mallett SV contributed equally to this work in designed and coordinated the research and writing the manuscript.

**Institutional review board statement:** This work was reviewed and approved by the Royal Free London Research and Development department and was deemed not to require ethics approval.

**Informed consent statement:** All data retrieved from our transplant database was anonymised. The data has been previously collected (prior to the study commencing), and was collected as part of standard care. Only those persons involved in clinical care had access to our database. No patient interventions were performed as part of this study. As a result, it was deemed unnecessary to require patient consent.

**Conflict-of-interest statement:** There are no conflicts of interest to declare.

**Data sharing statement:** Technical appendix, statistical code, and dataset available from the corresponding author at [s.thakrar1@nhs.net](mailto:s.thakrar1@nhs.net). Participant consent was not obtained but the presented data are anonymised and risk of identification is low.

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## Abstract

### AIM

To investigate the relationship between baseline platelet count, claus fibrinogen, maximum amplitude (MA) on thromboelastography, and blood loss in orthotopic liver transplantation (OLT).

### METHODS

A retrospective analysis of our OLT Database (2006-2015) was performed. Baseline haematological indices and intraoperative blood transfusion requirements, as a combination of cell salvage return and estimation of 300 mls/unit of allogenic blood, was noted as a surrogate for intraoperative bleeding. Two groups: Excessive transfusion (> 1200 mL returned) and No excessive transfusion (< 1200 mL returned) were analysed. All data analyses were conducted using IBM SPSS Statistics version 23.

### RESULTS

Of 322 OLT patients, 77 were excluded due to fulminant disease; redo transplant or baseline haemoglobin (Hb) of < 80 g/L. One hundred and fourteen (46.3%) were classified into the excessive transfusion group, 132 (53.7%) in the no excessive transfusion group. Mean age and gender distribution were similar in both groups.

Baseline Hb ( $P \leq 0.001$ ), platelet count ( $P = 0.005$ ), claus fibrinogen ( $P = 0.004$ ) and heparinase MA ( $P = 0.001$ ) were all statistically significantly different. Univariate logistic regression with a cut-off of platelets  $< 50 \times 10^9/L$  as the predictor and Haemorrhage as the outcome showed an odds ratio of 1.393 (95%CI: 0.758-2.563;  $P = 0.286$ ). Review of receiver operating characteristic curves showed an area under the curve (AUC) for platelet count of 0.604 (95%CI: 0.534-0.675;  $P = 0.005$ ) as compared with AUC for fibrinogen level, 0.678 (95%CI: 0.612-0.744;  $P \leq 0.001$ ). A multivariate logistic regression shows United Kingdom model for End Stage Liver Disease ( $P = 0.006$ ), Hb ( $P = 0.022$ ) and Fibrinogen ( $P = 0.026$ ) to be statistically significant, whereas Platelet count was not statistically significant.

### CONCLUSION

Platelet count alone does not predict excessive transfusion. Additional investigations, *e.g.*, claus fibrinogen and viscoelastic tests, provide more robust assessment of bleeding-risk in thrombocytopenia and cirrhosis.

**Key words:** Thrombocytopenia; Cirrhosis; Haemostasis; Fibrinogen; Liver transplantation

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**Core tip:** Current literature describing bleeding risk in thrombocytopenia and cirrhosis does not take into account the impact of fibrinogen. The minimal platelet count to form a clot with normal strength is unknown, and would be influenced by fibrinogen. Viscoelastic testing, particularly maximum amplitude (MA, thromboelastography) or maximum-clot-firmness (MCF, thromboelastometry), reflects platelet-fibrinogen interaction and allows assessment of clot strength. Low platelet count and low fibrinogen levels lead to low MA/MCF and correlate strongly with increased bleeding tendency. Assessment of platelet count alone does not accurately predict bleeding, but is useful in conjunction with other indices such as claus fibrinogen and MA/MCF.

Thakrar SV, Mallett SV. Thrombocytopenia in cirrhosis: Impact of fibrinogen on bleeding risk. *World J Hepatol* 2017; 9(6): 318-325 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i6/318.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i6.318>

### INTRODUCTION

Thrombocytopenia is a common finding in patients with advanced liver disease. In most instances it is well tolerated but it is traditionally thought to increase the likelihood of surgical or traumatic bleeding. Moderate thrombocytopenia (defined as platelet count  $< 50 \times 10^9/L$ ) occurs in approximately 13% of those with liver disease and is associated with significant morbidity<sup>[1]</sup>. A number of factors contribute to thrombocytopenia in

liver disease, including low thrombopoietin levels, and sequestration of platelets in hypersplenism as a result of portal hypertension<sup>[2]</sup>.

Derangements of other haematological indices in cirrhosis include prolongation of prothrombin time (PT), prolongation of activated thromboplastin time (APTT) and dysfibrinogenaemia. Conventionally, these changes were thought to lead to an increased bleeding risk. Over the last 10 years, however, a new paradigm of haemostasis in liver disease has been described. There is now considered to be a "rebalancing" with a reduction in pro-coagulant molecules being accompanied by a reduction in anticoagulant molecules. Thrombin generation is normal, or even increased and patients with cirrhosis are now considered to have an elevated risk of thrombosis rather than have complications of bleeding<sup>[3]</sup>.

Standard tests of coagulation such as PT and APTT do not accurately reflect coagulation status *in vivo*, as they cannot assess cellular contributions or the effects of anticoagulant molecules. *In-vitro* studies in cirrhosis have shown a compensatory increase in levels of Von Willebrand Factor (vWF) - a platelet adhesion protein and reductions in ADAMTS-13, the cleavage enzyme responsible for the breakdown of vWF<sup>[4]</sup>. Additionally, platelet hyperactivity has been reported in cholestatic liver disease<sup>[5]</sup>. A systematic review evaluating platelet function concluded that in patients with cirrhosis, primary haemostasis is not defective<sup>[6]</sup>.

Whole blood viscoelastic testing provides valuable information about dynamic clot formation. It measures changes in clot tensile strength with time and is used in goal-orientated algorithms to target transfusion. Clot strength is assessed by maximum amplitude (MA) or maximum clot firmness (MCF) and is influenced by both platelet count and by fibrinogen level. MA or MCF can be maintained in the face of low platelet counts by normal or increased levels of fibrinogen<sup>[7]</sup>. Whole blood global viscoelastic tests such as thromboelastography (TEG®) or thromboelastometry (ROTEM®) may provide more clinically relevant information about coagulation profiles in liver disease. Increasingly observed blood transfusion free orthotopic liver transplantation (OLT) suggests that conventional tests of coagulation are inadequate in predicting bleeding.

Studies of low platelet count in cirrhosis suggest that thrombin generation may be reduced in cases of severe thrombocytopenia<sup>[8]</sup>. *In-vitro* studies, however, have shown that a platelet count of  $20-30 \times 10^9/L$  is likely to be adequate to initiate haemostasis and generate enough thrombin to allow normal MA on TEG<sup>[9]</sup>. Despite a reduction in thrombin production, clot strength is likely to be adequate if the appropriate substrates for clot formation are present. Moderate reductions in platelet count, therefore, do not necessarily indicate an increased risk of bleeding in liver disease.

British Haematology Society guidelines for the use of platelet transfusions and consensus guidelines for percutaneous image guided interventions<sup>[10,11]</sup> recommend the prophylactic transfusion of platelets to a count of  $> 50$



$\times 10^9/L$  prior to liver biopsy to prevent complications of bleeding. In view of current knowledge of coagulation and haemostasis in cirrhosis, the objectives of this study were to investigate the relationship between baseline platelet count, claus fibrinogen, MA on TEG and the volume of blood transfused in patients undergoing orthotopic liver transplantation.

## MATERIALS AND METHODS

### Study design

A retrospective study of patients who had undergone OLT at the Centre for Hepatobiliary Surgery, Royal Free London between 2006 and 2015 was conducted. The cohort of patients reviewed had transplantation for chronic end stage liver disease, with or without hepatocellular carcinoma. Data from their intraoperative course was retrieved from a database formed as part of standard care. Those with acute fulminant liver failure, paracetamol overdose or redo transplantation were excluded. Patients with starting haemoglobin of less than 80 g/L were also excluded in view of an increased risk of intraoperative blood transfusion. Data was anonymised and institutional research and development departmental approval was obtained for its use.

Patient demographic data, baseline haematological results, number of packed red cell units transfused intra-operatively and volume of cell salvaged blood returned to patients was retrieved electronically.

### Measurements

Baseline variables were retrieved from the OLT database and included patient characteristics such as age, gender, diagnosis and severity scoring with United Kingdom model for End Stage Liver Disease (UKELD score). Baseline clinical measurements were point-of-care (*i.e.*, Medical diagnostic testing at the point of care) samples taken at the time of anaesthesia for liver transplantation from arterial catheters and measurements included haemoglobin concentration (Hb) and platelet count by pocH-100i full blood count analyser (Sysmex Europe GmbH). TEG variables were from TEG<sup>®</sup> 5000 (Haemonetics, Braintree, MA, United States). United Kingdom TD in particular MA on heparinase TEG was assessed. Heparinase TEGs were used for analysis to remove any influence that may have been exerted by endogenous heparinoids and to standardize results. Laboratory Clauss fibrinogen levels using ACL-TOP 700 (Werfen, United Kingdom) were obtained prior to transplantation and did not exceed 24 h prior to anaesthetic start time. All assays are controlled and monitored using laboratory quality assurance processes.

As a surrogate for intraoperative blood loss, an estimation of 300 mL of blood in a packed red cell unit given to patients was made, and the volume summated with cell salvage return volume to give a total volume of blood returned. Patients were divided into 2 groups according to total volume of blood returned:  $\leq 1200$  mL

(no excessive transfusion) and  $> 1200$  mL (excessive transfusion).

### Statistical analysis

Descriptive statistics were performed on baseline variables and comparisons made between excessive transfusion (ET) and no excessive transfusion (NET) groups. Univariate logistic regression was performed for each variable independently as the predictor, and ET as the binary outcome. Receiver operating characteristic (ROC) curves for baseline platelet count and for baseline claus fibrinogen were also constructed and area under the curves calculated. Binomial logistic regression with each variable as the predictor and ET as the outcome was also performed. Predicted probabilities from the binomial logistic regression were used for further ROC analysis. The relationship between platelet count and blood volume returned as well as fibrinogen and blood volume returned was further investigated by linear regression modeling. All data analyses were conducted using IBM SPSS Statistics version 23. All statistical analyses were reviewed by Ms Fatima Jichi, a trained biostatistician with the department of Biostatistics, University College London.

## RESULTS

### Baseline demographics

Results for 323 patients were reviewed and of these 37 patients had either acute, fulminant liver failure or a redo-liver transplant and were excluded. A further 40 patients had a baseline Hb less than 80 g/L and were also excluded. Of the remaining 246 patients, 114 (46.3%) had excessive transfusion and 132 (53.7%) had no excessive transfusion.

Mean patient ages were 53 years ( $\pm 1$  SD 10.04 years) and were similar in ET and NET groups. The gender distribution of patients was 72.8% male and 27.2% female with a similar divide in both groups. Mean UKELD was 56 ( $\pm 10.55$ ) in the ET group and 51 ( $\pm 5.08$ ) in the NET group ( $P \leq 0.001$ ). Liver disease due to Infection (33.3%) or Alcohol (29.6%) was the commonest aetiology. Interestingly, primary sclerosing cholangitis (PSC) was more common in the NET group (18.9%) vs the ET group (7.9%).  $\chi^2$  analysis of aetiologies in both groups revealed  $\times \chi^2 = 18.81$ ,  $P = 0.016$ . Baseline Hb, platelet count, claus fibrinogen and hep MA were all statistically significantly different between the two groups (Table 1).

A comparison of patient demographics and baseline measurements between those with platelet count  $< 50$  and those  $\geq 50 \times 10^9/L$  is described in Table 2. Baseline fibrinogen was statistically significantly different between those with low platelet count (mean =  $1.78 \pm 0.62$ ) and those without ( $2.45 \pm 1.15$ ,  $P \leq 0.001$ ). Baseline hep MA was also significantly different in the 2 groups ( $35.28 \pm 9.49$  vs  $47.85 \pm 11.93$ ,  $P \leq 0.001$ ). The total volume of blood returned was not significantly different between

**Table 1** Baseline demographics for patients with chronic liver disease who have undergone orthotopic liver transplantation<sup>1</sup>

	Excessive transfusion	No excessive transfusion	Total	P value
<i>n</i>	114 (46.3%)	132 (53.7%)	246	
Age (yr)				
Mean	53.12	52.72	52.91	0.757
SD	9.68	10.38	10.04	
Gender				
Female	28 (24.6%)	39 (29.5%)	67 (27.2%)	0.381
Male	86 (75.4%)	93 (70.5%)	179 (72.8%)	
UKELD				
Mean	56.11	51.11	53.02	< 0.001
SD	10.55	5.08	6.22	
Diagnosis				
ALD	44 (38.6%)	29 (21.9%)	73 (29.6%)	
Infectious	40 (35.1%)	42 (31.8%)	82 (33.3%)	
NASH	4 (3.5%)	6 (4.5%)	10 (4.1%)	
PSC	9 (7.9%)	25 (18.9%)	34 (13.8%)	
PBC	3 (2.6%)	6 (4.5%)	9 (3.7%)	
AIH	4 (3.5%)	3 (2.3%)	7 (2.8%)	
Wilson's	1 (0.8%)	0 (0%)	1 (0.4%)	
Haemochromatosis	1 (0.8%)	0 (0%)	1 (0.4%)	
Misc	8 (7.0%)	21 (15.9%)	29 (11.8%)	
Hb (g/L)				
Mean	101.90	109.71	106.09	< 0.001
SD	16.89	15.38	16.53	
Platelets ( $\times 10^9/L$ )				
Mean	83.18	107.29	96.12	0.005
SD	55.03	75.14	67.53	
Median	67	84.5	76.0	
IQR	42.5	77.5	61.25	
Fibrinogen (g/L)				
Mean	1.96	2.60	2.3	0.004
SD	0.91	1.15	1.09	
Hep MA (mm)				
Mean	42.15	47.71	45.14	0.001
SD	12.68	11.87	12.54	
Total blood returned (mL)				
Mean	3323	487	1802	
SD	2536	419	2251	

<sup>1</sup>Excessive transfusion defined as blood volume returned > 1200 mL. SD: Standard deviation; UKELD: United Kingdom end stage liver disease score; ALD: Alcoholic liver disease; NASH: Non-alcoholic steato-hepatitis; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; Hb: Haemoglobin; Hep MA: Maximum amplitude on heparinase thromboelastography.

the 2 groups ( $P = 0.69$ ).

A logistic regression analysis (Table 3) was performed to ascertain the independent effects of age, gender, UKELD, baseline Hb, baseline platelet count, baseline platelet count <  $50 \times 10^9/L$  or  $\geq 50 \times 10^9/L$  as a binary value, baseline claus fibrinogen level and baseline heparinase MA on likelihood of excessive transfusion. UKELD ( $P \leq 0.001$ ), HB ( $P \leq 0.001$ ), platelet count ( $P = 0.007$ ), claus fibrinogen ( $P \leq 0.001$ ) and Hep MA ( $P = 0.001$ ) were all statistically significant. A cut off value of platelet count less than 50 was not a good predictor of excessive transfusion ( $P = 0.286$ ).

Review of ROC curves showed an area under the curve (AUC) for platelet count of 0.604 (standard error: 0.036; 95%CI: 0.534-0.675;  $P = 0.005$ ). AUC for fibrino-

**Table 2** Comparison of baseline demographics according to baseline platelet count cut off value of  $50 \times 10^9/L$ 

	Platelet count < 50	Platelet count $\geq 50$	P value
<i>n</i>	53 (21.5%)	193 (78.5%)	
Age (yr)			
Mean	51.23	53.37	0.13
SD	10	9.04	
Median	52.38	55.26	
Range	52	50	
Gender			
Female	40 (75.5%)	139 (72%)	0.62
Male	13 (24.5%)	54 (28%)	
UKELD			
Mean	54.16	52.72	0.09
SD	5.06	6.47	
Diagnosis			
ALD	10 (19%)	63 (33%)	
Infectious	30 (56%)	52 (27%)	
NASH	3 (6%)	7 (4%)	
PSC	5 (9%)	29 (15%)	
PBC	1 (2%)	8 (4%)	
AIH	2 (4%)	5 (2%)	
Wilson's	0	1 (0.5%)	
Haemochromatosis	0	1 (0.5%)	
Misc	2 (4%)	27 (14%)	
Hb (g/L)			
Mean	106.34	106.03	0.89
SD	15.08	16.94	
Fibrinogen (g/L)			
Mean	1.78	2.45	< 0.001
SD	0.62	1.15	
Hep MA (mm)			
Mean	35.28	47.85	< 0.001
SD	9.49	11.93	
Total blood returned (mL)			
Mean	1692.13	1831.6	0.69
SD	1426.72	2431.54	
Median	1500	1110	
Range	6305	21237	

SD: Standard deviation; UKELD: United Kingdom end stage liver disease score; ALD: Alcoholic liver disease; NASH: Non-alcoholic steato-hepatitis; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; Hb: Haemoglobin; Hep MA: Maximum amplitude on heparinase thromboelastography.

gen level was 0.678 (standard error: 0.034; 95%CI: 0.612-0.744;  $P \leq 0.001$ ) (Figure 1).

A multivariate logistic regression with all covariates with  $P \leq 0.1$  from univariate logistic regression added to a model, shows UKELD ( $P = 0.006$ ), Hb ( $P = 0.022$ ) and Fibrinogen ( $P = 0.026$ ) to be statistically significant. Platelet count was not statistically significant (Table 4).

The AUC from ROC curve analysis of predicted probabilities from multivariate logistic regression was 0.749 (standard error: 0.032; 95%CI: 0.686-0.812;  $P \leq 0.001$ ), suggesting that variables need to be considered together to better predict excessive transfusion (Figure 2).

Further investigation of the relationship between baseline platelet count and total volume of blood returned to patients showed that for every 1 point increase in platelet count, a 4.9 mL ( $P = 0.19$ ) reduction in total

**Table 3** Univariate logistic regression

	Odds ratio	95%CI	P value
Age	1.004	0.979-1.029	0.76
Gender	1.288	0.730-2.271	0.382
UKELD	1.130	1.076-1.188	< 0.001
Hb	0.97	0.954-0.986	< 0.001
Platelet count	0.994	0.990-0.998	0.007
Platelets (Binary cut off < 50 and $\geq$ 50)	1.393	0.758-2.563	0.286
Fibrinogen	0.523	0.388-0.703	< 0.001
Hep MA	0.963	0.942-0.984	0.001

UKELD: United Kingdom end-stage liver disease score; Hb: Haemoglobin; Hep MA: Maximum amplitude on heparinase TEG.

**Table 4** Multivariate logistic regression with covariates  $P < 0.1$  from univariate logistic regression

	OR	95%CI	P value
UKELD	1.081	1.023-1.143	0.006
Hb	0.977	0.958-0.997	0.022
Platelets	0.999	0.994-1.004	0.700
Fibrinogen	0.682	0.487-0.955	0.026
Hep MA	0.986	0.957-1.015	0.338

OR: Odds ratio; UKELD: United Kingdom end-stage liver disease score; Hb: Haemoglobin; Hep MA: Maximum amplitude on heparinase TEG.

**Table 5** Relationship between baseline platelet count and baseline fibrinogen and blood returned to patients (linear regression)

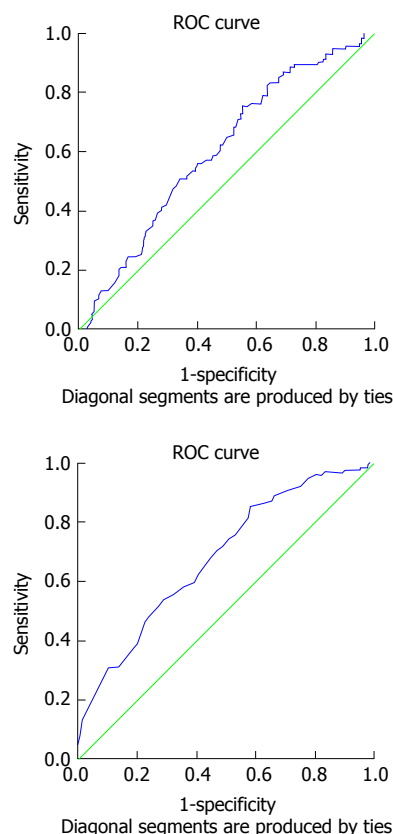
Model	Regression coefficient B	95%CI	P value
Platelet count	2280.72-4.99	-9.142-0.829	< 0.001
Fibrinogen	3014.63-525.95	-776.88-275.02	< 0.001

blood volume returned was achieved. For every 1-point increase in fibrinogen level, however, a reduction of 525.95 mL ( $P \leq 0.001$ ) of blood returned to the patient was achieved (Table 5).

## DISCUSSION

### Principle findings

Guidelines recommend the prophylactic transfusion of platelets to achieve a count of  $50 \times 10^9/L$  prior to invasive procedures such as liver biopsy. Understanding of haemostasis in the cirrhotic population has altered with the concept of a "rebalanced" haemostatic profile in liver disease. This study evaluated differences in baseline platelet count, fibrinogen levels, viscoelastic tests and blood transfusion requirements in those undergoing OLT for chronic liver disease. Patients in our study were divided according to whether they received excessive blood transfusion or not. On comparison, the 2 groups were well matched for gender and age, although UKELD was found to be significantly different. Severity of liver disease, in the form of Childs-pugh score and Model for



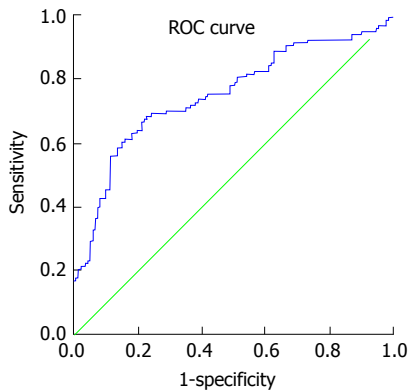
**Figure 1** Receiver operating characteristics for baseline platelet count and baseline claus fibrinogen. A: Platelets; B: Fibrinogen; ROC: Receiver operating characteristic.

end stage liver disease (MELD) scoring, is associated with a prediction of increased intraoperative transfusion requirement<sup>[12]</sup>. This may explain the difference in UKELD between the 2 groups.

It was interesting to note a higher preponderance of PSC as the aetiology of liver disease in those not requiring excessive transfusion. Hypercoagulable haemostatic profiles have been described for those with biliary cirrhotic disease and in general this population does not have thrombocytopenia or low fibrinogen<sup>[5]</sup>.

A statistically significant difference in baseline Hb, platelet count, Clauss fibrinogen and MA on heparinase TEG was observed between those who received excessive transfusion vs those who did not. This highlights an association with bleeding risk and indicates that possibly all of these measurements would be useful in predicting increased blood transfusion requirements.

Logistic regression performed to evaluate the probability of excessive transfusion with each variable shows clearly that a platelet threshold value of  $50 \times 10^9/L$  is not a good predictor of blood transfusion in this population. Although there has been a previously described association between a reduction in thrombin generation with a reduction in platelet count<sup>[8]</sup>, the cut off value of platelets requiring transfusion in cirrhosis is likely to lie significantly below  $50 \times 10^9/L$  described in guidelines. One small prospective study of liver biopsy in severe thrombocytopenia associated



**Figure 2** Receiver operating characteristics with predicted probabilities from multivariate regression. ROC: Receiver operating characteristic.

with haematological malignancy suggests the likely cut off value lies below  $30 \times 10^9/\text{L}$ <sup>[13]</sup>.

Much of the literature describing bleeding risk in cirrhosis and thrombocytopenia does not take into account fibrinogen level. The minimal platelet count required for normal clot strength is unknown and is markedly affected by fibrinogen. MA on TEG is a composite reflection of platelet-fibrinogen interaction and can be used to assess clot strength. Assessment of MA shows that even in the face of a low platelet count, adequate clot strength may still be achieved if fibrinogen is normal or raised. A combination of low platelet count and low fibrinogen level always results in low MA and is strongly associated with an increased bleeding tendency<sup>[14,15]</sup>. Platelet count alone is not a true indicator of clot strength; therefore, if baseline platelet count is low, assessment of MA is useful in guiding whether to replace fibrinogen or to transfuse platelets. Thrombocytopenia predominately leads to a reduction in blood clot strength displayed as MA on TEG<sup>[16]</sup>, but fibrinogen also contributes to clot firmness. The effect of the administration of fibrinogen concentrates in thrombocytopenia, at a count of  $30 \times 10^9/\text{L}$ , in the pig model has been studied. Velik-Salchner *et al.*<sup>[17]</sup> showed an improvement in impaired clot formation and a reduction in blood loss in thrombocytopenia with the addition of fibrinogen.

The impact of fibrinogen on bleeding risk can be observed in the results of this study. Baseline claus fibrinogen level is likely to have a greater protective effect than the other baseline haematological variables (OR: 0.52; 95%CI: 0.388-0.705;  $P \leq 0.001$ ). Similarly, Odds ratios for fibrinogen on multivariate analysis are the lowest when compared with other variables (0.682, 95%CI: 0.487-0.955) (Table 4). On comparison of AUCs on ROC curve analysis, baseline fibrinogen level is a better predictor of excessive transfusion than platelet count. Interestingly, linear regression analysis shows a 525.95 mL reduction in blood returned to patients with each 1-unit increase in baseline fibrinogen level (*i.e.*, 1 g of fibrinogen factor concentrate) (Figure 1). In comparison, 1 pool of platelets (one adult therapeutic dose) increases platelet count by  $20 \times 10^9/\text{L}$ <sup>[11]</sup>, equating to a 99.8 mL reduction in blood transfusion if the linear

model is used. ROC analysis of predicted probabilities on multivariate analysis show an AUC greater than that of platelet count alone, indicating a better predictive value on assessing all the demographic and haematological variables simultaneously (Figure 2).

### Strengths and weaknesses of the study

Although there are a number of *in-vitro* investigations into the associations between thrombocytopenia and fibrinogen concentration and clot strength, there is a lack of evidence relating to the influence of the two *in vivo*. There is also a lack of substantial evidence to validate a cut off value for prophylactic platelet transfusion in the cirrhotic population. This study highlights the contribution of fibrinogen in reducing the risk of excessive blood transfusion, and therefore bleeding risk.

Excessive transfusion was used as a surrogate for intraoperative bleeding in this study. Measurement of blood loss in suction and weight of swabs would provide more accurate information with regard to blood loss, but this information was unavailable retrospectively. Furthermore, OLT is complex surgery with other influences on haemorrhage apart from the haemostatic picture. These include presence of portal hypertension and varices, difficult operative dissection with multiple adhesions, surgical technique (*i.e.*, Caval replacement surgery or "piggy back" technique for reperfusion) and the volume of fluid given to the patient<sup>[12,18]</sup>. Additionally, baseline low haemoglobin values increase the likelihood of requiring intraoperative blood transfusion. Low haematocrit also has an impact on laminar flow in blood vessels and therefore a disturbance in primary haemostasis may occur in anaemia<sup>[19]</sup>. We excluded those with baseline haemoglobin of  $< 80 \text{ g/L}$  for this reason.

Although the results of our study point to the usefulness of measuring baseline claus fibrinogen in conjunction with platelet count and assessment of TEG, we are unable to assess for specific cut off values for baseline platelet count and fibrinogen level. Results would require validation against external data sets to allow for cut off values, requiring further prospective research.

### Implications of study

The transfusion of platelets is not without risk. Complications of platelet transfusion include allergic or anaphylactic reactions, haemolytic and non-haemolytic transfusion reactions, transfusion related acute lung injury and septic transfusion reactions of bacterial origin<sup>[20]</sup>. Furthermore, in liver transplantation, platelets have been shown to be involved in ischaemic reperfusion injury by interactions with activated sinusoidal endothelium and induction of apoptosis. Perioperative platelet transfusion has been identified as an independent risk factor for adverse post-operative outcomes<sup>[21]</sup>. In a large retrospective analysis of patients undergoing cardiac surgery, those receiving platelets were at an increased risk of postoperative infection, stroke and multiorgan failure<sup>[22]</sup>.

The availability of platelets, largely due to short storage life, can also be limited. By demonstrating a lack



of association between excessive blood transfusion and a threshold platelet count of  $50 \times 10^9/L$ , the question of unnecessary prophylactic platelet transfusion arises. If an increase in fibrinogen levels by 1 g reduces the volume of blood transfused significantly, the usefulness of fibrinogen concentrate rather than platelet transfusion should be considered. Fibrinogen concentrate appears to have a better safety profile than cryoprecipitate and fresh frozen plasma, particularly when considering the risk of blood borne infection. Other advantages of its use include the accuracy and rapidity of its administration<sup>[23]</sup>.

In conclusion, further prospective evaluation to assess for the true baseline platelet count at which bleeding risk is increased needs to be performed. Studies have described an increase likelihood of bleeding associated with invasive procedure with low platelet counts ( $< 75 \times 10^9/L$ ), but it is important to note that these studies failed to assess the contribution of fibrinogen to clot strength in cases of thrombocytopenia<sup>[24]</sup>. Additional haematological indices such as Clauss fibrinogen and the use of viscoelastic testing may provide a more robust assessment of bleeding risk in thrombocytopenia associated with cirrhosis.

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## COMMENTS

### Background

Thrombocytopenia is a common finding in patients with advanced liver disease. Concomitantly an increase in value of conventional tests of coagulation including prothrombin time and activated partial thromboplastin time occurs. A new paradigm of haemostasis in liver disease has been described and there is now considered to be a "rebalancing" of haemostasis in cirrhosis. As a result, the authors investigated the relationship between platelet count, Clauss fibrinogen, thromboelastography, and blood loss in orthotopic liver transplantation (OLT).

### Research frontiers

Much of the literature describing bleeding risk in cirrhosis and thrombocytopenia does not take into account fibrinogen level. There have been recent studies that show the effect of the administration of fibrinogen concentrates in thrombocytopenia, at a count of  $30 \times 10^9/L$ , in the pig model. Velik-salchner *et al* showed an improvement in impaired clot formation and a reduction in blood loss in thrombocytopenia with the addition of fibrinogen. Further studies evaluating the contribution of fibrinogen to clot strength in thrombocytopenia in humans are required.

### Innovations and breakthroughs

Currently, there are limited studies evaluating the contribution of fibrinogen to bleeding risk. The increased use of thromboelastography and in particular the evaluation of clot strength with other haematological indices could provide a more robust method of evaluating bleeding risk and requires further investigation.

### Applications

The transfusion of platelets does not come without risks to patients. The

availability of this valuable resource is also limited. The study highlights that platelet count alone is not a true indicator of clot strength and the threshold at which bleeding occurs in the face of a normal fibrinogen level needs further evaluation. Many current guidelines suggest prophylactic transfusion of platelets up to a count of  $50 \times 10^9/L$ , when in fact the threshold for bleeding risk is likely to be lower.

## Peer-review

Interesting study with respectable sample size. This article evaluated retrospectively the association between coagulation parameters and need for transfusion in patients undergoing OLT. The study is well written with a clear message.

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## Sarcopenia and non-alcoholic fatty liver disease: Is there a relationship? A systematic review

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### Abstract

#### AIM

To perform a systematic review to evaluate the incidence and prevalence of non-alcoholic fatty liver disease (NAFLD) in adult patients with sarcopenia.

#### METHODS

Randomized clinical trials, cross-sectional or cohort studies including adult patients (over 18 years) with sarcopenia were selected. The primary outcomes of interest were the prevalence or incidence of NAFLD in sarcopenic patients. In the screening process, 44 full-text articles were included in the review and 41 studies were excluded.

#### RESULTS

Three cross-sectional studies were included. The authors attempted to perform a systematic review, but due to the differences between the studies, a qualitative synthesis was provided. The diagnosis of NAFLD was made by non-invasive methods (image methods or any surrogate markers) in all three evaluated studies. All the studies suggested that there was an independent association between sarcopenia and NAFLD.

#### CONCLUSION

Sarcopenia is independently associated with NAFLD and possibly to an advanced fibrosis.

**Key words:** Metabolic syndrome; Obesity morbid; Sarcopenic obesity; Steatohepatitis; Skeletal muscle

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**Core tip:** The aim of the present study was to perform a systematic review evaluating the incidence and prevalence of non-alcoholic fatty liver disease (NAFLD) in adult patients with sarcopenia. Randomized clinical trials, cross-sectional or cohort studies including adult patients (over 18 years) with sarcopenia were selected. The primary outcomes of interest were the prevalence or incidence of NAFLD in sarcopenic patients, and three cross-sectional studies were finally included. There was an independent association between sarcopenia and NAFLD in all the studies. In conclusion, sarcopenia is independently associated with NAFLD and possibly to an advanced fibrosis.

Tovo CV, Fernandes SA, Buss C, de Mattos AA. Sarcopenia and non-alcoholic fatty liver disease: Is there a relationship? A systematic review. *World J Hepatol* 2017; 9(6): 326-332 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i6/326.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i6.326>

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as a set of liver diseases that can range from simple steatosis to steatohepatitis (NASH), which can progress to fibrosis or even cirrhosis<sup>[1]</sup> and complications such as hepatocellular carcinoma<sup>[2,3]</sup>. It will soon become the most common liver disease worldwide<sup>[4]</sup>, with an estimated prevalence in the general population of Western countries about 20% to 30%<sup>[5]</sup>. In specific populations, its prevalence can be much higher and may reach 90% in morbidly obese patients eligible for bariatric surgery, 69% in type 2 diabetes mellitus patients and 50% in dislipidemic ones<sup>[4]</sup>. In our experience, the prevalence of NASH when obese individuals without diabetes mellitus with high aminotransferases levels were evaluated in a nutrition outpatient clinic was 88%<sup>[6]</sup>. On the other hand, when we evaluated morbidly obese patients submitted to bariatric surgery, the prevalence of steatosis was 90.4% and NASH 70.4%<sup>[7]</sup>. NAFLD patients present higher mortality than the general population, being the cardiovascular disease the most common cause of death. In patients presenting NASH, however, the mortality is associated more often to hepatic causes<sup>[4]</sup>.

Sarcopenia is well characterized by the progressive loss of strength and skeletal muscle mass, generally associated with functional limitations, morbidity, and mortality<sup>[3,8,9]</sup>. The European consensus on definition and diagnosis of sarcopenia recommends using the low muscle mass and muscle function (strength or performance)

for its diagnosis. Assessment of different stages of sarcopenia may help to establish the best treatment to be administered in different contexts and set appropriate recovery targets<sup>[9]</sup>.

There is some concern about whether NAFLD results in sarcopenia through the activation of myostatin in the skeletal muscle, or if is sarcopenia the initial abnormality resulting in the activation of the stellate cells with fibrogenic properties in the liver. Considering the hypothesis that myostatin increases adipose tissue mass that will result in the decrease of adiponectin secretion, the original defect may actually begin in the skeletal muscle<sup>[10]</sup>.

Sarcopenia may occur simultaneously with obesity, particularly the accumulation of visceral fat, which can be related to inflammation, insulin resistance (IR), and further reduction in the skeletal muscle mass, consequently causing muscle catabolism<sup>[11]</sup>. In some conditions, lean body mass is lost while fat mass may be preserved or even increased<sup>[12]</sup>; this state is called sarcopenic obesity<sup>[9]</sup>. The prevalence of sarcopenic obesity increases with age, depending on definitions and reference populations<sup>[13-15]</sup>.

Although sarcopenia has been independently related to an increased risk of NAFLD and advanced fibrosis, and that sarcopenia may be associated with worse liver related clinical outcomes, this is an understudied issue, and its role on NAFLD or NASH has not been fully established<sup>[16]</sup>. The aim of this study was to perform a systematic review identifying original studies that evaluated the association between sarcopenia and NAFLD in adults.

## MATERIALS AND METHODS

### Protocol and registration

This systematic review was registered at the international prospective register of systematic reviews platform (PROSPERO) (<https://www.crd.york.ac.uk/PROSPERO/>), number CRD42015027083. This study followed the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses<sup>[17]</sup>.

### Eligibility criteria

Randomized clinical trials (RCTs), cross-sectional or cohort studies including adult patients (over 18 years) with sarcopenia were selected.

The primary outcomes of interest were the prevalence or incidence of NAFLD in sarcopenic patients, liver fibrosis and NASH activity index assessed by biopsy or non-invasive methods. Studies in which one or more of these outcomes were assessed were included in the present systematic review.

### Search and study selection

The search for eligible studies was performed in PubMed, Lilacs, EMBASE and Cochrane in October, 2016, without a limiting period. The search strategy included the following set of keywords: "Sarcopenia"(Mesh) OR "Sarcopenia"



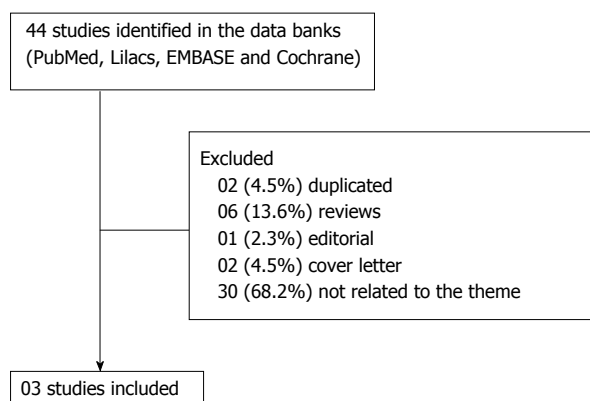


Figure 1 Screening process.

OR "Loss of skeletal muscle" OR "Loss of muscle mass and strength" OR "Reduced muscle mass and strength" OR "Intra-abdominal fat" OR "Muscle wasting" OR "Sarcopenic obesity" and (additional keyword). The last gap was changed at each search using the keywords "Non-alcoholic fatty liver disease"(Mesh) OR "Non-alcoholic fatty liver disease" OR "NAFLD" OR "NASH" OR "Non-alcoholic fatty liver disease" OR "Nonalcoholic fatty liver disease" OR "Nonalcoholic fatty liver" OR "Nonalcoholic fatty livers" OR "Nonalcoholic steatohepatitis" OR "Nonalcoholic steatohepatitides" OR "Fatty liver index". The searches were performed without limiting the types of articles (RCTs, clinical trial, comparative study). The selection of eligible studies was performed by title and abstract reading. When abstracts regarding subjects or outcomes of interest were not clear, the full text of the article was read.

### Data collection process

Data was collected by two independent investigators for the following variables: Design of the study, age and sex of participants, and the presence of NAFLD. The methodological quality assessment criteria followed the guidelines according to the study design - CONSORT<sup>[18]</sup> or STROBE<sup>[19]</sup>.

## RESULTS

In the initial screening process (Figure 1), 44 full-text articles were included in the present review, of which 41 studies were finally excluded, remaining three cross-sectional studies for analysis. The authors attempted a systematic review with meta-analysis, but due to the variance amongst the three studies, a qualitative synthesis is provided. The main results of the studies with the respective comparisons within and between groups (when available) are shown in Table 1.

The diagnosis of NAFLD was made by non-invasive methods (image methods or any surrogate markers) in all three evaluated studies. The liver attenuation index (LAI) was evaluated by computed tomography in the study of Hong *et al.*<sup>[15]</sup>. The fatty liver index (FLI) was

calculated from waist circumference, body mass index, gamma-glutamyl transpeptidase and triglyceride levels in the study of Moon *et al.*<sup>[20]</sup>. The NAFLD fibrosis score (NFS), hepatic steatosis index and the liver fat score were non-invasive scores used in the studies of Lee *et al.*<sup>[16]</sup>.

The diagnosis of sarcopenia was defined by the skeletal muscle mass index (SMI) as follow: Total skeletal muscle mass (kg)/weight (kg) × 100, and was evaluated by dual energy X-ray absorptiometry (DXA) in three of the studies<sup>[15,16]</sup> or by bioelectric impedance analysis (BIA) in one<sup>[20]</sup>.

Moon *et al.*<sup>[20]</sup> evaluated the effects of skeletal muscle mass to visceral fat area ratio by BIA on NAFLD (diagnosed using FLI). Of all the 9565 individuals who underwent a routine health examination, 1848 (19.3%) presented NAFLD (FLI ≥ 60). The group with low FLI showed the lowest visceral fat area and highest skeletal muscle mass, and the SMI presented inverse correlations with FLI. In the multivariate analysis, skeletal muscle mass to visceral fat ratio was negatively associated with FLI. Considering the quartiles of the skeletal muscle mass to visceral fat ratio, the highest one showed the lowest risk of NAFLD, adjusted for age, gender, diabetes mellitus, hypertension, C-reactive protein and lipid profile (odds ratio, 0.037).

The study of Hong *et al.*<sup>[15]</sup> performed a cross-sectional analysis between sarcopenia and NAFLD in the Korean Sarcopenic Obesity Study, a prospective observational cohort study. The authors included 452 healthy adults by LAI (evaluated by computed tomography), used as a parameter for the diagnosis of NAFLD. Both SMI and LAI were negatively correlated with the homeostasis model assessment of insulin resistance ( $P < 0.001$ ). After using the multiple logistic regression analysis, the odds ratio for NAFLD was 5.16 in the lowest quartile of SMI (adjusting for potential confounding factors).

Lee *et al.*<sup>[16]</sup> used a representative sample of 15132 subjects from the Korea National Health and Nutrition Examination Surveys (2008-2011), a population-based study. Non-invasive scores as the body mass index, aspartate aminotransferase/alanine aminotransferase ratio and diabetes mellitus (BARD) and fibrosis-4 (FIB-4) were used to define advanced fibrosis in subjects with NAFLD. The prevalence of NAFLD in non-sarcopenic patients ranged from 4% to 14% (non-obese) and from 50% to 72% (obese), depending on the hepatic steatosis score employed. The prevalence of NAFLD in sarcopenic patients ranged from 9% to 30% (non-obese) and from 61% to 83% (obese). The SMI was inversely correlated with the NAFLD predicting scores ( $P < 0.001$ ). Sarcopenic subjects had an increased risk of NAFLD regardless of obesity (odds ratio 1.55-3.02;  $P < 0.001$ ) or metabolic syndrome (odds ratio 1.63-4.00;  $P < 0.001$ ) than those non-sarcopenic. Furthermore, it was demonstrated an independent association between sarcopenia and NAFLD when analysed by multiple logistic regression analysis.

**Table 1** Characteristics and outcomes of the included studies

Ref.	Year	Design of the study	Sample size	Mean age ( $\pm$ SD)	Gender	Method of diagnosis of sarcopenia	Independent variable	Method of diagnosis of NAFLD	Frequency of NAFLD	Results of the studies
Hong <i>et al</i> <sup>[15]</sup>	2014	Cross-sectional	452	49.5 $\pm$ 10.3	285 women (63.1%)	DXA	SMI/weight (quartiles)	CT (LAI)	Prevalence	OR of having NAFLD by quartiles of SMI after adjusting for potential confounding factors: OR = 5.16 (95% CI: 1.63-16.33)  $P$ = 0.041 after adjustment for age, sex, smoking status, physical activity, HOMA-IR, hsCRP and 25[OH]D levels
Lee <i>et al</i> <sup>[16]</sup>	2015	Cross-sectional	15132	49.7 $\pm$ 16.5	9515 women (62.9%)	DXA	SMI: < 32.2% for men and < 25.5% for women	HSI, CNS and LFS BARD and FIB-4 for advanced fibrosis	Prevalence: 22%-29%	Sarcopenic <i>vs</i> non-sarcopenic patients according to the NAFLD assessment method: OR = 1.18-1.22 (95% CI: 1.02-1.39)  $P$ < 0.001 when adjusted for age, sex, regular exercise, HOMA-IR, smoking and HT
Moon <i>et al</i> <sup>[20]</sup>	2013	Cross-sectional	9565	47 $\pm$ 10.3	5293 men (55.3%)	BIA multi frequencies	SVR (quartiles)	Surrogate marker: FLI $\geq$ 60	Prevalence: 19.32%	OR for NAFLD among the quartiles of SVR using multiple logistic regression analysis: OR = 0.037 (95% CI: 0.029-0.049)  $P$ < 0.001 when adjusted for age, sex, total cholesterol, low-density lipoprotein cholesterol, DM, HTN, hsCRP

BIA: Bioelectric impedance analysis; CNS: Comprehensive NAFLD score; CT: Computed tomography; DM: Diabetes mellitus; DXA: Dual energy X-ray absorptiometry; FLI: Fatty liver index; HOMA-IR: Homeostasis model of insulin resistance; hsCRP: High sensitivity C-reactive protein; HSI: Hepatic steatosis index; HTN: Systemic hypertension; LAI: Liver attenuation index; LFS: Liver fat score; NAFLD: Non-alcoholic fatty liver disease; 25[OH]D: 25-hydroxyvitamin D; OR: Odds ratio; SMI: Skeletal muscle mass index; SVR: Skeletal muscle mass to visceral fat area ratio.

Among the individuals with NAFLD, the lower the SMI, the more chance of advanced fibrosis when compared with the non-sarcopenic ( $P$  < 0.001).

## DISCUSSION

In the present review, all the studies<sup>[15,16,20]</sup> concluded that there was an independent association between sarcopenia and NAFLD. The association of sarcopenia with NAFLD seems to be independent of IR<sup>[15,16]</sup> or obesity<sup>[16]</sup>. However, it is not possible to establish whether the association between sarcopenia and NAFLD is a cause or an effect. The skeletal muscle is now recognized as an endocrine organ secreting myokines, and this fact may help to understand its role in the pathogenesis of NAFLD<sup>[21]</sup> as well as contribute to the development of effective therapeutic options<sup>[10]</sup>.

The association between fat accumulation in the liver and in the muscle has recently been established. The fat content in the paravertebral muscles analyzed by computed tomography may be correlated with aging and steatosis, and a reduction in muscle fat may be associated with an decrease of the liver fat content<sup>[22]</sup>.

Insulin resistance and metabolic syndrome has been consistently associated with sarcopenia and NAFLD, as both conditions may share pathophysiological mecha-

nisms<sup>[23-26]</sup>. However, the association between sarcopenia and NAFLD seems to be independent of IR, raising the possibility that the loss of muscle mass may contribute to the development of NAFLD<sup>[27]</sup>.

The study of Moon *et al*<sup>[20]</sup> showed that the FLI was lower in the group with higher skeletal muscle mass, and the group with NAFLD (high FLI) presented lower SMI and higher visceral fat area when compared with the lower FLI group, suggesting that the incidence of NAFLD increases as the muscle mass relative to visceral fat decreases. Therefore, this fact could support a favorable role for skeletal muscle in IR and in the development of NAFLD.

Hong *et al*<sup>[15]</sup> evaluated the relationship between sarcopenia and NAFLD, demonstrating a higher risk of NAFLD in those with lower muscle mass after adjusting for confounding factors as IR and inflammation. The individuals with sarcopenia presented more metabolic syndrome, higher C-reactive protein levels and higher body fat mass when compared to those without sarcopenia.

The study of Lee *et al*<sup>[16]</sup> compared sarcopenic and non-sarcopenic patients within obese and non-obese groups of patients. The analysis made it possible to control the effect of obesity on NAFLD and it was the only study that clearly presented an association of sarcopenia

and hepatic steatosis. The prevalence of NAFLD in non-obese sarcopenic patients was more than twice as high as in non-obese non-sarcopenic patients. The proportion of increase in the prevalence of NAFLD comparing obese sarcopenic patients and obese non-sarcopenic patients was remarkably lower. This demonstrates the strong association of sarcopenia and NAFLD in non-obese patients, as well as with fibrosis.

It is worth noting that all three studies included representative samples and performed differing methods of analysis of the outcome, *i.e.*, the relationship between sarcopenia and NAFLD. Even though all three presented multivariable logistic regression analysis, the predictive models were different in all of them, illustrating the complexity and lack of consensus on the factors affecting NAFLD risk. Regardless the model, all of them showed increased risk of NAFLD in the presence of sarcopenia.

More recently, Lee *et al.*<sup>[28]</sup> investigated whether sarcopenia was associated with significant liver fibrosis in the same population. Liver fibrosis was assessed by non-invasive scores as Forns, FIB-4 and NFS. It was observed that sarcopenia was significantly associated with significant liver fibrosis (odds ratio 0.52-0.67;  $P < 0.01$ ) in subjects with NAFLD, independently of obesity and IR.

As possible limitations of the studies, the use of a cross-sectional design limits the possibility to infer causality between skeletal muscle mass loss and NAFLD or NASH<sup>[15,16,20]</sup>, and there was no information regarding the use of smoking status or alcohol consumption<sup>[15]</sup>, which may allow for a bias. Also, no study performed liver biopsy to establish the diagnosis of NAFLD, considered the gold standard in the respective diagnosis<sup>[4,9,15,20]</sup>. Furthermore, the BMI of the patients included in the studies was not so high, varying from 21.4<sup>[20]</sup> to 27.9<sup>[16]</sup>, characterizing overweight and not obesity, and being lower than the BMI of the occidental population<sup>[29]</sup>. This point may be explained by the local ethnic characteristics (all three studies reviewed are Korean studies), limiting the external validity of such studies.

The European consensus<sup>[9]</sup> defined that the CT scan and the magnetic resonance imaging are considered the gold standard to estimate muscle mass. DXA is considered the preferred alternative method, and BIA is a portable alternative to DXA. All the three studies included in the present analysis used the gold standard methods for the diagnosis of sarcopenia, being BIA<sup>[20]</sup> or DXA<sup>[15,16]</sup>.

Of the three articles included in the present systematic review, only the one of Lee *et al.*<sup>[16]</sup> reported the exclusion of approximately 25% of the patients because of missing information about the main variables evaluated (skeletal muscle mass and NAFLD).

Two additional studies were published in 2016, however they were excluded of the present systematic review because of the different primary outcomes of interest. The first was the cross-sectional study of Kim *et al.*<sup>[30]</sup>, evaluating 3739 Korean people, showing that the risk of NAFLD is associated with a low SMI independent

of metabolic risk factors, and may differ according to the age or menopausal status. The other study, of Koo *et al.*<sup>[31]</sup>, evaluated 309 Korean subjects, where the prevalence of sarcopenia was 8.7%, 17.9% and 35.0% in subjects without NAFLD, with NAFLD and with NASH respectively ( $P < 0.001$ ).

There is an independent association between sarcopenia and NAFLD and possibly to an advanced fibrosis. A higher skeletal muscle mass may have a beneficial effect in the prevention of NAFLD, which might be explored by future standardized experimental studies.

## COMMENTS

### Background

Non-alcoholic fatty liver disease (NAFLD) is becoming the most common liver disease worldwide, presenting a higher mortality than the general population. Sarcopenia has been related to an increased risk of NAFLD and advanced fibrosis, and may be associated with worse liver related clinical outcomes. However, this is an understudied issue, and its role on NAFLD has not been fully established. The aim of this study was to perform a systematic review identifying original studies that evaluated the association between sarcopenia and NAFLD in adults.

### Research frontiers

Sarcopenia may occur simultaneously with obesity, particularly the accumulation of visceral fat, which can be related to inflammation, insulin resistance and further reduction in the skeletal muscle mass, consequently causing muscle catabolism. In some conditions, lean body mass is lost while fat mass may be preserved or even increased; this state is called sarcopenic obesity. The skeletal muscle is now recognized as an endocrine organ secreting myokines, and this fact may help to understand its role in the pathogenesis of NAFLD as well as contribute to the development of effective therapeutic options.

### Innovations and breakthroughs

In the present review, all the studies concluded that there was an independent association between sarcopenia and NAFLD. The association of sarcopenia with NAFLD seems to be independent of insulin resistance or obesity. However, it is not possible to establish whether the association between sarcopenia and NAFLD is a cause or an effect.

### Applications

The association between fat accumulation in the liver and in the muscle has just recently been established. The fat content in the paravertebral muscles analyzed by computed tomography may be correlated with aging and steatosis, and a reduction in muscle fat may be associated with an decrease of the liver fat content.

### Terminology

Dual energy X-ray absorptiometry and bioelectric impedance analysis are methods of diagnosis of sarcopenia. Computed tomography using liver attenuation index, as well as the comprehensive NAFLD score, the hepatic steatosis index, the liver fat score and the fatty liver index are non-invasive methods of diagnosis of NAFLD.

### Peer-review

This review is timely as there is emerging evidence and understanding of the association between NAFLD and sarcopenia.

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# Vitamin E for the treatment of children with hepatitis B e antigen-positive chronic hepatitis: A systematic review and meta-analysis

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## Abstract

### AIM

To assess vitamin E efficacy, defined as its ability to induce hepatitis B e antigen (HBeAg) seroconversion, in children with HBeAg-positive persistent hepatitis.

### METHODS

In July 2016, we extracted articles published in MEDLINE and the Cochrane Library using the following search terms: "chronic hepatitis B", "children", "childhood", "therapy", "treatment", "vitamin E", "tocopherols", "tocotrienols". Only randomized controlled trials (RCTs) published in English language were collected.

### RESULTS

Three RCTs met inclusion criteria and were considered in the present meta-analysis. Overall, 23/122 children in the treatment group underwent HBeAg seroconversion vs 3/74 in the control group (OR = 3.96, 95%CI: 1.18-13.25,  $P = 0.025$ ).

### CONCLUSION

Although our meta-analysis has several limits, including the very small number of available studies and enrolled children with HBeAg positivity-related hepatitis, it suggests that vitamin E use may enhance the probability to

induce HBeAg seroconversion in these patients. Further well designed and adequately sized trials are required to confirm or deny these very preliminary results.

**Key words:** Hepatitis B; Pediatric hepatology; Viral hepatitis; Immunology

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**Core tip:** Treatment of chronic hepatitis B in children is still an area of uncertainty. Vitamin E, based on immunostimulatory anti-inflammatory activity, has been evaluated in the treatment of pediatric hepatitis B virus infection. These few experiences seem to be encouraging as they suggest a potential role of vitamin E in inducing HBeAg seroconversion, but they need to be confirmed in well-designed and adequately-sized trials.

Fiorino S, Bacchi-Reggiani ML, Leandri P, Loggi E, Andreone P. Vitamin E for the treatment of children with hepatitis B e antigen-positive chronic hepatitis: A systematic review and meta-analysis. *World J Hepatol* 2017; 9(6): 333-342 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i6/333.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i6.333>

## INTRODUCTION

Chronic hepatitis B virus (HBV) infection continues to represent a very serious health problem worldwide, both in adults and in children<sup>[1]</sup>, despite several efforts to prevent its spread and to reduce its disease burden, such as vaccination programs<sup>[2]</sup>, the use of safe injection techniques and blood donor screening<sup>[3]</sup>, as well as the introduction, in our therapeutic arsenal, of new and more advanced and effective antiviral treatments<sup>[4]</sup>. The clinical relevance of this pathogen depends on its ability to insidiously induce, in a large proportion of infected individuals, a necro-inflammatory hepatic disease with different patterns of severity and course, including cirrhosis and hepatocellular carcinoma. Several factors have been demonstrated to influence the prevalence<sup>[5]</sup>, severity and outcome of patients with HBV-related chronic hepatitis. In particular, ethnicity, mode of acquisition and mainly, age at the time of HBV infection represent the major risk factors for the development of a persistent liver disease<sup>[6]</sup>. Whereas approximately 90%-95% of acutely HBV-infected immunocompetent adults experience a self-limiting hepatitis with the establishment of protective long-lasting immunity, and only the remaining 5%-10% develop chronic hepatitis, neonatal transmission of HBV causes a higher rate of chronic infection<sup>[7]</sup>. Approximately 90% of infected children in highly endemic countries, where vertical transmission from mother to child is predominant, become persistent carriers<sup>[8]</sup>. According to the current knowledge, the natural history of long-lasting HBV infection is chara-

cterized by three chronological phases, including immune tolerance, immune clearance and low replication status.

Despite a large series of available studies, long-term outcomes of HBV infection acquired in infancy are still unclear. However, the spontaneous or therapy-induced hepatitis B e antigen (HBeAg) seroconversion with HBe antibody development is generally considered a key event in patients with long-lasting HBV-related infection<sup>[9]</sup>, because it is often accompanied by remission of liver disease and confers a favorable course over a long-term follow-up<sup>[10]</sup>. After HBeAg loss, serum HBsAg persists, but serum aminotransferase levels usually decrease, reaching normal or low values and a significant reduction in HBV replication is observed in a large part of the subjects undergoing HBeAb development<sup>[11]</sup>. Nevertheless, in some of these patients, the achievement of HBeAg seroconversion is not associated with the improvement of hepatic disease, rather these individuals still present with liver damage with different grades of severity and course and they are at higher risk of developing complications later in life<sup>[12]</sup>. Persistent HBV-related infection is considered as the result of an impaired immune response against this pathogen, consequently the boosting of antiviral immune response has become an innovative strategy in the attempt to obtain the remission of the infection, which still occurs at very low rate<sup>[13,14]</sup>. Starting from this assumption, a randomized controlled pilot trial was performed in 2001 in adult patients with HBV-related hepatitis, showing beneficial effects in anti-viral activities in these individuals<sup>[15]</sup>.

Therefore, on the basis of these findings, we aimed to perform a systematic review and meta-analysis to identify and summarize the current evidence on the potential efficacy of vitamin E in the treatment of children with HBeAg-positive persistent hepatitis, defined as its ability to induce HBeAg loss and HBeAb development.

## MATERIALS AND METHODS

### Search strategy

A systematic computer-based search of published articles, according to the Preferred Reporting Items for Systematic reviews and Meta-Analysis Statement, issued in 2009<sup>[16]</sup>, was conducted through Ovid interface, in order to identify relevant studies on the vitamin E use for the treatment of children with HBeAg-positive chronic hepatitis. The literature review was performed in July 2016. The following electronic databases were used: MEDLINE (1950 to June 30, 2016), the Cochrane Library (until the second quarter of 2016) and EMBASE (1980 to June 30, 2016) for all relevant articles. The search strategy and the search terms were developed with the support of a professional research librarian. The text word used for the search were identified by means of controlled vocabulary, such as the National Library of Medicine's MESH (Medical Subject Headings) and Keywords. The used MESH terms and keywords were: "chronic hepatitis B", "children", "childhood", "therapy", "treatment", "vitamin E", "tocopherols", "tocotrienols". The PubMed "related articles"

feature as well as the reference lists of retrieved articles was also searched to find additional pertinent studies. If a study was considered potentially eligible by either of the two reviewers, the full-text of this study was further evaluated. Full-text assessment was performed according to eligibility criteria developed to systematically include studies into this review. Selected studies were considered eligible if all the following predefined criteria were met: (1) the research was designed to assess the tolerability and efficacy of vitamin E use for the treatment of children with HBeAg-positive chronic hepatitis; (2) the research studies were designed as randomized studies; (3) the studies were reported in the English language, as peer-reviewed, full-text publications, whereas articles that were not published as full reports, such as conference abstracts, case reports, and editorials were excluded; and (4) sufficient data for the evaluation of HBeAg seroconversion rate were available (Table 1).

### Study selection

Data extraction: Two authors (Leandri P and Loggi E), independently and in a parallel manner, performed the literature search, identified and screened relevant articles, based on title or title and abstract. If a study was considered potentially eligible by either of the 2 reviewers, the full article was collected for further assessment. Other two authors (Bacchi-Reggiani ML and Andreone P) independently extracted and tabulated all relevant data from included studies by means of a standardized method, according to the Cochrane handbook section 7.3a checklist of domains. The following information was obtained from each study, by means of a predefined data extraction form, including: First author's name, study design, inclusion and exclusion criteria, year of publication, country of origin, ethnicity, number of cases and controls, diagnostic methods for HBV markers and genome detection. We contacted the authors of the three studies to obtain additional information, including children's seroconversion rate by age groups and by HBV genotype. Unfortunately, access to patients' database was possible only for one study, whereas the above mentioned data, concerning the other two trials, were no longer available for the time period elapsed since their publication. The accuracy of data collection was checked by SF and any disagreements concerning the results were settled by consensus between all authors.

**Quality score assessment:** Three investigators (Fiorino S, Bacchi-Reggiani ML and Andreone P) independently evaluated the quality of the selected studies, on the basis of the Jadad scale<sup>[17]</sup> (Table 2). It includes the assessment of the following three items: Randomization (2 points as maximum score), blinding (2 points as maximum score) and an account for all patients (1 point as maximum score).

Any disagreement was resolved by discussion with the other authors. Total score ranges from 0 to 5. In-

cluded studies were classified into higher quality ( $\geq 3$ ) and lower quality ( $< 3$ ), on the basis of the total scores.

### Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) were used as the summary statistic. The pooled OR was calculated with both fixed effect (inverse variance weighting) and random effect (Der Simonian and Laird) models. To avoid the exclusion of one among the eligible studies<sup>[18]</sup>, a 0.5 zero-cell correction was used. The variability, expressed in percentage across studies and attributable to heterogeneity beyond chance, was estimated with  $I^2$  statistic. We assessed the extent of small study effects by Egger's test.  $P$ -values  $\leq 0.05$  were considered significant for all included studies. Statistical analyses were carried out using STATA/SE version 14.1 (STATA Corp., College Station, TX, United States).

## RESULTS

### Study selection

We searched in MEDLINE, EMBASE, and Cochrane Library to retrieve works assessing vitamin E use in children with HBeAg-positive chronic hepatitis, our systematic review identified 2471 potential studies, and 2458 of them were excluded after a preliminary review of the titles and/or abstracts. The full texts of the remaining 13 articles were retrieved for a more detailed assessment. Of these, 10 studies did not meet the eligibility criteria, as described, because they were reviews, clinical studies carried out in adults or they were not published in the English language. Therefore, three studies were selected and included in the current meta-analysis. A flow-chart of the study search and selection process is shown in Figure 1.

### Study characteristics

Three randomized controlled studies, assessing the use of tocopherols in children with HBV-related chronic infection and rate of HBeAg seroconversion, met inclusion criteria and were considered in the present meta-analysis<sup>[18-20]</sup>.

Overall, the three trials involved 122 children, randomized to receive treatment according to an intention-to-treat protocol with different doses of vitamin E, and 74 controls. One study was performed in Turkey, one in Germany and one in Italy. The baseline characteristics of these included studies and their participants are summarized in Table 1. All considered studies described serological assays, which were employed to detect viral infection markers. Serum samples were tested for the presence of both viral antigens and host antiviral antibodies, using both enzyme-linked immunosorbent assay or radioimmunoassay, as well as of HBV-DNA, by means of liquid hybridization<sup>[18]</sup> or real time PCR<sup>[19,20]</sup>.

Overall, according to the Jadad scale, two trials were classified into higher quality<sup>[19,20]</sup> and one into lower quality<sup>[18]</sup> studies (Table 1).

The end-point of this meta-analysis was to estimate the potential antiviral efficacy of vitamin E, defined as its



**Table 1** Characteristic of included studies evaluating the use of vitamin E in children with hepatitis B e antigen-positive chronic hepatitis

First author/ year	Study design/ country of origin	Sample size	Treat and VE dose	Treat period/ follow-up	End-points	Main results	Quality score <sup>3</sup>	Tolerability
Dickici B, 2007	PRT (1:1) Turkey	58 enrolled children in the immune-tolerant phase (1) 30 treated patients M/F: 21/9 (2) Age (yr): (9.0 ± 3.8) No data concerning age 28 untreated patients M/F: 23/5 Age (yr): (8.5 ± 4.5) No data available for children's seroconversion rates by age groups and by HBV genotype	100 mg/d <i>vs</i> no treatment	3 mo/6 mo	HBV-DNA clearance HBeAg seroconversion	No antiviral-effects induced by vitamin E treatment	1	No side effects
Gerner P, 2008	PRT (3:1) Germany	92 enrolled children # (1) 69 in treatment group (2) 23 in placebo group 76 children completed the study (1) 56 in treatment group M/F: 34/22 Age (yr): 10.4 (2) 20 in placebo group M/F: 12/8 Age (yr): 11.8 No data available for children's seroconversion rates by age groups and by HBV genotype	From 200 to 600 IU/d depending on body weight <i>vs</i> placebo	6 mo/12 mo	HBV-DNA clearance HBeAg loss HBeAg seroconversion	VE may induce HBeAg seroconversion, but further studies are required	5	Well-tolerated Self-limited gastroenteritis cases
Fiorino S, 2016	PRT (1:1) Italy	46 enrolled patients (1) 23 in treatment group (18 in immune- tolerant phase and 5 in immune-reactive group) (2) 23 in placebo group (17 in immune-tolerant phase and 6 in immune- reactive group) 40 children completed the study  (1) 20 in treatment group M/F: 15/5 Age (yr): (11.9 ± 3.8) (2) 20 in placebo group M/F: 16/4 Age (yr): (10.2 ± 3.5) HBeAg seroconversion in vitamin E Age (yr)/number pts/ genotype 2/0 3/0 4/1 (D <sup>1</sup> ) 5/0 6/2 (1A, 1D)	15 mg/kg per day <i>vs</i> no treatment	12 mo/ 12 mo	(1) safety and tolerability (2) HBeAg loss/anti- HBe seroconversion (3) efficacy of VE in inducing: (1) $\geq 2 \log_{10}$ sustained decrease in serum HBV-DNA <i>vs</i> baseline	VE may induce HBeAg seroconversion, but further studies are required	3	Generally good safety profile Self-limited gastroenteritis (nausea, vomiting, upper abdominal pain, diarrhoea), headache, fatigue Adverse events: ALT flare

7/0
8/3 (1C, 2D)
9/0
10/2 (1C, 1D)
11/0
12/1 (1D)
13/6 (1A, 1C, 4D <sup>2</sup> )
14/3 (1A <sup>1</sup> , 1D, 1NA <sup>1</sup> )
15/0
16/2 (1D, 1F)
17/3 (3D <sup>3</sup> )
HBeAg seroconversion in the control group
Age (yr)/number pts/ genotype
2/1 (B)
3/0
4/1 (D)
5/1 (A)
6/1 (D)
7/0
8/3 (1C, 2F <sup>1</sup> )
9/1 (D)
10/1 (C)
11/5 (1A, 2C, 2D)
12/2 (1A, 1E)
13/2 (1B, 1D)
14/4 (2A, 2NA)
15/1 (D)
16/0
17/0

<sup>1</sup>Identifies 1 patients undergoing HBeAb seroconversion in a subgroup of patients; <sup>2</sup>Identifies 2 patients, who underwent HBeAg seroconversion; <sup>3</sup>According to the Jadad scale. PRT: Prospective randomized trial; IT: Immune-tolerant; IR: immune-reactive; VE: Vitamin E; CVR: Complete virological response, defined as sustained HBeAg loss/anti-HBe seroconversion together with serum HBV-DNA reduction < 2000 IU/mL; ALT: Alanine-aminotransferase; NA: Not available; HBeAg: Hepatitis B e antigen; M: Male; F: Female.

ability to induce HBeAg loss and HBeAb development. A pooled estimate was performed on the basis of the considered studies. Overall, we observed that vitamin E use induced HBeAg seroconversion in 18.8% patients (23 seroconversion among 122 patients in the treated children vs 3 among 74 controls, OR = 3.96, 95%CI: 1.18-13.25,  $P = 0.025$ ,  $I^2 = 0.0\%$ ) (Figure 2). Children's seroconversion rates by age groups and by HBV genotype were available only for one of the three trials (Table 1).

### Sensitivity analysis and publication bias risk

To evaluate the effect of each individual study on the overall meta-analysis estimate, one study at a time was excluded, but the exclusion of any single research did not cause a significant alteration of the final decision and did not suggest the possibility of publication bias, but the number of considered studies is very small and this factor suggests caution in data interpretation.

## DISCUSSION

The natural history of HBV-related persistent infection in childhood as well as the indications for the appropriate use of the antiviral therapy still represent an area of considerable uncertainty in this subset of patients, although an expert panel consensus<sup>[21]</sup> and some meta-analyses<sup>[22,23]</sup> have been proposed to guide physicians in the treatment decision-making process. As no definitive guidelines exist,

according to available recommendations, the criteria for the management of these subjects in the real-life clinical setting have to be carefully evaluated before initiating any type of antiviral therapy. In particular, treatment is considered appropriate in HBeAg-positive children with persistently elevated (about 1.5-2.0 times the laboratory upper limit of normal), or exceeding 60 IU/L serum alanine aminotransferase and moderate/severe inflammation/fibrosis on liver biopsy<sup>[8]</sup>. Some drugs, such as interferon- $\alpha$  (IFN- $\alpha$ )<sup>[24]</sup>, lamivudine<sup>[25]</sup>, entecavir<sup>[26]</sup>, adefovir<sup>[27]</sup> and tenofovir<sup>[28]</sup> have been approved for these patients. However, their use is limited by important side effects, risk of resistance development, high costs and, mainly, age of the treated subjects<sup>[29]</sup>. Most of these antiviral compounds have been licensed for children over 12 years old and only IFN- $\alpha$  as well as entecavir have been approved for a pediatric population aged between 2 and 18 years<sup>[24,26]</sup>. In addition, to date, no therapy is considered appropriate in HBeAg-positive children in the immune tolerance phase. A risk of resistance development to antiviral drugs exists in this group of patients<sup>[21]</sup>. To date, on the basis of these results, a long-term follow-up is the only approach currently applied to these children and further therapeutic approaches or treatment schedules are required<sup>[30]</sup>.

Therefore, according to preliminary observations on the beneficial role of vitamin E as therapy in patients with chronic hepatitis B in a randomized controlled pilot

**Table 2** Jadad scale is a procedure for assessing the methodological quality or risk of bias in randomized controlled trials, adapted from Jadad *et al.*<sup>[17]</sup>

Item	Maximum points	Description
Randomization	2	1 point if randomization is mentioned 1 additional point if the method of randomization is appropriate 1 point has to be deducted if the method of randomization is inappropriate (minimum 0)
Blinding	2	1 point if blinding is mentioned 1 additional point if the method of blinding is appropriate 1 point has to be deducted if the method of blinding is inappropriate (minimum 0)
An account of all patients	1	The fate of all patients in the trial is known, if no data are reported the reason is stated

trial several years ago<sup>[15]</sup>, we had the aim to understand whether this compound could exert useful and safe therapeutic effects against HBV in childhood. We performed a systematic review and meta-analysis to search studies available in the literature assessing vitamin E ability to induce HBeAg seroconversion in children with HBeAg-positive persistent hepatitis. We found only three trials<sup>[18-20]</sup> meeting inclusion criteria for our meta-analysis, but, surprisingly, two of them reported high rates of HBeAg loss and HBeAb development<sup>[19,20]</sup>.

In particular, in Fiorino's trial a very high percentage of children (7/23, 30.4%), receiving vitamin E supplementation for 12 mo with a follow-up period of further 12 mo, obtained HBeAg clearance vs 1/23 (4.3%), in the control arm at the end of the follow-up period (24 mo)<sup>[20]</sup>. However, it has to be considered that a substantial percentage of HBeAg loss (23.2%) was also observed in the group of vitamin E-treated children for 6 mo with an additional follow-up period of 12 mo in comparison to the placebo arm (8.7%) in Gerner's research<sup>[19]</sup>. HBeAg seroconversion rates obtained in the above mentioned studies are higher in comparison to those observed in trials enrolling patients who were treated with nucleotide/nucleoside analogues. A recent research performed by Chan *et al.*<sup>[31]</sup>, using TDF (tenofovir disoproxil fumarate) or TDF + FTC (emtricitabine) in adult immune-tolerant patients, showed low percentage of HBeAg loss and HBeAb development (5% and 0% respectively) over 192 wk. The reasons explaining these high seroconversion rates in children supplemented with vitamin E in comparison to subjects treated with TDF analogs, mainly in Fiorino's study, are not completely understood and deserve further investigations. Nevertheless, some very interesting issues have to be considered: (1) different mechanisms of vitamin E and nucleotidic/nucleosidic analogue action; (2) duration of the treatment period; and (3) vitamin E dosage used.

Nucleos(t)ide analogues exert antiviral activities by means of well-known direct as well as indirect mechanisms: (1) suppression of HBV replication mainly through the inhibition of reverse transcription process in the viral lifecycle<sup>[32]</sup>; (2) partial restoration of the impaired

Potentially relevant citations identified through literature search,  $n = 2471$   
 PubMed,  $n = 2456$   
 Cochrane Library  $n = 15$   
 Key words: "chronic hepatitis B", "children", "childhood", "therapy", "treatment", "vitamin E", "tocopherol", "tocotrienol"  
 Limits: Search restricted to peer-reviewed, full-text publications, written in English language (articles not published as full reports, such as conference abstracts, case reports, and editorials were excluded)

Excluded articles based on title and abstract:  $n$

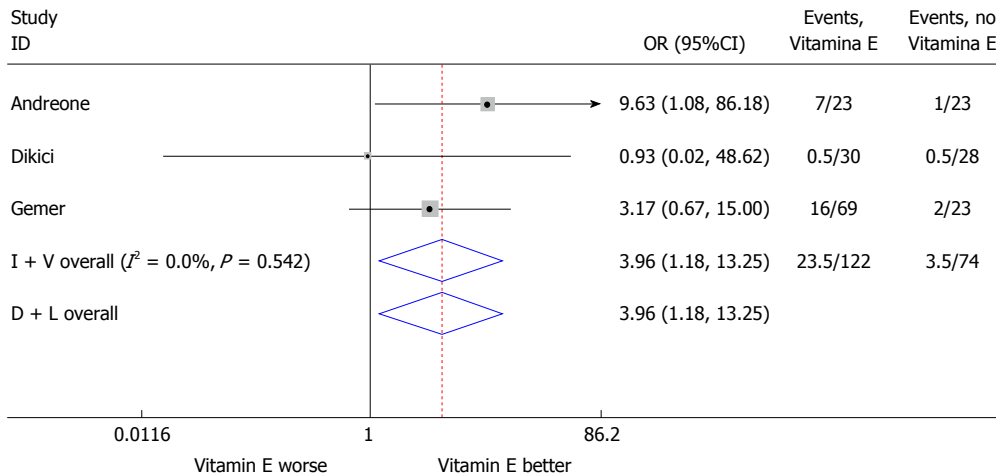
Potentially relevant articles:  $n = 13$

Articles excluded:  $n = 10$   
 Reviews:  $n = 6$   
 Not published in English language:  $n = 1$   
 Irrelevant:  $n = 3$

Articles included in the study:  $n = 3$   
 Randomized controlled trials: 3

**Figure 1** Study flow diagram concerning vitamin E use in children with hepatitis B e antigen positive chronic hepatitis.

immune response, as shown by significant reductions in the percentages of CD4<sup>+</sup>CD25<sup>high</sup> T regulatory cells, programmed death-ligand 1 (PD-L1) receptor expression on CD4<sup>+</sup> T cells and pro-inflammatory cytokine production<sup>[33,34]</sup>. Therefore, antigen-viral burden reduction is associated with the improvement of anti-HBV activity of immune cells. According to our current knowledge, vitamin E exerts its beneficial effects by means of similar mechanisms. It is well known that vitamin E plays critical roles for normal cellular functions<sup>[35]</sup>. This essential lipid-soluble compound, acting as a free radical "scavenger", exerts potent antioxidant effects<sup>[35]</sup> in all cellular membranes<sup>[36]</sup> and contributes to their protection from oxidative injury<sup>[37,38]</sup>. In addition, *in vitro* and *in vivo* studies have suggested that vitamin E is also able to improve immune-system functions<sup>[39]</sup>, mainly by increasing cell-mediated-immunity<sup>[40,41]</sup>. Mechanisms involved in these complex processes remain poorly understood, but, a recent systematic review has summarized its possible intracellular targets and suggested its potential direct antiviral or immunostimulating activities against HBV<sup>[42]</sup>. Vitamin E is able to influence the transcriptional function of some cellular genes by directly interacting with their promoter sequences<sup>[42-44]</sup> and it may modulate the post-transcriptional regulation of protein synthesis<sup>[45-47]</sup>. In particular, very preliminary studies have demonstrated that vitamin E is able to regulate the expression profiles of some microRNAs, but it is conceivable that it interacts with a larger number of these molecules<sup>[41,43,44]</sup>. In par-



**Figure 2** The relationship between vitamin E use and hepatitis B e antigen seroconversion in published studies. The area of each black square is proportional to the statistical size and the centre of the square is placed at the point of estimate. Error bars indicate 95% CIs for the estimate for each study. I + V: Inverse variance method; D + L: DerSimonian and Laird method.

particular, it has been reported that vitamin E increases the expression of 8 oxidative stress-associated miRs, including miR-16, miR-21, miR-122, miR-125b, miR-146a, miR-155, miR-181a, miR-223<sup>[44]</sup>. The tight modulation of the synthesis of these miRs, due to their pleiotropic effects, might increase the protective immune response against the virus. Some microRNAs, which are modulated by vitamin E, possess a double function. They are required not only to preserve the normal function of the immune system<sup>[48]</sup>, mainly of the cell-mediated arm<sup>[49]</sup>, and to prevent the development of a redundant and abnormal inflammatory response<sup>[50,51]</sup>, but they also exert a direct anti-HBV activity<sup>[52]</sup>. These premises may help us to explain the positive preliminary results of our meta-analysis. It is conceivable to think that vitamin E supplementation may contribute to the improvement of the anti-HBV activity of the immune system by means of a direct anti-viral action both by decreasing its replication abilities and by boosting the host's immune cell responses. The slow but progressive decline of HBV replication associated with HBeAg loss/HBeAb seroconversion, as described in 2 of the 3 studies included in our meta-analysis, corroborates the hypothesis that vitamin E acts as an immunomodulator resulting in a global antiviral activity. Interestingly, a delayed response has already been reported, with the use of immunomodulatory drugs for the treatment of adults with CHB<sup>[53]</sup>. In Fiorino's study, HBeAg seroconversion was also observed in additional 7 of 11 previously non-responder patients in the vitamin E supplemented group, with an extended follow-up for an additional period of 12 mo<sup>[20]</sup>. This observation underlines the importance of the duration of the treatment period and might contribute to explaining, at least in part, the absence of children responding to vitamin E therapy in Dickici's study<sup>[18]</sup>. In this trial, the period of supplementation with this compound was only three months long, in addition the dosage used was equal to 100 mg/d for three months, probably not enough to induce an improvement of anti-

viral immune responses. Therefore, both dosage and duration of vitamin E supplementation represent very important factors that must be considered when its anti-viral efficacy is evaluated. Taking into considerable account all the described factors, our study seems to suggest that vitamin E use may effectively enhance the probability to induce HBeAg seroconversion in these patients, even in children in the immune-tolerant phase. However, it has to be considered that our meta-analysis has several limits. First, the very small number of available studies enrolling children with HBeAg positivity-related hepatitis, as well as the small size of these trials may increase publication and selection biases; second, one research study has been carried out in Asia<sup>[18]</sup> and 2 studies in Europe<sup>[19,20]</sup>, it is not known whether differences in response rates, following vitamin E use, may exist among children belonging to different ethnicities or depending on the different prevalence of HBV genotypes; third, the study design did not include the use of placebo in control groups in two studies<sup>[18,20]</sup>; fourth, it has to be taken into account that HBeAg seroconversion rate in children in the immunetolerant phase is < 2% among children younger than 3 years and 4%-5% among older children<sup>[54]</sup> and that data concerning HBV genotypes as well as children's seroconversion rates by age groups were available only for one of the studies considered for the meta-analysis. Therefore, this limit precludes further proper assessments of the potential vitamin E benefits in children under 14 years as well as of its potential efficacious dose. However, in the study by Fiorino *et al.*<sup>[20]</sup>, patients who responded to vitamin E treatment were respectively 4 (1 child), 13 (2 children), 14 (2 children), and 17 (2 children) years old at the enrollment (Table 1).

In addition, it has to be considered that some meta-analyses<sup>[55,56]</sup> have reported that long-term administration of vitamin E, at dosages exceeding 150 UI once a day, is associated with serious negative outcomes, such as an increase in all-cause mortality. However, although the conclusions of these studies are rather questionable<sup>[57-60]</sup>



because of the meta-analytic approaches used and no severe side effects have been described in the three included trials, with the exception of some adverse events, represented by flares of transaminases, these results suggest caution in the generalization of vitamin E administration in the pediatric population before confirmation of the effectiveness and safety of this compound. Certainly, vitamin E, which was administered at high dosage to the children in the reported trials, has to be considered as a drug, with possible benefits as well as with potential risks. Therefore, to date these limiting factors prevent the formulation of definitive recommendations on the role of this type of treatment in children with HBeAg-positive hepatitis. However, to our knowledge this is the first attempt to quantitatively assess the therapeutic effects and efficacy of vitamin E in the treatment of these subjects. These patients suffer from a viral-related liver disease, a condition associated with a deficiency in immune system responses. Therefore, the use of vitamin E in patients with HBV chronic infection might represent an approach combining the direct antiviral effect of nucleoside/nucleotide analogues on HBV replication together with the immune-enhancing role of this fat-soluble compound. Therefore, further well-designed and adequately-sized trials are required to confirm or deny these preliminary, but apparently very interesting and promising, results with the aim to establish the potentially useful dose of vitamin E to induce HBeAg seroconversion.

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## COMMENTS

### Background

Hepatitis B virus (HBV) chronic infection in childhood continues to represent a very important clinical problem in several countries worldwide and the treatment indications for this subset of patients are still an area of considerable uncertainty. Clear-cut guidelines are lacking, therefore, to date, therapeutic decisions are only based on the consensus of expert panels.

### Research frontiers

A defective immune response is considered a crucial factor in maintaining a persistent infection in subjects with HBV. Drugs able to restore an adequate immune activity could contribute to promote an effective control of this infection.

### Innovations and breakthroughs

In the real-life clinical setting, a wide proportion of children with persistent HBV infection remain untreated and a conservative approach is generally applied in these subjects. Therefore, the introduction of additional therapies and different strategies in clinical practice is required. Since several years ago, vitamin E has emerged as a compound with a large spectrum of immune-stimulatory activities and its use could improve the defective immune activity detectable in several diseases, including chronic viral infections. This research field could be very

interesting, but this kind of application requires caution. Some reports, although rather questionable, report possible harmful effects, when this compound is used at high dose.

## Applications

Vitamin E administration could represent one among the options for the treatment of children with HBV-chronic infection, either as mono-therapy in the developing countries with limited economic resource or, for the future, in combination with standard antiviral drugs. The decrease in viral replication and antigen burden as well as the restoration of an adequate immune function might represent an effective approach for the management of patients with HBV persistent infection.

## Peer-review

The authors underwent a meta-analysis to evaluate the role of vitamin E administration to children with immune tolerant HBV infection. Results showed a nearly four-fold likelihood of achieving HBeAg seroconversion in those children receiving vitamin E. Sensitivity analysis showed that exclusion of any of the three studies did not change results.

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## Hyperammonemia crisis following parturition in a female patient with ornithine transcarbamylase deficiency

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**Author contributions:** Kido J and Nakamura K designed the report; Kido J, Kawasaki T, Mitsubuchi H, Kamohara H, Ohba T, Matsumoto S and Endo F collected the patient's clinical data; Kido J and Nakamura K analyzed the data and wrote the paper.

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### Abstract

Ornithine transcarbamylase deficiency (OTCD) is an X-linked disorder, with an estimated prevalence of 1 per 80000 live births. Female patients with OTCD develop metabolic crises that are easily provoked by non-predictable common disorders, such as genetic (private mutations and lyonization) and external factors; however, the outcomes of these conditions may differ. We resuscitated a female patient with OTCD from hyperammonemic crisis after she gave birth. Hyperammonemia after parturition in a female patient with OTCD can be fatal, and this type of hyperammonemia persists for an extended period of time. Here, we describe the cause and treatment of hyperammonemia in a female patient with OTCD after parturition. Once hyperammonemia crisis occurs after giving birth, it is difficult to improve the metabolic state. Therefore, it is important to perform an early intervention before hyperammonemia occurs in patients with OTCD or in carriers after parturition.

**Key words:** Brain image; Delivery; Glutamine; Amino acid; Ornithine transcarbamylase deficiency; Urea cycle disorders; Uterus; Hyperammonemia



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**Core tip:** Hyperammonemia crisis after parturition in patients with ornithine transcarbamylase deficiency (OTCD) is often fatal and difficult to predict. It is important to perform early intervention before hyperammonemia occurs in patients with OTCD or in carriers after parturition.

Kido J, Kawasaki T, Mitsubuchi H, Kamohara H, Ohba T, Matsumoto S, Endo F, Nakamura K. Hyperammonemia crisis following parturition in a female patient with ornithine transcarbamylase deficiency. *World J Hepatol* 2017; 9(6): 343-348 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i6/343.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i6.343>

## INTRODUCTION

Urea cycle disorders (UCDs) are one of the most common inherited metabolic diseases in Japan, with an estimated prevalence of 1 per 50000 live births. The urea cycle is the metabolic pathway that eliminates excess endogenous and exogenous nitrogen from the body by modifying ammonia into urea, thereby reducing its toxicity. This cycle comprises 6 different enzymes, including ornithine transcarbamylase (OTC; EC 2.1.3.3).

OTC deficiency (OTCD; Mc Kusick No. 311250) is an X-linked disorder, with an estimated prevalence of 1 per 80000 live births<sup>[1]</sup>. The traditional treatment for OTCD is a low-protein diet. Sodium benzoate and/or sodium phenylbutyrate are significant as an alternative pathway therapy<sup>[2,3]</sup>.

In female patients with OTCD, metabolic crises can be easily provoked by non-predictable common disorders, such as genetic (private mutations and Lyonisation) and external factors, and sometimes may be fatal.

We resuscitated a female patient with OTCD, who maintained a relatively stable condition using a self-restricted protein diet, from hyperammonemic crisis after parturition. Hyperammonemia after giving birth in a female patient with OTCD can be fatal<sup>[4]</sup>, and this type of hyperammonemia persists for an extended period of time. If patients with OTCD develop hepatic coma with hyperammonemia  $\geq 300$   $\mu\text{mol/L}$ , hemodialysis as well as treatments that target alternative nitrogen metabolism pathways and arginine or/and citrulline treatments should be used to control blood ammonia levels immediately to avoid damage to the brain<sup>[5-7]</sup>. We discontinue hemodialysis and control blood ammonia levels using the alternative pathway therapy, and arginine or/and citrulline treatments when their blood ammonia levels decrease to  $< 180$   $\mu\text{mol/L}$ <sup>[8,9]</sup>. Hemodialysis is excellent for the removal of ammonia in the body; however, this treatment does not suppress the production of ammonia and removes useful medications

from the body. Moreover, although a high-caloric infusion that largely consists of glucose is important, the early administration of essential amino acids and low-protein is useful for preventing protein catabolism in the body and for controlling ammonia levels<sup>[10]</sup>.

Here, we discuss the cause and treatment of hyperammonemia in a female patient with OTCD after parturition.

## CASE REPORT

A 37-year-old female patient who was diagnosed with late-onset OTCD was followed by her doctor, and was introduced to our institute after pregnancy. She was the first child of nonconsanguineous parents and had no family history of hyperammonemia. She presented with seizures at the age of 7 and developed hyperammonemia following the administration of valproic acid as treatment for the seizure. She was diagnosed with OTCD by liver biopsy examination (liver OTC enzyme activity that was 30% the level of healthy patients) (Table 1). She had visited the emergency room previously due to hyperammonemia and had undergone hemodialysis for impaired consciousness at the age of both 18 years and 21 years. Despite this, she followed a self-restricted protein diet and L-carnitine treatment. She naturally became pregnant at the age of 37 years and delivered an unaffected male baby by vacuum extraction at 41 wk and 1 d of gestation. Her blood ammonia value was 68  $\mu\text{mol/L}$  at delivery, 59  $\mu\text{mol/L}$  at 10 h after delivery, and 54  $\mu\text{mol/L}$  at 24 h after delivery. She developed hyperammonemia (194  $\mu\text{mol/L}$ ) 4 d after delivery, but was discharged 6 d after delivery because her blood ammonia level decreased to 60  $\mu\text{mol/L}$  following arginine and citrulline treatment. Upon discharge, she consumed a hamburger at a fast food restaurant and 250 g of beef at her home. She was hospitalized on an emergency basis at midnight because of hyperammonemia (180  $\mu\text{mol/L}$ ) with impaired consciousness (Table 2). Blood ammonia levels instantly decreased to 82  $\mu\text{mol/L}$  by the continuous administration of arginine. She then underwent hemodialysis and continuous hemodiafiltration under respirator management in the intensive care unit because her blood ammonia level increased to 339  $\mu\text{mol/L}$ , with a grade III hepatic coma. Moreover, she received a high-calorie infusion (2500 kcal/d), arginine (80 mg/kg per day), citrulline (150 mg/kg per day), sodium benzoate (150 mg/kg per day), and sodium phenylbutyrate (140 mg/kg per day) as an alternative pathway therapy. Following this, her hyperammonemia improved.

Although extubation was attempted because of stable blood ammonia levels following these treatments (Figure 1), she was reintubated because she could not maintain respiration. Her ammonia levels increased again to 210  $\mu\text{mol/L}$  after experiencing physical stress during extubation, before gradually decreasing to 60  $\mu\text{mol/L}$ . She developed sepsis and was managed at the

**Table 1** Data at diagnosis

	Serum AA (nmol/mL)	Uric AA (nmol/mg Cre)	Cerebrospinal fluid AA (nmol/mL)
Amino acids			
Glutamine	1754.6	282.9	534.1
Glutamic acid	90.8	277.9	TR
Ornithine	60.3	TR	5.8
Citrulline	19.7	ND	ND
Arginine	54.4	ND	14.2
Lysine	162.8	ND	15.9
Urinary orotic acid	13.3 µg/mg Cre		
Liver enzyme assay	Patient (µmol/mg protein/min)	Control	
CPS1	0.024	0.023-0.074	
OTC	0.17 (30% of the control)	0.51-1.51	

AA: Amino acids; Cre: Creatinine; TR: Trace; ND: Not determined; CPS1: Carbamoyl-phosphate synthetase 1; OTC: Ornithine transcarbamylase.

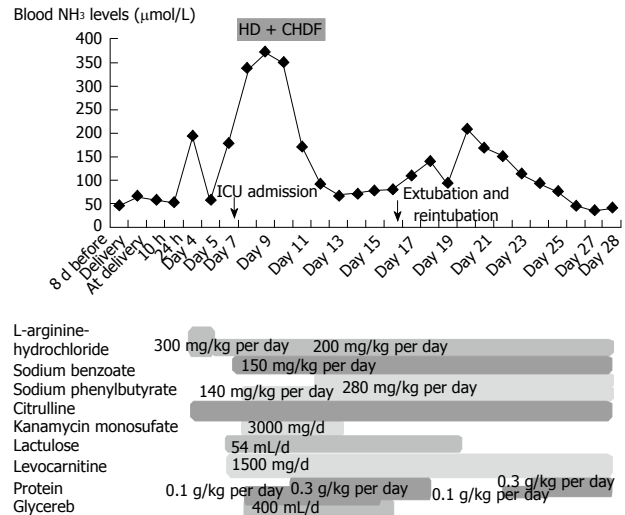
**Table 2** Laboratory data upon admission

AST	28 (IU/L)
ALT	34 (IU/L)
γGTP	19 (IU/L)
LDH	369 (IU/L)
ALP	338 (IU/L)
CHE	219 (IU/L)
T-Bil	0.6 (mg/dL)
TP	6.8 (g/dL)
Alb	3.3 (g/dL)
BUN	12.3 (mg/dL)
Cre	0.44 (mg/dL)
NH <sub>3</sub>	180 (µmol/L)
Amy	90 (IU/L)
CK	111 (IU/L)
CRP	0.3 (mg/dL)
WBC	12000 (/µL)
Hb	10.7 (g/dL)
Plt	26.4 × 10 <sup>4</sup> (/µL)
PT	112 (%)
APTT	97 (%)
P-FDP	37.2 (µg/mL)
Fib	347 (mg/dL)
AT III	134 (%)

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γGTP: γ glutamyl transpeptidase; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; CHE: Choline esterase; T-Bil: Total bilirubin; TP: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; Cre: Creatinine; Amy: Amylase; CK: Creatine kinase; CRP: C-reactive protein; WBC: White blood cell count; Hb: Hemoglobin; Plt: Platelets; PT: Prothrombin time; APTT: Activated partial thromboplastin time; P-FDP: Plasma fibrin degradation products; Fib: Fibrinogen; AT III: Antithrombin III.

intensive care unit for 43 d.

The cause of hyperammonemia for this case was considered to be: (1) physical stress experienced during vaginal parturition and fasting while withstanding pain for a prolonged period of time; (2) excessive protein intake after discharge; and (3) metabolic changes during puerperium following anabolism for the repair of the parturient canal, including the uterus, after delivery and



**Figure 1** Blood ammonia levels after delivery. The patient was admitted on emergency basis because of impaired consciousness 7 d after delivery (day 7) and was intubated. She then underwent HD and CHDF under ICU management (from day 8 to day 12). HD: Hemodialysis; CHDF: Continuous hemodiafiltration; ICU: Intensive care unit.

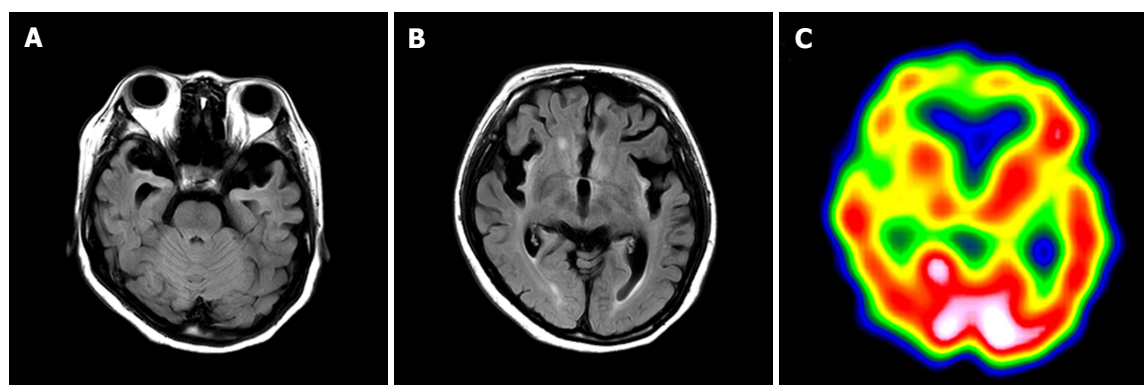
catabolism for producing maternal milk.

Presently, she uses medication and has been able to raise her child following hospital discharge despite her brain magnetic resonance imaging and single-photon emission computed tomography indicating atrophy of the bilateral frontal and temporal lobes with decreased bold flow (Figure 2).

## DISCUSSION

We resuscitated a female patient with late-onset OTCD who developed hyperammonemia crisis after giving birth. We previously reported the long-term outcome for patients with OTCD in Japan<sup>[8,9,11]</sup>. The expected survival rate at 35 years of age in late-onset OTCD patients was less than 30% for both male and female patients according to data obtained from 1978-1995<sup>[8]</sup>, and 89% for male patients and 84% for female patients according to data obtained from 1999-2009<sup>[9]</sup>. The more recent long-term outcome improved compared to previous outcomes due to improved medication, hemodialysis, and liver transplantation; however, the long-term survival of patients is not guaranteed. These patients always have the potential to develop hyperammonemia due to metabolic stress during infection, surgery or delivery, even if the hyperammonemia is resolved at the time of onset and their medical state is well controlled thereafter.

It has been reported that female patients with OTCD are likely to develop hyperammonemia that persists for 6 to 8 wk at 3 to 14 d after delivery<sup>[12,13]</sup>. Although the cause of hyperammonemia after delivery in female patients with OTCD is not clear, it is considered to be related to increased protein load for collagen catabolism following involution of the uterus<sup>[14]</sup>. We also consider that hyperammonemia is related to metabolic changes in



**Figure 2** Brain magnetic resonance imaging and single-photon emission computed tomography 7 mo after delivery. A and B: FLAIR demonstrates atrophy of the bilateral frontal and temporal lobes, and a high signal for the cortex and subcortex on both sides of insula, the ventral temporal lobe and the frontal lobe bottom. The signal is elevated bilaterally in the putamen, caudate nucleus, and front globus pallidus; C: Single-photon emission computed tomography shows bilaterally decreased blood flow in the frontal lobe, ventral temporal lobe, basal ganglia, and thalamus.

their bodies during puerperium.

She developed hepatic coma that required respiratory management, which required 40 d to resume breathing without assistance and to regain improved level of consciousness. She continued to produce maternal milk a month after the onset of hyperammonemia, and hyperammonemia persisted even after maternal milk production stopped. The cause of long-term hyperammonemia is not only puerperium. Mitochondrial abnormalities within the liver of patients with OTCD has been previously demonstrated<sup>[15]</sup>, and hyperammonemia itself disrupts the function of mitochondria<sup>[16]</sup>. Therefore, it has been considered that the impaired mitochondria in cases of hyperammonemia aggravates OTCD and results in long-term hyperammonemia.

In this case, her blood ammonia levels remained at 60  $\mu\text{mol/L}$  from during pregnancy to 3 d after delivery and elevated at 4 d after delivery. She did not receive sodium benzoate or sodium phenylbutyrate, which are utilized as alternative pathway therapies, because her blood ammonia levels soon decreased after arginine and citrulline treatment. We consider that even female patients with OTCD who do not receive sodium benzoate or sodium phenylbutyrate therapy are recommended to start receiving sodium benzoate or sodium phenylbutyrate treatment from the start of labor to immediately after delivery, if they have developed severe hyperammonemia<sup>[17]</sup>. Benzoate is conjugated with glycine to form hippurate, which is rapidly excreted in the urine. For each mole of benzoate administered, 1 mole of nitrogen is removed. Phenylbutyrate is activated to the CoA ester, which is metabolized by  $\beta$ -oxidation in the liver to form phenylacetyl-CoA, which is then conjugated with glutamine. The resulting phenylacetylglutamine is excreted into the urine, and 2 moles of nitrogen are excreted for each mole of phenylbutyrate<sup>[5]</sup>.

Therefore, it is effective for OTCD patients who have never received sodium phenylbutyrate or sodium benzoate to preliminarily receive such a medication when the blood ammonia level is expected to increase

during excessive stress, such as during delivery. Because ammonia is a final product in amino acid metabolism, it is believed that there is a time lag from stress load to the increased blood ammonia levels. It may be effective to measure blood glutamine levels to predict whether the blood ammonia levels will increase or to estimate the change in body condition in patients with OTCD because glutamine is a supplier of ammonia and one of the markers of the medical condition<sup>[18,19]</sup>. Furthermore, the blood value is high, even in OTCD carriers<sup>[20]</sup>. Moreover, it may be useful to adjust the amount of sodium phenylbutyrate or sodium benzoate based upon blood glutamine levels.

The patient should have been monitored without discharge from our hospital because hyperammonemia crisis was likely to develop anytime within 2 wk after parturition and she could not consume protein-rich food in our hospital. Moreover, we consider that hyperammonemia crisis could have been avoided if she had received sodium benzoate or sodium phenylbutyrate therapy immediately after delivery. Caesarean section and the halting maternal milk production might contribute to preventing a hyperammonemia crisis.

Moreover, this patient was a candidate for liver transplantation (LT) since she was susceptible to developing hyperammonemia crisis for non-predictable common disorders. If female patients with OTCD who have had hyperammonemia crisis are going to be married and give birth, LT before pregnancy may be a treatment option because LT prevents recurrent hyperammonemia attacks and contributes to improved quality of life in patients with UCD<sup>[21-24]</sup>.

In conclusion, we treated a female patient with late-onset OTCD, who developed hyperammonemia crisis after delivery. Hyperammonemia crisis after delivery in patients with OTCD is often fatal and difficult to predict. It is important to perform early intervention before hyperammonemia occurs in patients with OTCD or carriers after delivery. Once hyperammonemia crisis occurs following parturition, it is difficult to improve the

metabolic state of the patient.

## COMMENTS

### Case characteristics

Hyperammonemia crisis with hepatic coma after delivery.

### Clinical diagnosis

Hyperammonemia crisis with hepatic coma.

### Differential diagnosis

Carbamoyl-phosphate synthetase 1 deficiency, arginosuccinate synthetase deficiency, arginosuccinate lyase deficiency and arginase 1 deficiency were considered to be a differential diagnosis.

### Laboratory diagnosis

Increased blood glutamine and urinary orotic acid levels as well as impaired liver ornithine transcarbamylase enzyme activity.

### Imaging diagnosis

Atrophy of the bilateral frontal and temporal lobe, as indicated by brain magnetic resonance imaging.

### Treatment

Arginine, citrulline, sodium benzoate, sodium phenylbutyrate, and hemodialysis.

### Related reports

A peak ammonia concentration less than 180  $\mu\text{mol/L}$  was shown to be a marker of a good neurodevelopmental prognosis, and a peak ammonia concentration of more than 360  $\mu\text{mol/L}$  was a marker of a bad prognosis.

### Term explanation

UCDs: Urea cycle disorders; OTCD: Ornithine transcarbamylase deficiency; LT: Liver transplantation.

### Experiences and lessons

It is important to perform early intervention before hyperammonemia occurs in patients with OTCD or carriers following parturition.

### Peer-review

Hyperammonemia after delivery in a female patient with OTCD can be fatal. In this case report, authors have discussed the cause and treatment of hyperammonemia in a female patient with OTCD after delivery. This case indicates that it is important to perform early intervention before hyperammonemia occurs in patients with OTCD or carriers after delivery.

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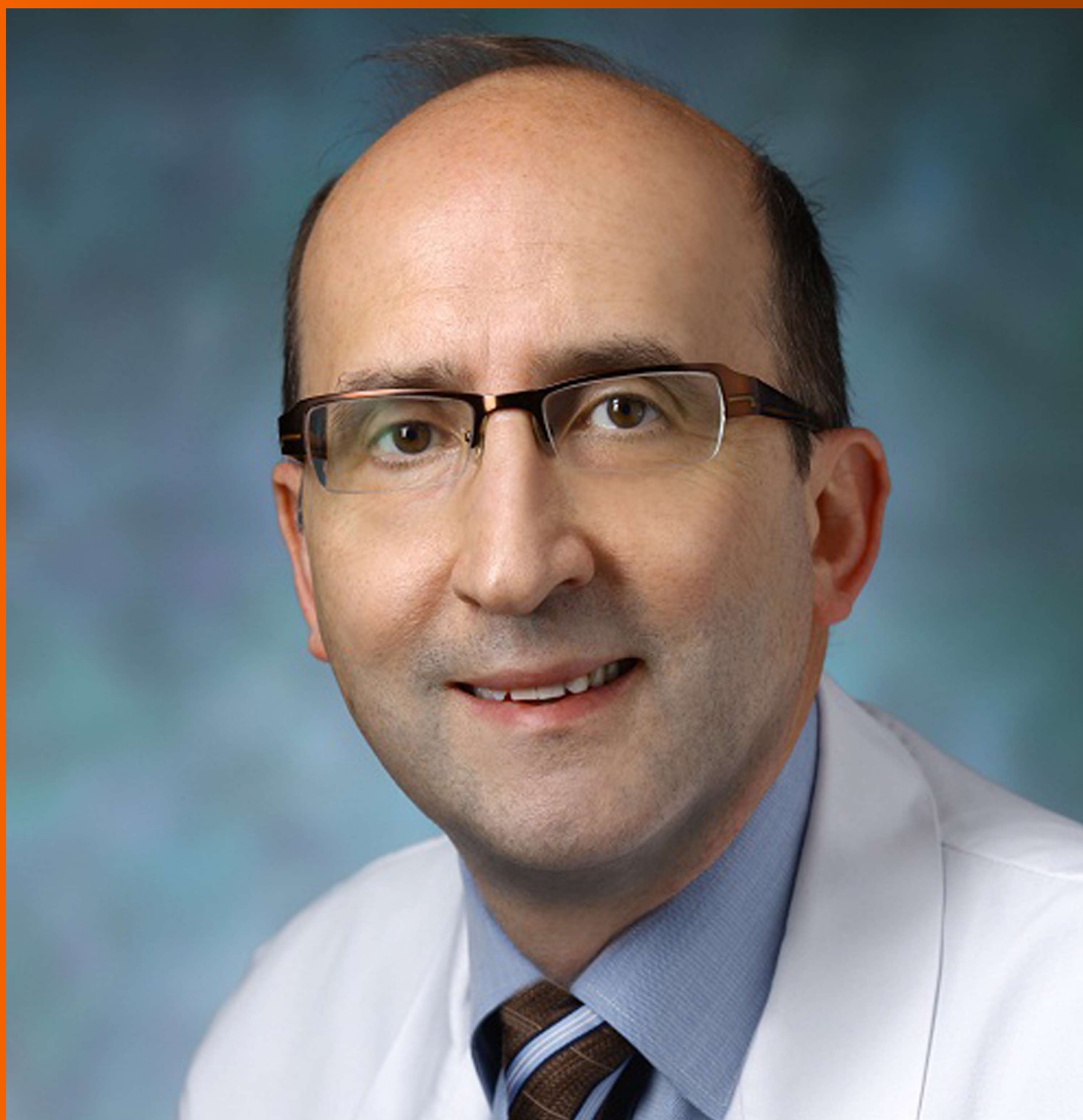
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## Parkin in cancer: Mitophagy-related/unrelated tasks

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### Abstract

Dysfunctional mitochondria may produce excessive reactive oxygen species, thus inducing DNA damage, which may be oncogenic if not repaired. As a major role of the PINK1-Parkin pathway involves selective autophagic clearance of damaged mitochondria *via* a process termed

mitophagy, Parkin-mediated mitophagy may be a tumor-suppressive mechanism. As an alternative mechanism for tumor inhibition beyond mitophagy, Parkin has been reported to have other oncosuppressive functions such as DNA repair, negative regulation of cell proliferation and stimulation of p53 tumor suppressor function. The authors recently reported that acute ethanol-induced mitophagy in hepatocytes was associated with Parkin mitochondrial translocation and colocalization with accumulated 8-OHdG (a marker of DNA damage and mutagenicity). This finding suggests: (1) the possibility of Parkin-mediated repair of damaged mitochondrial DNA in hepatocytes of ethanol-treated rats (ETRs) as an oncosuppressive mechanism; and (2) potential induction of cytoprotective mitophagy in ETR hepatocytes if mitochondrial damage is too severe to be repaired. Below is a summary of the various roles Parkin plays in tumor suppression, which may or may not be related to mitophagy. A proper understanding of the various tasks performed by Parkin in tumorigenesis may help in cancer therapy by allowing the PINK1-Parkin pathway to be targeted.

**Key words:** Cancer; Ethanol; Liver; Mitophagy; Parkin; PINK1; 8-OHdG

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**Core tip:** A large number of studies have found that the impaired Parkin function or downregulation of expression may induce cancer initiation and progression *via* mitophagy-related/unrelated mechanisms. Thus, there is a growing belief that Parkin may have tumor suppressor effects. Based on literature and on the authors' recent publications regarding animal models of alcohol abuse, this paper highlights the various roles of Parkin in the suppression of oncogenesis. Proper understanding of Parkin functions may have therapeutic implications in the treatment of various cancers.

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Mutations in the Parkin gene are frequently associated with Parkinson's disease (PD). They lead to defects in autophagic clearance of damaged mitochondria *via* mitophagy, resulting in the characteristic neuronal loss observed in PD<sup>[1]</sup>. Parkin-mediated mitophagy is characterized by accumulation of PINK1 on the outer mitochondrial membrane (OMM) of damaged mitochondria and subsequent mitochondrial translocation of Parkin and ubiquitination of numerous OMM proteins, followed by clearance of these organelles *via* the microtubule-associated protein light chain 3 (LC3)-mediated autophagic machinery<sup>[1,2]</sup>. Parkin-mediated ubiquitination of OMM proteins stimulates the recruitment of different LC3 interacting region-containing autophagy receptors which bind ubiquitin-tagged OMM proteins, including p62, optineurin and NBR1<sup>[2]</sup>. Dysfunctional mitochondria can transform cells and promote tumorigenesis, suggesting that mitophagy may function as a tumor suppressor mechanism<sup>[2]</sup>. A number of recent studies have investigated the involvement of mitophagy in tumor suppression, with results including the finding that insufficient mitophagy resulted in oncogenic formation in heterogeneous thyroid Hürthle cell tumors<sup>[3]</sup>. However, a growing body of evidence suggests that Parkin also plays a role in cancer as a putative tumor suppressor. Parkin<sup>-/-</sup> mice exhibited enhanced hepatocyte proliferation associated with upregulation of endogenous follistatin, resulting in the induction and progression of hepatocellular carcinoma (HCC)<sup>[4]</sup>. Upon autophagy activation the Atg4 cysteine protease first cleaves pro-LC3 at the C-terminus, thus forming LC3- I. Induction of Atg7 conjugates phosphatidylethanolamine (PE) to LC3- I, forming LC3- II (essential form of LC3 for mitophagosome formation). The Atg5/12/16 complex also acts as an E3 ligase, promoting PE conjugation to LC3<sup>[2]</sup>. Mice with systemic mosaic deletion of Atg5 and liver-specific Atg7<sup>-/-</sup> mice develop benign liver adenomas<sup>[5]</sup>. Parkin deficiency results in overexpression of its substrates, mitotic defects, genomic instability and tumorigenesis<sup>[6]</sup>. Downregulation of Parkin protein has been observed in HCC, whereas Parkin overexpression inhibits the migration and invasion of multiple cancer cells<sup>[7]</sup>. Parkin has been reported to contribute to the functions of p53 - another tumor suppressor - *via* regulation of the energy metabolism (especially the Warburg effect) and antioxidant defense<sup>[8]</sup>. Paradoxically, in some cases Parkin activity may be required for KRAS-driven tumors to maintain mitochondrial quality control and buffer oxidative stress, making it a pro-survival protein<sup>[7]</sup>. KRAS mutant pancreatic adenocarcinoma has been reported to rely on autophagy and mitophagy to supply bioenergetic intermediates for the TCA cycle. Mitophagy

also appears to be a prosurvival mechanism in immortal baby mouse kidney epithelial cells ectopically expressing oncogenic HRAS or KRAS by removing damaged mitochondria<sup>[9]</sup>.

Seitz and Stickel<sup>[10]</sup> reported that animal models of alcohol abuse have clearly identified ethanol as a hepatic carcinogen *via* mechanisms related to excessive reactive oxygen species and acetaldehyde production, altered methylation and reduction of retinoic acid in hepatocytes. Recently the authors<sup>[11,12]</sup> and others<sup>[13]</sup> investigated Parkin-mediated hepatic mitophagy in animal models of acute and chronic alcoholism. The authors found that acute ethanol administration (5 g/kg) to adult rats enhanced hepatocyte mitophagy, which was associated with Parkin mitochondrial translocation and colocalization with accumulated 8-OHdG - a marker of oxidative nuclear and mitochondrial DNA (mtDNA) damage and mutagenicity<sup>[11,12,14,15]</sup>. Accordingly, Parkin co-localization with accumulated 8-OHdG in hepatocyte mitochondria of acute ETRs may be a signal for mitophagy induction *via* the triggering of Parkin mitochondrial translocation<sup>[12,16]</sup>. It may also be a stimulus for DNA repair and prevention of oncogenesis, as endogenous Parkin has a reported physical association with mtDNA<sup>[12,17]</sup> and translocates to nuclei interacting with proliferating cell nuclear antigen in cultured neuronal cells affected by oxidative DNA damage<sup>[18]</sup>. In addition, Parkin-deficient mice have been reported to show increased 8-oxoguanine in the cerebral cortex. Parkin's promotion of DNA repair may therefore be an important mechanism in the suppression of cancer and neurodegenerative diseases<sup>[18,19]</sup>. The authors' findings in animal models of ethanol-induced mitophagy may support the above-mentioned literature regarding the tumor suppressor roles of Parkin, which may or may not be mitophagy-related. Parkin has additionally been reported to regulate two additional cytoprotective mechanisms on cellular exposure to oxidative stress: (1) induction of mitochondrial-derived vesicle formation<sup>[12,16,20]</sup>; and (2) suppression of mitochondrial spheroid formation<sup>[11,21,22]</sup>. Further studies are needed to determine whether Parkin regulates these two mechanisms in cancer cells and to evaluate the impact of any such regulation on tumorigenesis<sup>[23]</sup>.

The authors believe that their recent publications on animal models of alcoholism and the work of others may provide evidence for Parkin-mediated oncosuppression, which may have implications in cancer therapy.

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## Future of liver disease in the era of direct acting antivirals for the treatment of hepatitis C

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### Abstract

Hepatitis C virus (HCV) infection has been a global health problem for decades, due to the high number of infected people and to the lack of effective and well-tolerated therapies. In the last 3 years, the approval of new direct acting antivirals characterized by high rates of virological clearance and excellent tolerability has dramatically improved HCV infection curability, especially for patients with advanced liver disease and for liver transplant recipients. Long-term data about the impact of the new direct acting antivirals on liver fibrosis and liver disease-related outcomes are not yet available, due to their recent introduction. However, previously published data deriving from the use of pegylated-interferon and ribavirin lead to hypothesizing that we are going to observe, in the future, a reduction in mortality and in the incidence of hepatocellular carcinoma, as well as a regression of fibrosis for people previously affected by hepatitis C. In the liver transplant setting, clinical improvement has already been described after treatment with the new direct acting antivirals, which has often led to patients delisting. In the future, this may hopefully reduce the gap between liver organ request and availability, probably expanding liver transplant indications to other clinical conditions. Therefore, these new drugs are going to change the natural history of HCV-related liver disease and the epidemiology of HCV infection worldwide. However, the global consequences will depend on treatment accessibility and on the number of countries that could afford the use of the new direct acting antivirals.

**Key words:** Direct acting antivirals; Hepatitis C; Liver transplantation; Liver fibrosis; Cirrhosis; Hepatocellular carcinoma

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**Core tip:** The approval of new direct acting antivirals with high rates of virological clearance and excellent tolerability has dramatically improved hepatitis C virus (HCV) infection curability, especially for patients with advanced liver disease and for liver transplant recipients. The aim of this review is to draw the possible future scenery in HCV-related liver disease, focusing our attention on the impact of second generation direct acting antivirals on liver fibrosis, hepatocellular carcinoma and liver transplantation.

Ponziani FR, Mangiola F, Binda C, Zocco MA, Siciliano M, Grieco A, Rapaccini GL, Pompili M, Gasbarrini A. Future of liver disease in the era of direct acting antivirals for the treatment of hepatitis C. *World J Hepatol* 2017; 9(7): 352-367 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i7/352.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i7.352>

## INTRODUCTION

Since its discovery, hepatitis C virus (HCV) has been a constant burden for global health, with 3 to 4 million new infections each year and an overall number of 130-170 million infected people in the world<sup>[1]</sup>. The prevalence of HCV infection has a large geographical variability, ranging from less than 1% to more than 10% in different regions<sup>[2,3]</sup>. In particular, 2.3 million of the chronically infected subjects have been estimated to reside in the United States, 1.5 in Japan and 11.5-19 in Europe<sup>[4]</sup>.

HCV infection becomes chronic in up to 50%-80% of cases, establishing a damage that may lead to cirrhosis and its complications [e.g., hepatocellular carcinoma (HCC), portal hypertension, liver decompensation and insufficiency] in approximately 10%-20% of patients<sup>[5,6]</sup>. Nevertheless, chronic HCV infection may be associated with extrahepatic manifestations, such as cryoglobulinemia and non-Hodgkin lymphoma, mainly caused by the continuous stimulation of the immune system<sup>[7,8]</sup>.

Non-pegylated interferon (IFN) or pegylated IFN (PEG-IFN) in combination with ribavirin (RBV) have been the main pharmacological agents for the treatment of HCV infection. However, only 30%-40% of subjects with genotype 1 HCV and 70%-90% of those with genotype 2 and 3 treated with PEG-IFN in association with RBV were able to reach a sustained virological response (SVR), defined as the absence of detectable levels of HCV-RNA 24 wk after the end of treatment<sup>[9-14]</sup>. In 2011 the association of the first-generation direct acting antivirals (DAAs) boceprevir and telaprevir with PEG-IFN and RBV increased the overall SVR rates to 68%-75% for naive patients and to 59%-88% for treatment-experienced patients, even if these regimens were dedicated just to the treatment of genotype 1 HCV infection<sup>[12,14,15]</sup>. However, the suboptimal response

rates, the long duration of treatment (24-48 wk) and the scarce tolerability of boceprevir and telaprevir, especially by cirrhotic patients, has heavily affected their clinical use and has led to search for new drugs<sup>[16]</sup>.

## SECOND-GENERATION DAAs

The second-generation DAAs are characterized by elevated SVR rates, good safety profiles, and more comfortable types of administration. They can be used or not in combination with RBV, depending on virological and disease-associated characteristics<sup>[17]</sup>. Sofosbuvir (SOF) has been the first new agent approved by the Food and Drug Administration (FDA) in December 2013 (Table 1 and Figure 1)<sup>[18]</sup>.

SOF targets HCV-RNA replication with a pangenotypic efficacy since it blocks the nucleotide polymerase NS5B, which is highly preserved among different HCV genotypes<sup>[19]</sup>. Treatment with SOF, either in combination with PEG-IFN plus RBV or with RBV alone has shown SVR rates above 85% at 12 wk after the end of treatment (SVR12)<sup>[20]</sup>. Successively, new DAAs for the treatment of HCV infection in association with SOF have been approved: Simeprevir (SMV, a NS3/4A protease inhibitor) and ledipasvir (LDV, a NS5A inhibitor) for genotype 1, and daclatasvir (DCV, a NS5A inhibitor) for genotype 3, reporting SVR12 rates > 90%<sup>[21-24]</sup>. More recently, the pangenotypic NS5A inhibitor velpatasvir has also been approved for HCV treatment in combination with SOF<sup>[25,26]</sup>.

The first antiviral regimen SOF-free was approved in July 2015 and includes paritaprevir (a NS3/4A protease inhibitor), ritonavir (a CYP3A inhibitor, used as a pharmacologic booster) and ombitasvir (a NS5A inhibitor), in association with dasabuvir (a non-nucleoside NS5B polymerase inhibitor), and is indicated for the treatment of genotype 1 (with dasabuvir) and 4 (without dasabuvir) HCV infection<sup>[27,28]</sup>. Successively, the FDA has approved another SOF-free antiviral regimen including elbasvir and grazoprevir<sup>[29]</sup>, and new drugs with pangenotypic efficacy are in final phase of study and will soon be available<sup>[30]</sup>.

The main advantage of the new DAAs-based antiviral regimens is the achievement of high SVR rates for all HCV genotypes within a short treatment period, together with the infrequent occurrence of side effects, usually of mild grade. Resistance-associated variants (RAVs) of the virus may exist prior to treatment, may persist for years after treatment and affect most frequently the NS3/5A viral protein; RAVs are associated with (but do not inevitably result in) treatment failure, which may occur in about 10%-15% of patients<sup>[31,32]</sup>.

The most ambitious result we might expect from the use of DAAs would be the reduction of liver cirrhosis-related complications, such as HCC development, and in the long-term period a decreased progression towards end-stage liver disease and a decreased need for liver transplant (LT), as well as the prevention of post-LT HCV infection recurrence<sup>[33]</sup>. Indeed, according to the latest data published by the World Health Organization in 2013, 5%-7% of infected subjects died from a

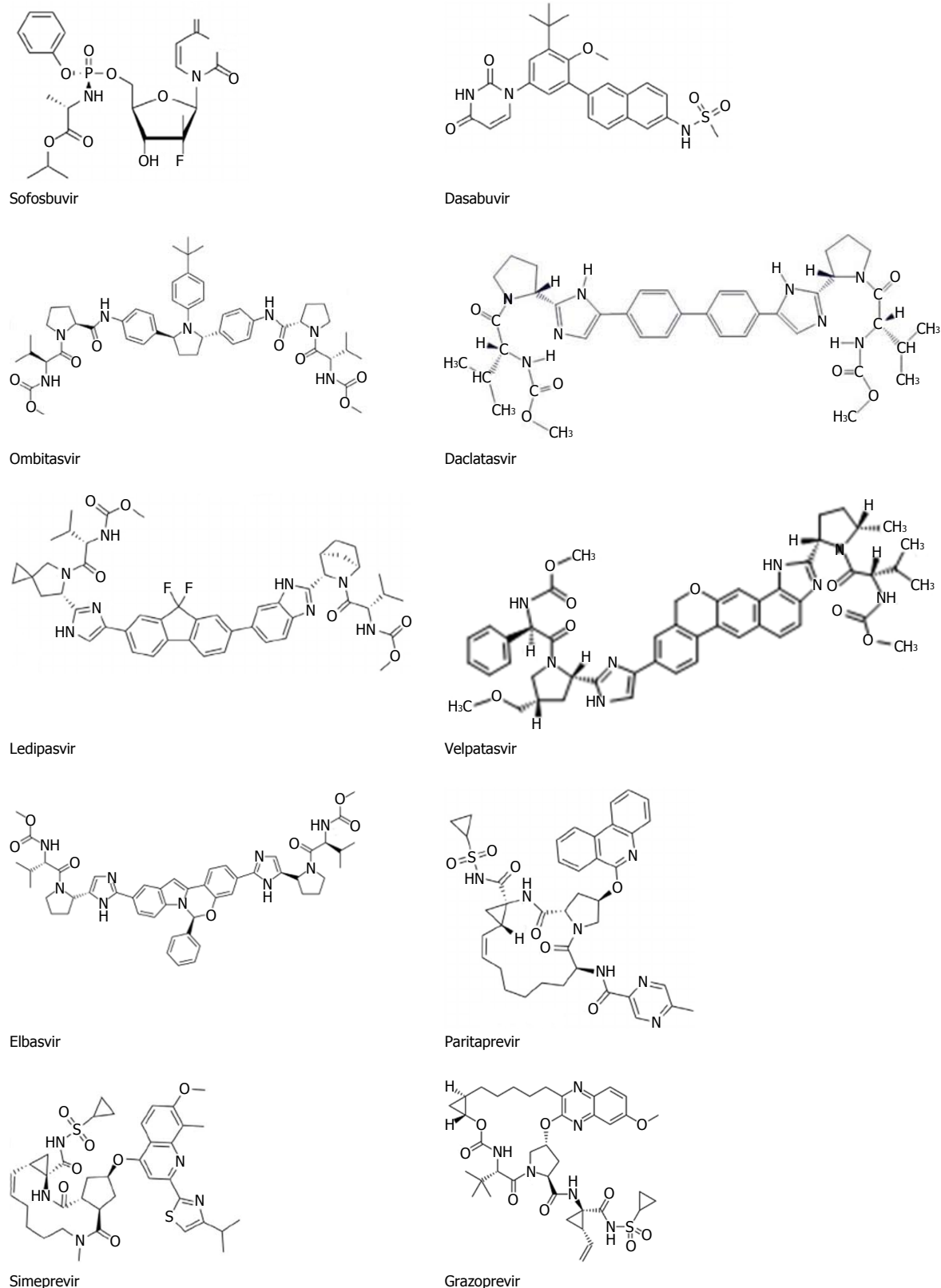


Figure 1 Second-generation direct acting antivirals molecules.

disease related to HCV<sup>[34]</sup>, with an estimated risk of liver failure of 10.4% and 26.5% in patients with F3 and F4 fibrosis, respectively<sup>[35]</sup>. HCV-associated liver disease represents the most common indication for LT and, in developed countries, is the most common etiological factor

of HCC, which is the third leading cause of cancer death worldwide<sup>[36-40]</sup>.

However, due to the relatively recent introduction of these new drugs, data about their impact on liver disease progression, complications and liver-related mortality are

**Table 1** Main features of antiviral targets and clinical indications of second-generation direct acting antivirals<sup>[17]</sup>

Molecule	Class	Target	Genotype	Associations
Sofosbuvir	Nucleotide polymerase inhibitor	NS5B RNA-dependent RNA polymerase	Pangenotypic	Ledipasvir Daclatasvir Simeprevir Velpatasvir
Dasabuvir	Non-nucleoside polymerase inhibitor	NS5B RNA-dependent RNA polymerase	Genotype 1	Ombitasvir + paritaprevir + ritonavir
Ombitasvir		NS5A	Genotype 1, 4	Paritaprevir + ritonavir with or without dasabuvir
Daclatasvir		NS5A	Genotype 1, 2, 3	Sofosbuvir
Ledipasvir		NS5A	Genotype 1, 4	Sofosbuvir
Velpatasvir		NS5A	Pangenotypic	Sofosbuvir
Elbasvir		NS5A	Genotype 1, 4	Grazoprevir
Paritaprevir		NS3/4A protease	Genotype 1, 4	Ombitasvir + ritonavir with or without dasabuvir
Simeprevir		NS3/4A protease	Genotype 1, 4	Sofosbuvir
Grazoprevir		NS3/4A protease	Genotype 1, 4	Elbasvir

scarce. Therefore, previously published data about the impact of SVR achieved with PEG-IFN-based regimens are the only available reference to evaluate the future positive effects that DAAs might produce on liver disease outcomes.

## IMPACT OF VIRAL ERADICATION ON LIVER CIRRHOSIS-ASSOCIATED MORBIDITY AND MORTALITY

Published data have demonstrated a correlation between the achievement of SVR and the reduction of HCV-related complications, liver disease severity and mortality (Table 2).

Veldt *et al.*<sup>[41]</sup> reported that among 286 subjects who achieved SVR and were followed-up for 5 years, only 1% experienced liver failure, with a survival similar to that of the general population. Another study including 721 patients with chronic hepatitis C reported a significantly lower annual mortality rate in subjects who had previously achieved SVR after IFN therapy compared to those who had not (0.44%/year, 1.98%/year and 3.19%/year for SVR, non-SVR and untreated patients, respectively;  $P < 0.0001$ ). The study also showed that viral clearance was able to reduce the hazard ratio for total deaths by 0.173<sup>[42]</sup>.

A meta-analysis including 129 trials for a total amount of 15067 patients has demonstrated that SVR achievement reduces the risk of LT requirement by 90%, and the risk of death by 60%-84%<sup>[43]</sup>. Nevertheless, viral clearance leads to a lower incidence of liver-related morbidity and death (0.62 and 0.61 among SVR patients, respectively, and 4.16 and 3.76 among non-SVR patients, respectively;  $P < 0.001$ )<sup>[44]</sup>. Recent data further confirmed that HCV infection resolution allows reduction in the incidence of liver decompensation<sup>[45]</sup>, all-cause mortality<sup>[46,47]</sup> and annual deaths rate (8.9% in SVR patients vs 26.0% in non-SVR patients;  $P < 0.001$ )<sup>[48]</sup>. This evidence was confirmed by an extensive review by Szabo *et al.*<sup>[34]</sup>; moreover, survival rates comparable to general population have been

reported after the achievement of SVR even in patients with well-compensated cirrhosis<sup>[49]</sup>. Although useful to figure out the long-term benefits expected from DAAs, the interpretation of data emerging from the use of IFN- or PEG-IFN-based regimens is limited by the selection of patients, since those affected by comorbidities were usually not suitable for treatment and were not included in outcomes analyses; moreover, the lack of homogeneous design and patients' stratification make it difficult to deduce general conclusions.

## IMPACT OF VIRAL ERADICATION ON LIVER FIBROSIS

Despite the positive impact of HCV infection eradication on patients' prognosis, few data about liver cirrhosis/fibrosis regression are available.

Regression of liver fibrosis as a result of viral clearance is supported by the reduction of inflammatory mediators that leads to apoptosis of myofibroblasts, and occurs by the inactivation of stellate cells. The down-regulation of inflammation, as well as microvascular remodelling, degradation of extracellular matrix and hepatocyte repopulation leads to the generation of new hepatic tissue<sup>[50,51]</sup>.

Cirrhosis regression has been reported in about 61% of cases after a median time of 3 years from the achievement of SVR (Table 2)<sup>[52]</sup>. Mallet *et al.*<sup>[53]</sup> observed the evolution of liver fibrosis in 96 patients treated with IFN or PEG-IFN with or without RBV, for a median follow-up of 118 mo. Although statistical significance was not reached, 18 subjects obtained a regression of fibrosis from METAVIR stage 4 to stage  $\leq 2$ . In another study, among 153 cirrhotic patients treated with IFN or PEG-IFN in combination or not with RBV for 24 or 48 wk, 75 (49%) had a regression of fibrosis after a mean time of  $21 \pm 4$  mo. In addition to SVR, factors independently associated with histology improvement were age  $< 40$  years ( $P < 0.001$ ) and body mass index  $< 27$  kg/m<sup>2</sup> ( $P < 0.001$ )<sup>[54]</sup>.

Other small studies reported variable rates of fibrosis regression after different time periods from viral



Table 2 Main studies highlighting the effects of hepatitis C virus antiviral therapy on patients' mortality, fibrosis regression and risk of hepatocellular carcinoma

Ref.	HCV genotype	Fibrosis stage	Treatment	SVR rate	Mortality (n, pts)	Survival	Other outcomes
Veldt <i>et al</i> <sup>[47]</sup> , 2007	G1: 280/474 (59%)	Ishak score 4: 120 (25%) Ishak score 5: 94 (20%) Ishak score 6: 265 (55%)	Duration of treatment, 26 wk (21-48) IFN: 131 (27%) IFN + RBV: 130 (27%) PEG-IFN: 10 (2.1%) PEG-IFN + RBV: 208 (43%)	142/280 (50.7%)	SVR: 2/280 (0.7%) Non-SVR: 24/280 (8.6%)	-	SVR associated with reduction in the hazard of events (adjusted HR = 0.21, 95%CI: 0.07-0.58; $P < 0.003$ )
Yoshida <i>et al</i> <sup>[61]</sup> , 1999	G1: 1177/2400 (49%) G2: 496/2400 (20.6%)	F0: 45 (1.9%) F1: 665 (27.7%) F2: 896 (37.7%) F3: 564 (23.5%) F4: 230 (9.6%)	IFN- $\alpha$ : 84% IFN- $\beta$ : 14% Combination of IFN- $\alpha$ and IFN- $\beta$ : 2%	789/2400 (32.8%)	-	-	Risk of HCC for IFN therapy: Adjusted risk ratio = 0.516, 95%CI: 0.358-0.742 ( $P < 0.001$ ); risk of HCC for SVR pts: risk ratio = 0.197, 95%CI: 0.099-0.392 ( $P < 0.002$ )
Veldt <i>et al</i> <sup>[41]</sup> , 2004	SVR G1: 112/286 (39.2%) Not specified: 174/286 (60.8%) Non-SVR G1: 21/50 (42%) Not specified: 29/50 (58%)	SVR: F4: 15 (5.2%) Non-SVR: F4: 11 (22%)	Recombinant IFN $\alpha$ 2a, $\alpha$ 2b, or natural IFN monotherapy for 39 wk	286	SVR 6/286 (2.1%) 3/50 (6%)	SVR group: Comparable with the general population	29% regression and 5% progression of fibrosis in SVR group
Maruoka <i>et al</i> <sup>[42]</sup> , 2012	Treated (577): G1: 383/577 (66.2%) G2: 144/577 (24.8%) Untreated (144)	Treated: F0: 15 (2.6%) F1: 290 (50%) F2: 132 (22.9%) F3: 82 (12.2%) F4: 58 (10.1%) Untreated: F0: 2 (1.4%) F1: 64 (44.4%) F2: 32 (22.2%) F3: 18 (12.5%) F4: 100%	IFN (not specified)	221/577 (38.3%)	Untreated: 37/144 (25.7%) Non-SVR 74/356 (20.8%) SVR 10/221 (4.5%)	-	Risk ratio of overall death and liver-related death reduced to 0.173 (95%CI: 0.075-0.402)
Bruno <i>et al</i> <sup>[49]</sup> , 2016	G1: 88/181 (48.6%)	F4: 100% CPT A5: 154/181 (85.1%) CPT A6: 27/181 (14.9%)	IFN mono-therapy or IFN (pegylated or not) + RBV	181	18/181 (9.9%)	-	-
Cardoso <i>et al</i> <sup>[44]</sup> , 2010	G1: 60% G2: 8% G3: 16% G4: 13%	F4: 54%	PEG-IFN + RBV: 252 (82%), PEG-IFN: 22 (7%), IFN + RBV: 33 (11%)	103/307 (33.5%)	21/307 (6.8%)	-	-
Tada <i>et al</i> <sup>[46]</sup> , 2016	G1: 1476/2743 (53.8%) G2: 789/2743 (28.3%) Unknown: 478/2743 (17.4%)	-	IFN (not specified)	587/2267 (25.9%)	137/2267 (6%)	-	-
Van der Meer <i>et al</i> <sup>[48]</sup> , 2012	G1: 340/498 (68.3%) G2: 48/498 (9.6%) G3: 88/498 (17.7%) G4: 22/498 (4.4%)	Ishak 4: 143/498 (27%) Ishak 5: 101/498 (19%) Ishak 6: 22/498 (4%)	IFN: 175 (33%) IFN + RBV: 148 (28%) PEG-IFN: 176 (33%) PEG-IFN + RBV: 176 (33%) IFN + RBV: 10/38 (26.3%) PEG-IFN + RBV: 28/38 (73.6%)	192/498 (38.5%)	SVR: 13 Non-SVR: 100	-	SVR reduced all-cause mortality (HR = 0.265, 95%CI: 0.14-0.49; $P < 0.001$ )
D'Ambrosio <i>et al</i> <sup>[50]</sup> , 2012	G1: 11/38 (28.9%) G2: 24/38 (63.2%) G3: 3/38 (7.9%)	Only cirrhotic patients	Duration of treatment 24 mo (24-48)	-	-	-	SVR reduced area of fibrosis by 2.3% ( $P < 0.0001$ ), with a median individual decrease of 71.8%

Mallet <i>et al</i> <sup>[53]</sup> , 2008	G1: 51/96 (53.1%)	F4: 100%	IFN or PEG-IFN, with or without RBV	39/96 (40.6%)	SVR: 4 (10.2%) Non-SVR: 17 (29.8%)	-	Regression of fibrosis (according to METAVIR score): Stage 4: 69 (71.9%); stage 3: 9 (9.4%); stage 2: 10 (10.4%); stage 1: 7 (7.3%); stage 0: 1 (1%) Reduction of portal inflammation ( $P < 0.0002$ ), piecemeal necrosis ( $P < 0.0004$ ), lobular necrosis ( $P < 0.0005$ ), fibrosis ( $P < 0.0008$ ) after SVR Reduction in fibrosis score in both groups: responders = -0.91 ( $P = 0.038$ ), non-responders = -0.48 ( $P = 0.021$ )
Reichard <i>et al</i> <sup>[54]</sup> , 1999	G1: 41/100 (41%) G2: 27/100 (27%) G3: 23/100 (23%) Mixed: 9/100 (9%)	F0-3: 22 F4: 4	IFN alpha2b: 73 Human leucocyte IFN alpha: 42	27/100 (27%)	-	-	
Arif <i>et al</i> <sup>[57]</sup> , 2003	Naive (52): G1a: 64% G1b: 19% G2: 6% G3: 10% G4: 1%	Naive Fibrosis score: $2.91 \pm 1.64$	IFN alpha2b Duration of treatment: 12-24 wk: 10 24 wk: 56 36 wk: 8 48 wk: 30	Naive 21/52 (40.4%) Experienced 18/79 (22.8%)	-	-	
George <i>et al</i> <sup>[58]</sup> , 2009	Experienced (79): G1a: 55% G1b: 26% G2: 7% G3: 10% G4: 2%	Fibrosis score: $2.83 \pm 1.62$ Fibrosis stage $\geq 2$ : 116 Fibrosis stage = 4: 16 According to Scheuer	IFN alpha2b + RBV: 146 (97%) PEG-IFN alpha2a + RBV: 4 (3%)	100%	-	1	39/49 (79.6%) reduction in fibrosis stage (according to Ishak score) 16/49 (32.6%) pts had 2 point or greater decrease in stage Decrease in fibrosis index score in SVR group compared with non-responders: From $0.33 \pm 0.06$ at baseline to $0.18 \pm 0.06$ at 72 wk vs from $0.41 \pm 0.03$ at baseline to $0.44 \pm 0.03$ at 72 wk ( $P < 0.001$ )
Poynard <i>et al</i> <sup>[59]</sup> , 2002	-	Standard: F0: 12 (15%) F1: 42 (54%) F3: 24 (31%) F4: 0 (0%) Reinforced: F0: 16 (18%) F1: 41 (47%) F3: 30 (35%) F4: 0 SVR: F0: 3 (2%) F1: 42 (23%) F2: 69 (37%) F3: 45 (25%) F4: 24 (13%) Non-SVR: F0: 3 (1%)	Standard: IFN alpha2a 3 MU TIW for 24 wk Reinforced: IFN alpha2a 6 MU daily for 12 d followed by thrice weekly for 22 wk, then 3 MU thrice weekly for 24 wk	Standard: 3/78 (3.8%) Reinforced: 14/87 (16%)	-	-	SVR group: Rate of fibrosis progression -0.28 $\pm$ 0.03 unit/year (regression) Non-SVR group: Rate of fibrosis progression: 0.02 $\pm$ 0.02 unit/year $P < 0.001$
Shiratori <i>et al</i> <sup>[60]</sup> , 2000	-	-	IFN alpha2a or IFN alpha2b or Natural IFN alpha weekly for 3 to 6 mo IFN alpha 6-7 times per wk for 8 wk	183/487 (37.6%)	-	-	

Pts: Patients; IFN: Interferon; PEG: Pegylated; SVR: Sustained virological response; HCC: Hepatocellular carcinoma; RBV: Ribavirin.

However, the neo-formed parenchyma derived from the generation of new liver tissue is different from the healthy one and is characterized by architectural and structural alterations<sup>[63]</sup>. At present, little is known about its functionality.

In HCV-infected subjects, the development of liver cirrhosis is the main oncogenic trigger for HCC<sup>[5,64,65]</sup>, though not the only one. Indeed, direct and indirect viral-related mechanisms may contribute to the growth of cancer cells, including the expression of viral proteins with oncogenic effect from infected cells, messy proliferation of non-infected hepatocytes responsive to the apoptotic boost and the oxidative stress caused by inflammation<sup>[66]</sup>.

HCV eradication may also reduce the risk of HCC recurrence after surgical treatment. A 63.4% cumulative recurrence rate has been reported in non-treated patients, compared to 63.2% in treated patients who did not achieve SVR and to 41.7% in the SVR group (non-treated vs SVR,  $P = 0.008$ ; SVR vs treated without SVR,  $P = 0.035$ )<sup>[69]</sup>. Mazzaferro *et al*<sup>[70]</sup> also found SVR as the only factor significantly reducing HCC late recurrence in HCV-pure (hepatitis B antiretrovirus negative) patients. A subsequent meta-analysis also reported a reduced rate of early recurrence in 51 patients undergoing surgical resection or percutaneous ablation, reporting a 30% reduction in HCC recurrence rate<sup>[71]</sup>. In addition, IFN therapy seems to exert beneficial effects, even when started before HCC curative treatments<sup>[72]</sup>.

DAAAs may likely modulate the expression of genes involved in the production of endogenous IFN $\gamma$ . In patients treated with SOF in association with RBV a reduction in

types I and II IFN in liver tissue and an increase of IFN- $\alpha$ 2 have been observed<sup>[75]</sup>. Conversely, other authors have reported a loss of intrahepatic immune activation by IFN- $\gamma$ , associated with normalization of the natural killer cells phenotype and function, consequent to DAAs treatment<sup>[76]</sup>. How these findings may be associated with DAAs treatment outcome still needs to be further elucidated.

Although the risk of HCC development is significantly reduced by viral clearance it is not completely eliminated, especially in cases of persistence of other cofactors promoting carcinogenesis. Toyoda *et al.*<sup>[77]</sup> reported that 18/522 patients who achieved SVR after IFN treatment developed HCC after a median follow-up of about 7.2 years (1.0-22.9 years), with an incidence of 1.2% and 4.3% at 5 years and 10 years, respectively. In the analysis, the presence of diabetes mellitus and advanced fibrosis (FIB-4 index  $\geq 2$ ) at 24 wk after SVR were correlated to an increased risk of developing HCC. Other data identified type 2 diabetes mellitus and total alcohol intake as independent risk factors for HCC development (HR = 2.77, 95%CI: 2.13-3.60,  $P < 0.001$  and HR = 2.13, 95%CI: 1.74-2.61,  $P < 0.001$ , respectively)<sup>[78]</sup>. In another study, among 232 SVR patients who underwent liver biopsy between 1992 and 2009, the development of HCC was definitively lower in the group with low-intermediate grade fibrosis (F0-F2 according to Metavir) than in that with F3-F4 grade (1.6% and 8%, respectively)<sup>[79]</sup>.

Data about the impact of DAAs treatment on HCC recurrence in previously treated patients and on the development of new HCC nodules have been recently published. It seems to be clear that these new antivirals are not able to modify the natural history of HCC in cirrhotic patients, and it has also been postulated that they may act as promoters, although other studies have not supported this hypothesis<sup>[80-87]</sup>. Probably, an investigation focused on the immunologic changes and the microenvironmental hepatic tissue alterations consequent to DAAs treatment may be worthwhile to quell this debate<sup>[88]</sup>.

## DAAs AND LT

HCV infection-associated cirrhosis and HCC account for 40% of all cases on the LT waiting list in the United States and for about 1/3 of LTs in cirrhotic patients<sup>[39,40]</sup>. HCV infection recurrence of the graft is universal and leads to cirrhosis in up to 20%-30% of recipients, being one of the most important causes of death and retransplantation<sup>[89,90]</sup>. The time course of post-LT HCV reinfection is faster than among immunocompetent individuals; cirrhosis can be histologically documented within 5 years after LT, and from that point on the first episode of decompensation may occur within less than 1 year<sup>[91]</sup>.

After HCV infection eradication, a 62%-84% decrease in 5-year mortality as well as a reduction by 90% of the risk of receiving LT have been reported<sup>[43]</sup>. This im-

provement in survival was observed in both sustained virological responders and relapsers<sup>[92]</sup>. The new available DAAs account for response rates higher than 90% and are better tolerated than either IFN and PEG-IFN, allowing for treatment of patients for whom the previous antivirals were contraindicated and who had low chances of response due to unfavourable virological or clinical conditions<sup>[93]</sup>. As patients who achieve SVR have a reduced risk of progression to cirrhosis and of developing its complications, the widespread use of the new DAAs will probably change the scenario of LT, potentially reducing the need for liver organs.

## DAAs treatment before LT

The aim of antiviral treatment in patients on the waiting list is to prevent the recurrence of HCV infection after LT. To reduce the risk of post-LT recurrence, the achievement of at least 30 d of HCV-RNA negativity before LT has been suggested<sup>[94,95]</sup>. However, whether it is necessary to continue antiviral therapy after LT in patients who received a very short course of therapy before transplantation is not yet clear<sup>[96]</sup>.

Furthermore, achieving SVR in waiting-list patients may directly impact the severity of liver disease, with possible delisting after treatment. A recent real-life multicentre study<sup>[97]</sup> including 103 decompensated cirrhotic patients listed for LT and treated with second-generation DAAs reported HCV eradication rates of 16% at 24 wk and of 35% at 48 wk after the beginning of treatment (Table 3). This was associated with delisting of 20% of patients at 48 wk from the end of treatment. The evidence of a significant improvement of liver function also comes from the SOLAR-1 study cohort A<sup>[98]</sup>, including cirrhotic patients with decompensated disease treated with LDV and SOF plus RBV. For this study, similar SVR rates (from 86% to 89%) were reported for Child-Pugh class B and C patients regardless of treatment duration, and this was associated with the improvement of model for end-stage liver disease (MELD) and Child-Pugh scores.

Other studies confirmed liver function amelioration after viral eradication in decompensated cirrhotic patients<sup>[26,98-104]</sup>; although in cases with more advanced liver impairment (Child-Pugh C, albumin lower than 3.5 g/dL, MELD > 20) and in the elderly worsening of liver function has also been reported<sup>[105,106]</sup>.

Delisting due to clinical improvement may therefore become frequent in the era of DAAs, making it possible to reserve LT only to patients who do not show significant benefit. Munoz<sup>[107]</sup> estimated that DAAs-induced reduction in MELD score down to the threshold of LT benefit may occur in 592-993 listed patients/year during the first year after treatment, and that approximately 213-515 donated livers/year may become available for redistribution to other patients.

The future impact of DAAs on indications for LT and on organ allocation policy may depend not only on the decreased number of HCV-infected recipients but also on the potential use of anti-HCV positive donors<sup>[108]</sup>. Indeed, DAAs might introduce a new era, in which anti-



Table 3 Main studies evaluating the effects of direct acting antivirals in patients with advanced cirrhosis and/or listed for liver transplantation

Ref.	HCV genotype	Fibrosis stage	Treatment	SVR rate	Observed improvement
Charlton <i>et al</i> <sup>[8]</sup> , 2015	Cohort A G1a: 74/108 (68.5%) G1b: 31/108 (28.7%) G4: 3/108 (2.8%)	Child A: 1/108 (1%) Child B: 65/108 (60.2%) Child C: 42/108 (38.9%)	LDV/SOF + RBV 12 or 24 wk	Child B: -12 wk 26/30 (87%) -24 wk 24/27 (89%)	-
Belli <i>et al</i> <sup>[9]</sup> , 2016	G1a: 20/103 (19.4%) G1b: 40/103 (38.8%) G2: 3/103 (3%) G3: 20/103 (19.4%) G4: 20/103 (19.4%)	Child A: 0 Child B: 46/103 (44.7%) Child C: 57/103 (55.3%)	SOF/RBV: 52/103 (50.4%) SOF/LDV ± RBV: 9/103 (8.7%) SOF/DCV ± RBV: 35/103 (33.9%) SOF/SMV ± RBV: 7/103 (6.8%)	SOF/RBV (24-48 wk): RVR 61% EVR 98%  SOF + 2 <sup>nd</sup> DAA (12-24 wk): RVR 67% EVR 98%	MELD: -3.4 points  Child: -2 points  Delisting: 20%
Munoz <i>et al</i> <sup>[10]</sup> , 2015	-	Only cirrhosis	SOF/LDV + RBV (12-24 wk): 230 DCV/SOF + RBV (12 wk): 56 GRZ/ELB (12 wk): 27 SOF/LDV/DCV ± RBV (12 wk): 220 LED/SOF + RBV 12 or 24 wk	SVR 84%	Improvement in refractory ascites that became treatable with diuretics MELD: -2.9 + -0.1 Child B to Child A: 35% Child C to Child B: 48%
Manns <i>et al</i> <sup>[11]</sup> , 2016	G1a: 50/107 (46.7%) G1b: 47/107 (43.9%)  G4: 10/107 (9.4%)	Child A: 2/107 (2%) Child B: 60/107 (56%)  Child C: 45/107 (42%)	Child B: 12 wk 20/23 (87%); 24 wk 22/23 (96%) Child C: 12 wk 17/20 (85%); 24 wk 18/23 (78%), 1/2 (50%) Genotype 4 Child B: 12 wk 2/3 (67%); 24 wk 100% Child C: 12 wk 0%	genotype 1 Child B: 12 wk 20/23 (87%); 24 wk 22/23 (96%) Child C: 12 wk 17/20 (85%); 24 wk 18/23 (78%), 1/2 (50%) Genotype 4 Child B: 12 wk 2/3 (67%); 24 wk 100% Child C: 12 wk 0%	MELD improvement in 72% Child B to Child A: 28%  Child C to Child B: 68%
Poordad <i>et al</i> <sup>[10]</sup> , 2016	G1a: 34/60 (56.7%) G1b: 11/60 (18.3%) G2: 5/60 (8.3%) G3: 6/60 (10%) G4: 4/60 (6.7%) Part 1 G1a: 27/30 (90%) G1b: 3/30 (10%) G1a: 159/267 (59.6%) G1b: 48/267 (18%) G2: 12/267 (4.5%) G3: 39/267 (14.6%) G4: 8/267 (3%) G6: 1/267 (0.3%) G1a: 29/101 (28.7%)	Child A: 12/60 (20%) Child B: 32/60 (53.3%) Child C: 16/60 (27.7%)  Only Child B cirrhosis  Child A: 16/267 (6%) Child B: 240/267 (89.9%) Child C: 11/267 (4.1%)  Child A: 15/101 (14.8%)	DCV/SOF + RBV 12 or 24 wk  GRZ/ELB 12 wk  SOF/VEL 12 or 24 wk SOF/VEL + RBV 12 wk	Child A: 11/12 (92%) Child B: 30/32 (94%) Child C: 9/16 (56%)  SVR 27/30 (90%)  SOF/VEL 12 wk: 75/90 (83%) SOF/VEL + RBV 12 wk: 82/87 (94%) SOF/VEL 24 wk: 77/90 (86%)	MELD improvement in 47% of pts Child improvement in 60% of pts  MELD improvement in 11/30 (36.7%) pts  MELD improvement in 51% of pts Child improvement in 47% of pts
Jacobson <i>et al</i> <sup>[9]</sup> , 2015					
Curry <i>et al</i> <sup>[12]</sup> , 2015					
Gray <i>et al</i> <sup>[10]</sup> , 2016			SOF/LDV ± RBV 12 wk	74.3%	No significant differences from baseline

Aquei <i>et al</i> <sup>[100]</sup> , 2015	G1b: 19/101 (18.8%) G1 (no subtype): 27/101 (26.7%) G2: 0	Child B: 67/101 (66.3%) Child C: 19/101 (18.8%)	SMV/SOF ± RBV 12 wk	RVR: 82/119 (69%) SVR 12: 92/118 (78%; Child A: 83%, Child B: 68%) (1 pts died after achieving SVR4)	Mortality rate 7.9% (6% Child B, 21% Child C)
	G3: 24/101 (23.8%) G4: 1/101 (1%) Mixed: 1/101 (1%)				
Saxena <i>et al</i> <sup>[101]</sup> , 2015	G1b: 82/119 (69%) G1b: 24/119 (20%) G1 (no subtype): G13/119 (11%)	Child A: 84/119 (70%) Child B: 34/119 (29%) Child C: 1/119 (1%)	SMV/SOF ± RBV 12 wk	Child A (37% with RBV): 91% Child B/C (35% with RBV): 73%	MELD improvement in 61/92 (66.4%) pts that achieved SVR 12 No significant differences from baseline
	1a: 98/160 (62%) 1b: 62/160 (38%)	Child A: 101/160 (65%) Child B: 49/160 (31%) Child C: 6/160 (4%)			

Pts: Patients; LDV: Ledipasvir; SOF: Sofosbuvir; RBV: Ribavirin; DCV: Daclatasvir; SMV: Simeprevir; RVR: Rapid virological response (HCV-RNA < 15 UI after 12 wk of treatment); GRZ: Grazoprevir; ELB: Elbasvir; VEL: Velpatasvir; FCH: Fibrosing cholestatic hepatitis.

HCV positive donors could be reconsidered as a potential source of liver grafts. Moreover, in case of HCV infection transmission from anti-HCV positive donors during LT, it may be easily cured<sup>[108]</sup>. Recent data suggest that LT outcomes for recipients who accept HCV-positive allografts could be comparable with those of recipients who received HCV-negative allografts<sup>[109,110]</sup>. Probably, in the future, histological evaluation may become crucial in the choice and the allocation of liver grafts from anti-HCV positive donors, overcoming the issue of previous or active HCV infection. However, these considerations are based on the universal adoption of screening policies for HCV infection, as well as on the widespread use of DAAs for HCV infection treatment, which is still limited by restricted accessibility.

### DAAs treatment after LT

DAAs have demonstrated unprecedented results in the treatment of LT recipients (Table 4).

The SOLAR-1 study, cohort B<sup>[98]</sup>, explored the efficacy of LDV and SOF plus RBV in the treatment of LT recipients without cirrhosis (group 3), with compensated cirrhosis (group 4), and with Child-Pugh B (group 5) and C cirrhosis (group 6). In groups 3 and 4 the SVR rates ranged from 96% to 98% independent of treatment duration; in group 5, SVR was achieved by 86% of patients who received 12 wk of treatment and by 88% of those who received 24 wk of treatment, and group 6, instead, had lower rates of SVR, being 60% and 75% in patients receiving 12 wk and 24 wk of treatment, respectively.

Another study with a similar design, the SOLAR-2, also reported excellent SVR rates in LT recipients with decompensated cirrhosis and genotype 1 or 4 HCV infection treated with LDV and SOF plus RBV for 12 wk or 24 wk<sup>[101]</sup>.

The ALLY-1 and the HCV-TARGET study confirmed good outcomes also for the combination regimens including SMV plus SOF with or without RBV and DCV plus SOF with RBV<sup>[100,111]</sup>.

Although these data may highlight that the achievement of SVR is more difficult in LT cirrhotic patients with more advanced liver impairment, the SOLAR-1 and -2 studies also reported an improvement in MELD and Child-Pugh scores in treated patients<sup>[98,101]</sup>. This was confirmed by a prospective, multicentre study in patients with post-LT hepatitis C recurrence treated with LDV and SOF plus RBV; the response rate was 96% in Child-Pugh A patients compared to 85% and 65% in Child-Pugh class B and C ones, respectively. However, an improvement in Child-Pugh class and MELD scores was observed in patients with decompensated cirrhosis who achieved SVR12<sup>[112]</sup>.

Therefore, liver function improvement consequent to antiviral treatment will hopefully reduce the need for retransplantation and the morbidity and mortality related to liver dysfunction and liver cirrhosis complications.

## HCV INFECTION AND DISEASE-RELATED COMPLICATIONS IN THE FUTURE

The future trend of HCV-related morbidity and mortality in the era of IFN-free antiviral regimens is difficult to predict, although encouraging prospects can be inferred by

**Table 4** Studies evaluating the effects of direct acting antivirals in liver transplant recipients

Ref.	HCV genotype	Fibrosis stage	Treatment	SVR12 rate	Observed improvement
Charlton <i>et al</i> <sup>[98]</sup> , 2015	Cohort B G1a: 164/229 (71.6%) G1b: 63/229 (27.5%) G4: 2/229 (0.9%)	No cirrhosis: 111/229 (48.5%) Child A: 51/229 (22.3%) Child B: 52/229 (22.7%) Child C: 9/229 (3.9%) FCH: 6/229 (2.6%)	LDV/SOF + RBV 12 or 24 wk	No cirrhosis: 12 wk 53/55 (96%) 24 wk 55/56 (98%) Child A: 12 wk 25/26 (96%) 24 wk 24/25 (96%) Child B: 12 wk 22/26 (85%) 24 wk 23/26 (88%) Child C: 12 wk 3/5 (60%) 24 wk 3/4 (75%) FCH: 12 and 24 wk 100%	-
Manns <i>et al</i> <sup>[101]</sup> , 2016	Cohort B G1a: 113/226 (50%) G1b: 86/226 (38%) G4: 27/226 (12%)	No cirrhosis: 101/226 (44.7%) Child A: 71/226 (31.4%) Child B: 40/226 (17.7%) Child C: 9/226 (4%) FCH: 5/226 (2.2%)	LDV/SOF + RBV 12 or 24 wk	Genotype 1: No cirrhosis: 12 wk: 42/45 (93%) 24 wk: 44/44 (100%) Child A: 12 wk: 30/30 (100%) 24 wk: 27/28 (96%) Child B: 12 wk: 19/20 (95%) 24 wk: 20/20 (100%) Child C: 12 wk: 1/2 (50%) 24 wk: 4/5 (80%) FCH: 12 and 24 wk: 100%  Genotype 4: No cirrhosis: 12 and 24 wk 100% Child A: 12 wk 3/4 (75%) 24 wk 100% Child B: 12 and 24 wk 100% Child C: 12 wk 0%	MELD improved in 58% Child B to A: 52% Child C to B: 60%
Poordad <i>et al</i> <sup>[100]</sup> , 2016	G1a: 31/53 (58.5%) G1b: 10/53 (18.9%) G2: 0 G3: 11/53 (20.7%) G4: 0 G6: 1/53 (1.9%)	F0: 6 F1: 10 F2: 7 F3: 13 F4: 16 ND: 1	DCV/SOF + RBV 12 and 24 wk	50/53 (94%)	-
Brown <i>et al</i> <sup>[111]</sup> , 2016	G1a: 87/151 (57.6%) G1b: 42/151 (27.8%) G1 (unspecified): 22/151 (14.6%)	Cirrhosis: 97/151 (64.2%)	SMV/SOF ± RBV	133/151 (88%) SMV/SOF 105/119 (88%) SMV/SOF + RBV 28/32 (88%)	-

LDV: Ledipasvir; SOF: Sofosbuvir; RBV: Ribavirin; DCV: Daclatasvir; SMV: Simeprevir; RVR: Rapid virological response (HCV-RNA < 15 UI after 4 wk of treatment); EVR: Early virological response (HCV-RNA < 15 UI after 12 wk of treatment); GRZ: Grazoprevir; ELB: Elbasvir; VEL: Velpatasvir; FCH: Fibrosing cholestatic hepatitis; HCV: Hepatitis C virus.

recent data and projections.

Sievert *et al*<sup>[113]</sup> recently analysed the future effects of the increase in SVR rates in the Australian population, taking into account three different models: Without increasing (first scenario) or increasing (second scenario) the number of treated patients and, finally, considering treatment prescription restricted to patients with fibrosis  $\geq$  F3 only (third scenario).

Applying the model of restricted prescription in the time period between 2015 and 2017, an estimated reduction by 51% of HCC development and by 56%

and 54% of compensated and decompensated cirrhosis could be expected in 2030, respectively, as well as a 56% decrease in mortality rates. The cumulative costs of HCV infection were reduced by 26% from the base case. If the time span was extended to all years, a 90% decrease in compensated and decompensated liver cirrhotic patients was expected by 2030, with a reduction of HCC by 84%. In absence of eligibility restriction, chronically infected people were estimated to reduce by 60% in 2030, with a slightly lower decrease of cases of cirrhosis and HCC and comparable

cumulative costs reduction.

A similar study conducted on the French population analysed the reduction in the need for LT associated with HCV infection treatment. Based on two main scenarios constructed by estimating the number of LT candidates between 2013-2022, the authors demonstrated that antiviral treatments will avoid 4425 transplants, reducing by 45% and by 88% the gap between liver organs request and availability for patients with decompensated cirrhosis and HCC, respectively. This will allow for satisfaction of the LT demand for patients affected by HCC within 2022, although (probably) the same results cannot be achieved for decompensated cirrhotic patients<sup>[114]</sup>.

Finally, Kabiri *et al.*<sup>[115]</sup> published a transition model analysis to predict the effect of HCV therapies in the United States. Compared to a scenario including new therapies but with limited treatment capacities and risk-based or birth-cohort screening, a scenario with universal screening and absence of treatment limitations was able to prevent 91000 cases of HCC, 128800 cases of decompensated cirrhosis, 153200 liver-related deaths, and 13400 LT. The authors concluded that HCV might be destined to become a rare disease within 2036.

Although the major limitation of these studies is represented by the correct estimation of treatment response rates, as well as by the quantification of treatment costs, which are in constant evolution, they may provide a useful projection of the evolution of HCV-related health and economic burden in the near future.

## CONCLUSION

The discovery of DAAs has radically changed the world scene of hepatitis C infection and its associated morbidity and mortality.

The current evolution and revolution of HCV antiviral treatment has increased the number of patients achieving viral eradication and, therefore, is going to reduce the incidence of cirrhosis, the rate of liver decompensation and HCC development, as well as patients' mortality. This will probably lead to a decrease in the need for LT, providing an adequate supply for nearly all patients with HCC and part of those with decompensated cirrhosis. The future widespread use of these new antivirals might also influence the policy of donor selection, leading to the expansion of the pool of available liver organs, since HCV infection may represent no more a contraindication for the use of liver grafts.

Although DAAs have made it possible to envisage a bright future in the fight against HCV-related liver disease, only long-term follow-up studies will allow for accurate quantification of the benefit obtained. The assessment of less evident effects of the new antivirals, such as microenvironmental and immunologic changes in the liver, is also mandatory to predict and avoid the occurrence of possible unexpected consequences.

Finally, the disparity in the use of DAAs throughout

the world caused by the high costs and the restricted availability makes it difficult to draw definitive conclusions about the future epidemiology and evolution of HCV-related liver disease worldwide.

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Basic Study

# Characterization of a new monoclonal anti-glypican-3 antibody specific to the hepatocellular carcinoma cell line, HepG2

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## Abstract

### AIM

To characterize the antigen on HepG2 cell that is specifically recognized by a new monoclonal antibody raised against human liver heparan sulfate proteoglycan (HSPG), clone 1E4-1D9.

### METHODS

The antigen recognized by mAb 1E4-1D9 was immunoprecipitated and its amino acid sequence was analyzed LC/MS. The transmembrane domain, number of cysteine residues, and glycosylation sites were predicted from these entire sequences. Data from amino acid analysis was aligned with glypican-3 (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The competitive reaction of mAb 1E4-1D9 and anti-glypican-3 on HepG2 cells was demonstrated by indirect immunofluorescence and analyzed by flow cytometry. Moreover, co-immunoprecipitation of mAb 1E4-1D9 and anti-glypican-3 was performed in HepG2 cells by Western immunoblotting. The recognition by mAb 1E4-1D9 of a specific epitope on solid tumor and hematopoietic cell lines was studied using indirect immunofluorescence and analyzed by flow cytometry.

### RESULTS

Monoclonal antibody 1E4-1D9 reacted with an HSPG isolated from human liver and a band of 67 kD was

detected under both reducing and non-reducing conditions. The specific antigen pulled down by mAb 1E4-1D9, having a MW of 135 kD, was analyzed. The results showed two sequences of interest, gi30722350 (1478 amino acid) and gi60219551 (1378 amino acid). In both sequences no transmembrane regions were observed. Sequence number gi30722350 was 99.7% showed a match to FYCO1, a molecule involved in induction of autophagy. Sequence number gi60219551 contained 15 cysteines and 11 putative glycosylation sites with 6 predicted N-glycosylation sites. It was also matched with all PDZ domain proteins. Moreover, it showed an 85.7% match to glypican-3. Glypican-3 on HepG2 cells competitively reacted with both phycoerythrin-conjugated anti-glypican-3 and mAb 1E4-1C2 and resulted in an increase of double-stained cell population when higher concentration of mAb 1E4-1D9 was used. Moreover, antigens precipitated from HepG2 cell by anti-glypican-3 could be detected by mAb 1E4-1D9 and vice versa. The recognition of antigens, on other solid tumor cell lines, by mAb 1E4-1D9 was studied. The results demonstrated that mAb 1E4-1D9 reacted with Huh7, HepG2, HT29, MCF7, SW620, Caco2, B16F1, U937, K562 and Molt4 cells. It was also found to be weakly positive to SW1353 and HL60 and negative to H460 and Hela cell lines.

### CONCLUSION

All findings show that mAb 1E4-1D9 specifically recognizes glypican-3. Moreover, a new partner molecule of glypican-3, FYCO1 is proposed based on the results from co-precipitation studies.

**Key words:** Monoclonal anti-glypican-3; Hepatocellular carcinoma; HepG2; Heparan sulfate proteoglycan; Co-immunoprecipitation

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**Core tip:** Heparan sulfate proteoglycan (HSPG) was isolated from human liver. Preliminary results showed that it was detected by rabbit anti-glypican. Monoclonal antibody, 1E4-1D9 was raised against human liver HSPG and its specific antigen was characterized. Amino acid sequence analysis revealed that the antigen recognized by mAb 1E4-1D9 specific molecule contained no transmembrane region. It has 15 cysteines and 11 putative glycosylation sites and 6 predicted N-glycosylation sites. The sequence matched to all PDZ domain proteins with an 85.6% match to glypican-3. Studies of co-expression and co-precipitation demonstrated that mAb 1E4-1D9 could compete with anti-glypican-3. It could also react with a various tumor cell lines including solid and hematopoietic cells. The findings suggested that the antigen recognized by 1E4-1D9 was glypican-3. Moreover, findings revealed that FYCO1 co-precipitated with glypican-3 using mAb 1E4-1D9, suggesting that FYCO1 is a partner molecule of glypican-3.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer-related deaths<sup>[1,2]</sup>. The majority of these cases occur in Asia and Africa. However, the incidence has also been rising in the developed world. Among liver cancer cases, 80% are HCC, which does not respond well to chemotherapy<sup>[3]</sup>. Early detection is difficult and there are poor outcomes to aggressive therapies<sup>[4,5]</sup>. Thus, early detection of HCC is a key goal in improving this poor prognosis. In addition, identification of novel molecular targets for development of diagnostic and therapeutic approaches remains of great interest. Glypican-3 is highly expressed in HCC and recently has been suggested as a good diagnostic marker for HCC<sup>[6-14]</sup>. In addition to effective early diagnosis, drugs targeting different mechanisms of action involving glypican-3 targeted antibody therapy are addressed<sup>[15]</sup>. To date, several clones of monoclonal antibodies specific to glypican-3 have been described<sup>[6,8,16-19]</sup>. These have not only been used as research tools and in diagnostic development, but some have been developed for preparing potential agents for HCC immunotherapy<sup>[17-21]</sup>. Moreover, silencing of glypican-3 was recently reported to induce apoptosis in HCC cell lines<sup>[22]</sup>. Thus, glypican-3 has great promise as an excellent molecular target for the diagnosis and therapy of HCC.

Glypican is a family of heparan sulfate proteoglycans (HSPGs) that are expressed on the extracellular membrane as a glycosylphosphatidylinositol (GPI)-anchored proteoglycan. These HSPGs regulate cellular signaling during morphogenesis, adult physiology and carcinogenesis by interaction with a multitude of extracellular matrix molecules including chemokines, growth factors or morphogens and their receptors<sup>[23-25]</sup>. Glypican is expressed in cell-, tissue- and development-specific patterns. Among the six members of the glypican family, glypican-3 has been studied most extensively<sup>[23,26,27]</sup>.

Since glypican-3 is an HSPG, it typically contains a heparan sulfate glycosaminoglycan chain (GAG), but in some instances a chondroitin sulfate (GAG) can also be found on glypican-3<sup>[23]</sup>. GAG chains carry negative charge, allowing glypican-3 to interact with basic growth factors and morphogens in the extracellular space. Glypican-3 has a 70-kD core protein which can be cleaved by furin generating two fragments of 40-kD N-terminal and 30-kD C-terminal<sup>[27]</sup>. The GPI anchor linking glypican-3 to the membrane can be cleaved by lipase (notum), releasing glypican-3 to extracellular matrix<sup>[28]</sup>. The

shedding of glypican-3 plays a role in regulating signaling of Wnts, hedgehogs, fibroblast growth factors, and bone morphogenetic proteins<sup>[23,26,29,30]</sup>. There has also been a report that soluble glypican-3 can inhibit HCC proliferation both *in vitro* and *in vivo*<sup>[31]</sup>. Therefore, glypican-3 can play both positive and negative role in cell growth depending on cell type<sup>[32,33]</sup>. Glypican-3 is expressed in a variety of tissues and acts as oncofetal protein. Among membrane HSPGs, glypican-3 is the only HSPG that is highly expressed on HCC tissue but it is usually not found in normal and in non-tumor liver tissues<sup>[34]</sup>. Previous findings indicate that glypican-3 stimulates *in vitro* and *in vivo* growth of HCC<sup>[26,35-39]</sup>. The mechanism in HCC growth promotion of glypican-3 is to regulate Wnt signaling as well as oncogenesis through insulin-like growth factor signaling pathway<sup>[40]</sup>. It was reported that, in primary HCC, sulfatase-2 (SULF2) enzyme with 6-O-sulfatase activity is up-regulated and associated to poor prognosis<sup>[41]</sup>. Increasing of SULF2 enhances the expression of glypican-3 *in vitro* and *in vivo*<sup>[42]</sup>.

The liver is a rich source of GAGs and the liver is known to be receptor of many molecules involved in diseases and in pathogen binding<sup>[43-46]</sup>. Recently, an HSPG was isolated from human liver. The analysis of its GAG component demonstrated that it was heparan sulfate, not heparin<sup>[47]</sup>. Digestion of liver HSPG with heparin lyase I, II, III yielded a core protein product that could be detected by anti-rat glypican with a band of approximately 61 kD. These results suggested that the HSPG isolated from human liver was a glypican.

Monoclonal antibodies were raised against liver HSPG. Two of the clones obtained are 1E4-1C2 and 1E4-1D9. The clone 1E4-1C2 specifically reacts with membrane molecules of various malignant cell lines, including solid tumor and hematopoietic cells in erythromyeloid series<sup>[48]</sup>. This antibody can differentiate between acute myeloid leukemia from normal blood cells and normal blast cells in bone marrow. Moreover, mAb 1E4-1C2 strongly reacts with HepG2 cells and inhibits cell proliferation in a dose dependent manner both *in vitro* and in an animal model<sup>[49]</sup>. Development of HepG2 cell-targeted drug delivery based on mAb 1E4-1C2 has also been studied<sup>[50]</sup>. Intensive characterization of mAb 1E4-1C2 and its specific antigen is in progress.

Our preliminary results of mAb 1E4-1D9 showed that it could react with HepG2. Together with the previous observations that liver HSPG was a glypican and that glypican-3 is up regulated in HCC, we hypothesized that antigen recognized by mAb 1E4-1D9 was glypican-3. The present study is aimed at characterizing the specific antigen on HepG2 cells recognized by mAb 1E4-1D9.

## MATERIALS AND METHODS

### Cell lines

HL60 cell line was a kind gift from Associate Professor, Dr. Songyot Anuchpreeda, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Huh7 was from Professor, Dr.

Pa-thai Yenchitsomanus, Faculty of Medicine Siriraj Hospital, Mahidol University. The other cell lines were purchased from ATCC.

### Reagents and reagent kits

OPI supplement, fetal bovine serum, 3,3-diamino benzidine (DAB), and SuperSignal™ West Pico Chemiluminescent Substrate were purchased from Sigma-Aldrich (St. Louis, MO, United States). All culture media were from Gibco (Life Technologies, NY, United States). Mouse IgG1 and phycoerythrin (PE) conjugated mouse IgG2a were purchased from Biolegend, CA, United States and anti-glypican-3 [clone 9C2, IgG1, immunogen: Recombinant human glypican-3 (amino acid 1-580)] was from Abcam (United Kingdom). PE conjugated anti-glypican-3 [clone 307801, IgG2a, immunogen: Recombinant human glypican-3 (amino acid 25-558)] was obtained from United States Biological Life Sciences, MA, United States. Fluorescein isothiocyanate (FITC) conjugated anti-mouse Igs and horseradish peroxidase (HRP)-conjugated anti-mouse Igs were purchased from Dako (CA, United States). Protein G agarose was purchased from Pierce (Rockford, IL, United States). IsoStrip was obtained from Roche (IN, United States). Other common reagents used in these studies were purchased from local reputable companies including PCL Holdings (Thailand) and Pacific Sciences (Thailand).

### Preparation and purification of mAb 1E4-1D9 antibody

The hybrid clone 1E4-1D9 was grown in OPI containing-Dulbecco's Modified Eagle's medium (DMEM)/high glucose supplemented with 10% fetal bovine serum to exponential phase. Cell culture supernatant was collected and mAb 1E4-1D9 was purified using protein G affinity agarose beads. Briefly, cell culture supernatant was diluted with binding buffer provided (1:1 v/v) before applying and allowed to flow completely into the resin. The column was then washed with binding buffer and eluted with the elution buffer provided. Fractions of 1 mL were collected and neutralized with neutralizing buffer (Tris-base, pH 8.0, 100 µL). Pooled purified mAb was dialyzed against phosphate buffered saline (PBS) pH 7.2, concentrated and aliquots were frozen. Isotype was determined using IsoStrip according to the manufacturer's directions.

### Determination of mAb 1E4-1D9 specificity to human liver HSPG

HSPG isolated from human liver<sup>[47]</sup> was diluted to 5 µg/mL with PBS, pH 7.2. Twenty µL of sample was mixed with 5 µL of 5 × sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 70 mmol/L sodium dodecylsulfate (SDS), 10% glycerol, 2% bromphenol blue) and non-reducing sample buffer, and boiled for 5 min. Sample was subjected to electrophoresis on 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 200 V for 45 min and blotted onto polyvinylidene difluoride (PVDF) membrane. Before probing with mAb 1E4-1D9, non-specific sites were blocked with 5% non-fat dried milk in tris-buffered saline (TBS) pH 7.4

(0.15 mol/L NaCl, 10 mmol/L Tris-base) for 1 h at room temperature on a rocking plate. The membrane was washed 3-times (10 min each) with TBS pH 7.4. Primary antibody (mAb 1E4-1D9, 100 µg/mL in 0.1% Tween-20 in; TBS-Tween) was added onto the membrane. The reaction was performed at room temperature for 1 h on a rocking plate. After completion, membrane was washed with TBS-Tween for 3-times (10 min each) on a rocking plate. The reaction was then detected with HRP-conjugated rabbit anti-mouse Igs for 1 h at room temperature on a rocking plate and washed. Finally, signal was then developed with DAB containing H<sub>2</sub>O<sub>2</sub>. Molecular weight (kD) was calculated from a plot of log molecular weight standard vs migration distance and a  $R^2 \geq 0.99$  was obtained.

### **Expression of mAb 1E4-1D9 on HepG2 cell lines**

HepG2 cells cultured in DMEM high glucose supplemented with 10% fetal bovine serum (FBS) grown to exponential phase. Cells were collected, washed twice with PBS, pH 7.2. Cell viability was checked by trypan blue dye exclusion assay and adjusted to  $4 \times 10^5$  cells/mL with PBS pH 7.2. Heat-inactivated normal human AB serum was added to the final concentration of 10% and incubated on ice for 30 min. An aliquot of cell suspension (50 µL) was added to an equal volume of various final concentrations of mAb 1E4-1D9. Mouse IgG1 and washing buffer [cold 1% bovine serum albumin (BSA)-PBS, 0.02% NaN<sub>3</sub>] were used as isotype and conjugated control, respectively. The reactions were incubated on ice for 30 min. After completion, cells were washed 3-times with washing buffer. Fifty microlitre of FITC-conjugated rabbit anti-mouse Igs (1:20 diluted in washing buffer) was added and reaction was incubated for another 30 min on ice. Following with 3-washes, cell pellet was suspended with 300 µL of 0.5% paraformaldehyde in PBS, pH 7.2 and analyzed by flow cytometer (Becton Dickinson, CA, United States).

### **Immunoprecipitation of mAb 1E4-1D9 specific antigen for amino acid analysis**

HepG2 cells grown to exponential phase were harvested, washed 5-times with PBS pH 7.2 (0.137 mol/L NaCl, 2.68 mmol/L KCl, 1.88 mmol/L NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 8.10 mmol/L Na<sub>2</sub>HPO<sub>4</sub>) and adjusted to  $1 \times 10^6$  cells/mL. One millilitre of lysis buffer (1% Brij58, 20 mmol/L Tris-HCl pH 7.5, 0.15 mol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 5 mmol/L iodoacetamide, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L pepstatin A, 10 mg/mL aprotinin) was added. The suspension was mixed thoroughly, centrifuged 12000 rpm at 4 °C for 30 min to pellet cell debris. HepG2 cell lysate was used as source of antigen precipitating by mAb immobilized protein G agarose beads.

Prior to immobilization of mAb, 50 µL of protein G was washed 3-times with binding buffer (0.2 mol/L NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.15 mol/L NaCl) followed by immunoprecipitation (IP) buffer (25 mmol/L Tris-base, 0.15 mol/L NaCl). One

hundred microlitres of mAb 1E4-1D9 (100 µg/mL) was then mixed individually with protein G agarose beads for 30 min at room temperature before washing (3-times) out non-bound mAb with IP buffer. Washing buffer was discarded and 100 µL of HepG2 lysate was added. The reaction was incubated at 4 °C overnight. After completion, the reaction was centrifuged, supernatant was discarded and beads were washed 6-times with IP buffer. Fifty microlitre of elution buffer (0.1 mol/L glycine, pH 3.0) was added and mixed for 5 min. Finally beads were pelleted down and eluate, containing specific antigen, was collected. This step was repeated twice. Collected supernatants were pooled and neutralized with 10 µL of neutralizing buffer (1 mol/L Tris-base, pH 8.0).

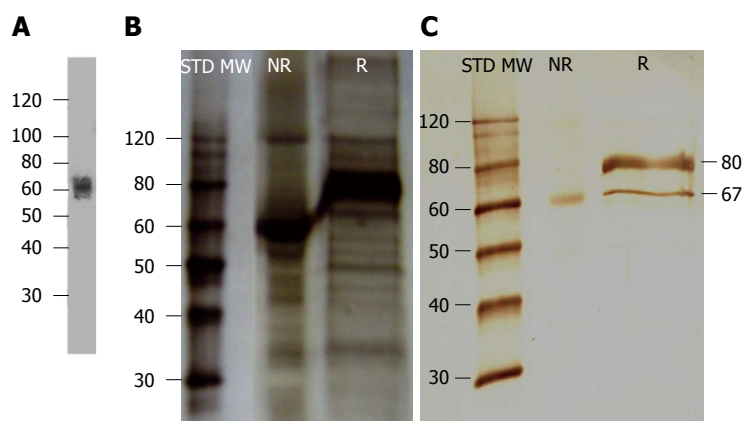
Twenty microlitres of eluate was mixed with 5 µL of  $5 \times$  sample buffer and separated on 10% SDS-PAGE at 200 V for 45 min before blotting onto PVDF membrane. Non-specific binding sites on the membrane were blocked with 5% non-fat dried milk in TBS pH 7.4 (0.15 mol/L NaCl, 10 mmol/L Tris-base) for 1 h at room temperature on a rocking plate. PVDF membrane was then washed, with 0.1% Tween-20 in TBS (TBS-Tween), 3-times for 10 min each on a rocking plate. Primary antibody, mAb 1E4-1D9 (1 mg/mL in 1%BSA TBS-Tween) was added to each membrane. The reaction was performed at 4 °C overnight. After completion, membrane was washed (3-times for 10 min each) with 0.1% Tween-20 in TBS (TBS-Tween) on a rocking plate. The reaction was then detected with HRP-conjugated rabbit anti-mouse Igs for 1 h at room temperature on a rocking plate. After washing out the excess antibody (3-times for 10 min each) with 0.1% Tween-20 in TBS (TBS-Tween), signal was visualized by SuperSignal™ West Pico chemiluminescent substrate. Molecular weight was calculated from standard molecular weight graph as previously mentioned.

The eluate was pooled and subject to electrophoresis in  $5 \times$  non-reducing sample buffer on 10% SDS-PAGE at 200 V for 45 min. Gel was stained with Coomassie Brilliant Blue to prepare mAb 1E4-1C2 specific antigens for amino acid sequence analysis. The band of interest, selected by as comparison to result on immunoblot, was cut and sent for amino acid analysis by LC-MS (HDMS Synaptat, Waters, MA, United States) at the National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand).

### **Co-expression of mAb 1E4-1D9 and anti-glypican-3 on HepG2 cells**

HepG2 cells in exponential phase were harvested and washed twice with PBS, pH 7.2. Cell viability was determined by trypan blue dye exclusion assay and adjusted to  $4 \times 10^5$  cells/mL with PBS pH 7.2. After blocking with heat-inactivated normal AB serum for 30 min on ice, an aliquot of cell suspension (50 µL) was added to equal volumes of various concentrations of mAb 1E4-1D9. Mouse IgG1 was used as isotype control. The reaction was incubated on ice for 30 min follow by 3-washes with cold washing buffer. Cell pellet was re-





**Figure 1** Liver heparan sulfate proteoglycan was detected by anti-rat glypican and mAb 1E4-1D9. A: Liver heparan sulfate proteoglycan (HSPG) was digested with heparin lyase I, II, III and probed with anti-rat glypican<sup>[47]</sup>; B: Silver stain of liver HSPG; C: Liver HSPG was reacted with mAb 1E4-1D9 and visualized by horseradish peroxidase-conjugated rabbit anti-mouse IgG following with 3,3-diamino benzidine/ $H_2O_2$  substrate.

suspended with 50  $\mu$ L washing buffer and added with equal volume of FITC-conjugated rabbit anti-mouse IgG (1:20 diluted in washing buffer). After incubating on ice for another 30 min, cells were washed 3-times and PE-conjugated anti-glypican-3 (1:10 diluted with washing buffer). PE conjugated mouse IgG2a was used as isotype control. The reaction was performed on ice for 30 min and washed 3-times. Finally, cells were suspended with 300  $\mu$ L of 0.5% paraformaldehyde in PBS, pH 7.2 and analyzed by flow cytometer.

#### Co-immunoprecipitation of mAb 1E4-1C2 and anti-glypican-3 on HepG2 cells

HepG2 cell lysate was prepared as mentioned above and was used as source of antigen. The three different antibody immobilized protein G agarose beads, anti-glypican-3, mAb 1E4-1D9, and mouse IgG1 (isotype control) were immobilized on protein G agarose beads as mentioned.

Twenty microlitres of eluates from mAb 1E4-1D9, anti-glypican-3, or mouse IgG1 immobilized protein G agarose beads was separated on 10% SDS-PAGE at 200 V for 45 min in non-reduced condition and blotted onto PVDF membrane. Three membranes were prepared. Non-specific binding sites on the membrane were blocked with 5% non-fat dried milk in TBS, pH 7.4 for 1 h at room temperature on a rocking plate. PVDF membrane was then washed, 3-times for 10 min, with TBS-Tween each on a rocking plate. Primary antibody [mAb 1E4-1D9, anti-glypican-3, or mouse IgG1 isotype control (100  $\mu$ g/mL in 1% BSA TBS-Tween)] was added to each individual membrane. The reaction was performed at 4  $^{\circ}$ C overnight. After completion, membrane was washed, 3-times for 10 min, with TBS-Tween each on a rocking plate. The reaction was then detected with HRP-conjugated rabbit anti-mouse IgG for 1 h at room temperature on a rocking plate. After washing (3-times for 10 min each) out the excess antibody with TBS-Tween, signal was then developed by SuperSignal<sup>TM</sup> West

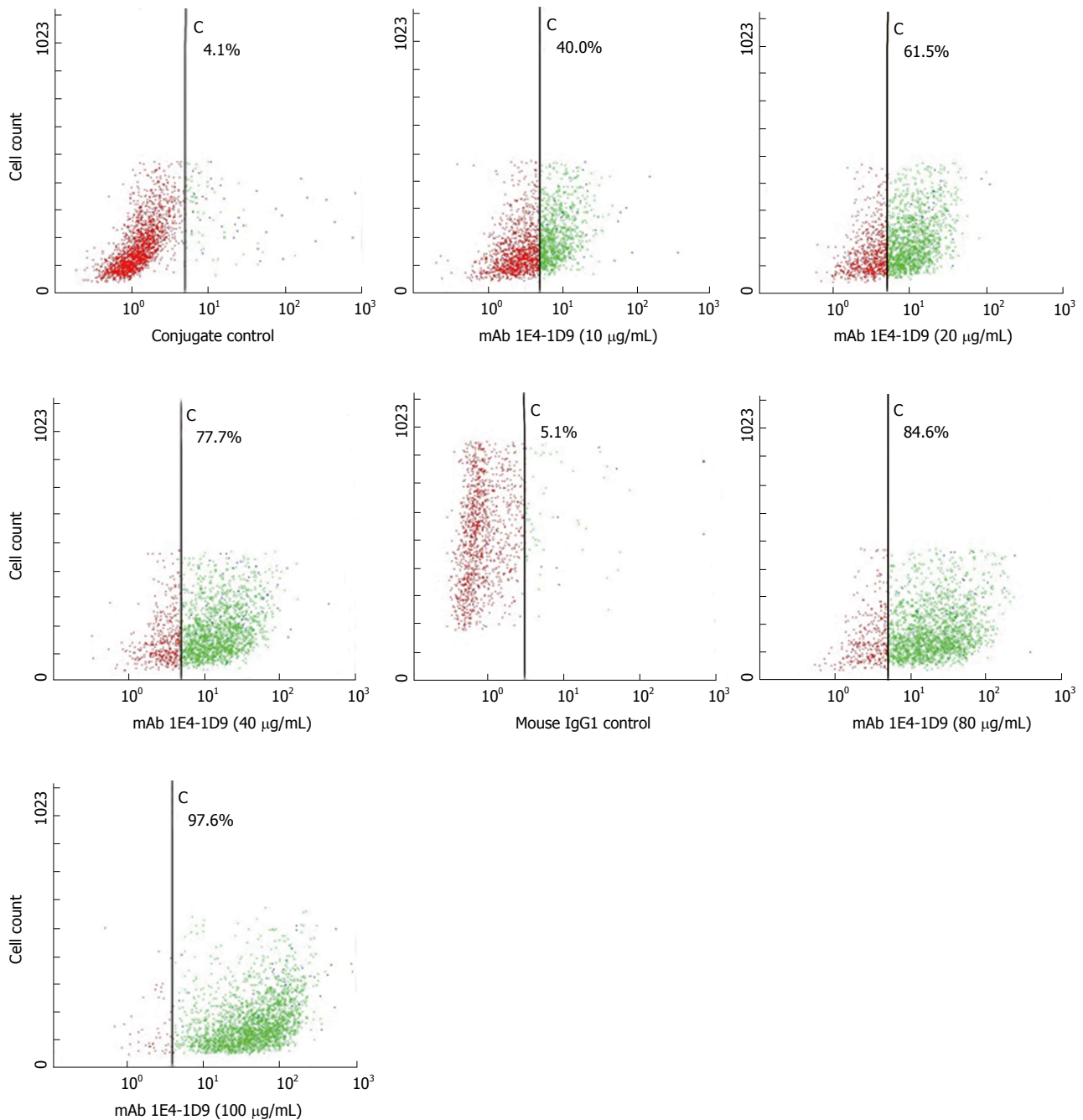
Pico chemiluminescent substrate and auto-radiographed. The molecular weight was calculated from a standard molecular weight plot as previously described.

#### Expression of mAb 1E4-1D9 on malignant cell lines

Solid tumor cell lines (Huh7, B16F1, HT29, Caco2, MCF7, SW620, SW1353, H460 and Hela) cultured in DMEM high glucose supplemented with 10% FBS and hematopoietic cell lines (HL60, K562, U937 and Molt4) cultured in RPMI-1640 were grown to exponential phase. Cells were collected, washed twice with PBS, pH 7.2. Cell viability was checked by trypan blue dye exclusion assay and adjusted to  $4 \times 10^5$  cells/mL with PBS pH 7.2. Heat-inactivated normal human AB serum was added to cell suspension to the final concentration of 10% and incubated on ice for 30 min. Aliquot of cell suspension (50  $\mu$ L) was added with an equal volume of mAb 1E4-1D9 (20  $\mu$ g/mL). Mouse IgG1 and washing buffer (cold 1%BSA-PBS, 0.02%  $NaNO_3$ ) were used as isotype control and conjugated control, respectively. The reactions were incubated on ice for 30 min. After completion, cells were washed 3-times with washing buffer. Fifty microlitres of FITC-conjugated rabbit anti-mouse IgG (1:20 diluted in washing buffer) was added and reaction was incubated for another 30 min on ice. Following 3-washes with washing buffer, the cell pellet was suspended with 300  $\mu$ L of 0.5% paraformaldehyde in PBS, pH 7.2 and was analyzed by flow cytometer (Becton Dickinson, CA, United States).

## RESULTS

Before any assay was performed, antibody isotype was determined using a commercial isotyping kit and it was confirmed that mAb 1E4-1D9 was an IgG1. The specificity to the immunogen was also studied by Western immunoblotting of liver HSPG and probed with mAb 1E4-1D9. The results demonstrated a band was detected at approximately 67 kD under both non-reducing and reducing conditions (Figure 1C). The molecular weight



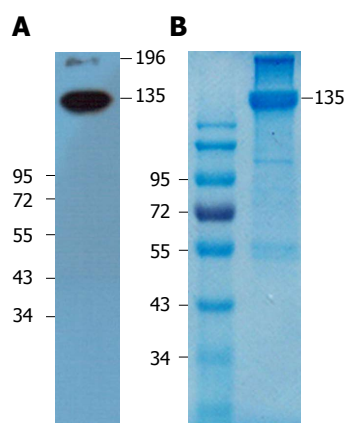
**Figure 2** HepG2 cells ( $4 \times 10^5$  cells/mL) were reacted with various final concentrations of mAb 1E4-1D9 (0-160 µg/mL) for 30 min on ice. Mouse IgG1 was used as isotype control. After washing, cells were stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse Igs (1:20) for 30 min on ice and washed. Finally, cells were suspended with 300 µL of 0.5% paraformaldehyde in phosphate buffered saline, pH 7.2 and analyzed by flow cytometry.

was close to that reported previously where liver HSPG was probed with rabbit anti-rat glypican<sup>[47]</sup> (Figure 1A). These results suggest that the epitope of mAb 1E4-1D9 is present in both folded and linear forms.

We next used indirect immunofluorescence to examine the expression of the antigen on HepG2 cells that reacts with mAb 1E4-1D9. mAb 1E4-1D9 reacted specifically to an antigen on HepG2 in concentration dependent manner (Figure 2). Moreover the highest expression of this antigen was observed during incubation (data not

shown) while HepG2 was in exponential phase was at day-4 of incubation. The specific antigen was immune-precipitated by mAb 1E4-1D9 immobilized protein G agarose beads. A band at 135 kD was visualized by immunoblotting (Figure 3A) and was cut from the gel (Figure 3B) and sent for analysis.

Amino acid analysis demonstrated the presence of two hypothetical sequences, gi30722350 (1478 amino acid) and gi60219551 (1378 amino acid). Neither sequence had a transmembrane region domain based on analysis



**Figure 3** Specific antigen was immunoprecipitated from HepG2 cell lysate by mAb 1E4-1D9-immobilized protein G agarose beads. Eluate was separated under non-reducing conditions in 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis at 200 V for 45 min. One gel was blotted onto polyvinylidene difluoride membrane and probed with mAb 1E4-1D9. A: The reaction was detected by HRP-conjugated rabbit anti-mouse IgG and signal was developed by SuperSignal™ West Pico Chemiluminescent Substrate; B: Another gel was stained with Coomassie Brilliant Blue and protein band of 135 kD was cut and sent for amino acid analysis (B).

by TMHMM software (Figure 4A). Data analysis also demonstrated the number of cysteine residues was 19 and 15 in gi30722350 and gi60219551, respectively. Moreover, gi30722350 contains two putative glycosylation sites while the latter, gi60219551 has 11 putative glycosylation sites at amino acid 139, 227, 377, 393, 577, 721, 891, 911, 1053, 1090 and 1243, respectively with 6 predicted N-glycosylation sites (amino acid 139, 227, 377, 577, 721 and 1053) (Figure 4B). Alignment of gi30722350 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) demonstrated that it was matched to all FYVE containing protein with 99.7% matched to FYCO-1 (data not shown). Interestingly, gi60219551 matched to a PDZ domain protein with 85.7% match to glypican-3 (Figure 4C).

Expression of mAb 1E4-1D9 together in competition with anti-glypican-3 was undertaken to verify that antigen specific to mAb 1E4-1D9 was glypican-3. However, prior to this experiment, the concentration of PE-conjugated anti-glypican-3 was optimized for maximum intensity detection by direct immunofluorescence. The result indicated that PE-conjugated anti-glypican-3 at dilution of 1:10 could specifically react to 97.8% of antigen on HepG2 cells (data not shown). Various final concentrations of mAb 1E4-1D9 were used to react with HepG2 cells followed by fixing with PE-conjugated anti-glypican-3 and analyzed by flow cytometry. The number of cells, in the upper right quadrant (positive both FL1 and FL2), increased in dose dependent manner while FL2 signal of the PE-conjugated anti-glypican-3 decreased (Figure 5). This indicates that mAb 1E4-1D9 is specific to glypican-3 on HepG2 since mAb 1E4-1D9 could compete with PE-conjugated anti-glypican-3 used. Moreover, it suggests that antigenic site of mAb 1E4-1D9 on glypican-3 may be at or close to N-terminal region

because immunogen of PE-conjugated anti-glypican-3 used was recombinant human glypican-3 (amino acid 25-558).

Co-immunoprecipitation of a specific antigen on HepG2 cells by mAb 1E4-1D9 and anti-glypican-3 was performed. Mouse IgG1 was used in parallel as an isotype control. Findings from experiments show that mAb 1E4-1D9 precipitated three interesting bands of 69, 115 and 130 kD (Figure 6B), which also reacted with anti-glypican-3 (Figure 6C). A protein band of 130 kD precipitated by anti-glypican-3 was clearly visualized by mAb 1E4-1D9 (Figure 6B). However, anti-glypican-3 itself showed less reaction (Figure 6C). Lysate was probed with anti-glypican-3 to verify that lysate contained glypican-3 and a band was observed at 115 kD (Figure 6D). Taken together this demonstrated that mAb 1E4-1D9 could react with antigen precipitated by anti-glypican-3 and vice versa.

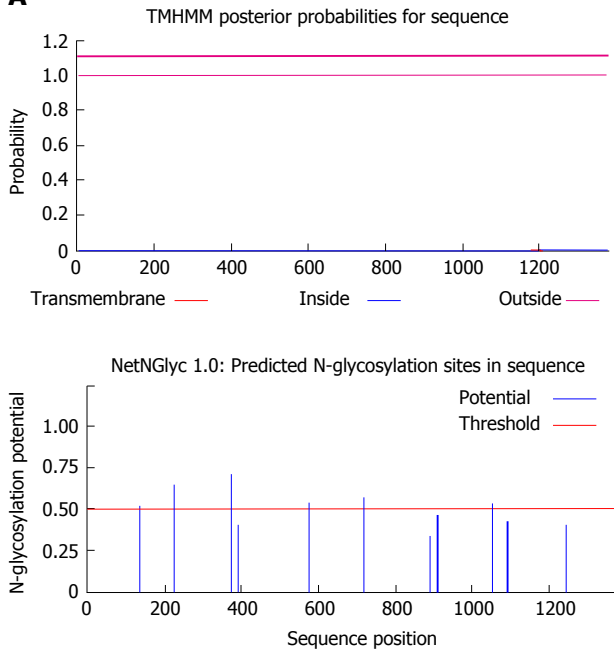
Expression of mAb 1E4-1D9 on other cells was studied by indirect immunofluorescence. We found that the antigen recognized by mAb 1E4-1D9 was expressed on a variety of cell lines tested including B16F1, Caco2, HT29, MCF7, SW620, K562, U937 and Molt4 (Figure 7). Some cells such as SW1353 and HL60 were weakly positive and some were negative (H460 and Hela cells).

## DISCUSSION

HCC is one of the most common cancers worldwide with a poor prognosis and a low 5-year survival rate. Thus, specific biomarkers have become increasingly important to identify HCC. Glypican-3 is upregulated and highly expressed in HCC but not in normal or non-malignant liver tissues. Glypican-3 has important roles in cell growth, differentiation and motility<sup>[33]</sup>. As a key molecule in relation to signaling with several growth factors and growth factor receptors, glypican-3 can regulate the proliferation of malignant cells both in negative and positive ways<sup>[32]</sup>. Therefore, antibodies specific to glypican-3 are of interest and many antibody-expressing clones have been developed. Some clones are used to prepare antibodies as tools to study the glypican-3 related cellular activities and some have been applied in tumor investigation and tumor-specific drug development<sup>[6,15,20,51]</sup>.

Our previous report demonstrated that HSPG isolated from human liver contained glypican<sup>[47]</sup>. Monoclonal antibody raised against human liver HSPG, mAb 1E4-1D9 was, thus, proposed to be specific to glypican-3, which is the only membrane HSPG that highly expressed by HCC<sup>[34]</sup>.

Probing of liver HSPG with mAb 1E4-1D9 resulted a band of 67 kD under both reducing and non-reducing conditions indicate that epitope of mAb 1E4-1D9 can be recognized in either the folded and linear forms. Amino acid analysis of band of 135 kD precipitated from mAb 1E4-1D9 afforded two hypothetical sequences, gi30722350 (1478 amino acid) and gi60219551 (1378

**A**

**B**

Name: Sequence gi\_60219551 Length: 1378

VGHNFIRSVLPEGPVGHSGKLFSGDELLEVNGITLLGENHQDVVNILKELPIEVTMVCCRRTPVPTTQSELDLGIQHIE 80  
 LEKSGKLGFSILDYQDPIDPASTVIIIRSLVPGGIAEKDGRLLPGDRLMFVNDVNLENSSLEEAVEALKGAPSGTVRIG 160  
 VAKPLPLSPEEGYVSAAKEDSFLYPHSCFEAGLADKPLFRADLALVGTNDADLVDESTFESPYSPENDSIYSTQASILSL 240  
 HGSSCGDGLNYSGLSPSPKDVIENTCDPVLDLHMSLEELYTQNLLQRQDENTPSVDISMGPASGFTINDYTPANAIEQ 320  
 QYECENTIVWTESHLPSVIESSAELPSVLPDSAGKGEYLLEQSSLAENAEVMLQNVSKESFERTINIAKGNSSLGMTV 400  
 SANKDGLGMIVRSIIHGGAISSRDGRIAGDCILSINEESTISVTNAQARAMLRHSLIGPDIKITYVPAEHLEEFKISLG 480  
 QQSGRVMALDIFSSYTGRIPELPEREEGEGESELQNTAYSNNWNQPRRVELWREPSKSLGISIVGGRMGSRSLNGEVM 560  
 RGIFIKHVLSDSPAGKNGTLKPGDRIVEAPSQSESEPEKAPLCSVPPPPSAFAEMGSDHTQSSASKISQDVKEDFGY 640  
 SWKNIRERYGTLTGELHMIIELEKGHSLGLSLAGNKDRSRMSVFIVGIDPNGAAGKDGRLLQIADELLEINGQILYGRSHQ 720  
 NASIIKCAPSKVKIIFIRNKDAVNQMAVCPGNAVEPLPSNSENQNKETEPTVTTSDAAVDLSSFKNVQHLELPKDQGG 800  
 LGIAISEEDTLGVIKSLTEHGAATDGRLLKVGDDQILAVDDEIVGVPIEFISLLKTAKMTVKLTIHAENPDSQAVPS 880  
 AAGAASGEKKNSQSLMVPQSGSPEPESIRNTSRSSSTPAIFASDPATCPIIPGCTTIEISKGRGTGLGLSIVGSDTLG 960  
 AIIHEVYEEGAAKCDGRLLWAGDQILEVNGIDLKATHDEAINVLRQTPQRVRLTYRDEAPYKEEEVCDTLTIELQKKP 1040  
 GKGLGLSIVGKRNDTGVFVSDIVKGGIADADGRLLMQGDQILMVNGEDVRNATQEAVAALLKCSLGTVTLEVGRKAGPFH 1120  
 SERRPSQSSQVSEGLSSFTPLSGSSTSESSSKKNALASEIQGLRTVEMKKGPDTSLGISIAGGVGSPLGDVPIFI 1200  
 AMMHPTGVAQAQTKLRVGDRIVTCGTSTEGMTHTQAVNLLKNASGSIEMQVWAGGDMSVVTGHQEPASSSLSFGLTS 1280  
 SSIFQDDLGPQCKSITLERGPDGLGFSIVGGYGSPhGLDPIYKTVFAKGAASEDGRLLKRGDQIIAVNGQSLEGVTHEE 1360  
 AVAILKRTKGTVTLMVLS

(Threshold = 0.5)

SeqName	Position	Potential	Jury	N-Glyc agreement result
1	Sequence	139 NSSL	0.5176	(6/9) +
2	Sequence	227 NDSI	0.6440	(8/9) +
3	Sequence	377 NVSK	0.7067	(9/9) ++
4	Sequence	393 NSSL	0.4068	(5/9) -
5	Sequence	577 NGTL	0.5366	(7/9) +
6	Sequence	721 NASS	0.5723	(7/9) +
7	Sequence	891 NSSQ	0.3354	(9/9) --
8	Sequence	911 NTSR	0.4600	(7/9) -
9	Sequence	1053 NDTG	0.5287	(6/9) +
10	Sequence	1090 NATQ	0.4311	(8/9) -
11	Sequence	1243 NASG	0.4047	(9/9) --



## C

CLUSTAL O(1.2.3) multiple sequence alignment

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gi|60219551
VGHHFIRSVLPEGPVGHSGKLFSGDELLEVNGITLLGENHQDVVNILKELPIEVTMVCCR
NP_001158090.1      -----MAGTVRTAC-----LVVAMLL-----
NP_001158091.1      -----MAGTVRTAC-----LVVAMLL-----
NP_001158089.1      -----MAGTVRTAC-----LVVAMLL-----
AAH35972.1          -----MAGTVRTAC-----LVVAMLL-----
NP_004475.1          -----MAGTVRTAC-----LVVAMLL-----
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gi|60219551      RTVPPTTQSELDSLGIQHIELEKSGKLGFSILDYQDPIDPASTVIIIRSLVPGGIAEKD
NP_001158090.1    -----SLDFPGQAQPPPPPDATCHQVRSFF-----
NP_001158091.1    -----SLDFPGQAQPPPPPDATCHQVRSFF-----
NP_001158089.1    -----SLDFPGQAQPPPPPDATCHQVRSFF-----
AAH35972.1        -----SLDFPGQAQPPPPPDATCHQVRSFF-----
NP_004475.1        -----SLDFPGQAQPPPPPDATCHQVRSFF-----
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gi|60219551      GRLLPGDRLMFVNDVNLNSSLEEAVEALKGAPSGTVRIGVAKPLPLSPEEGYVSAKEDS
NP_001158090.1    ---QRLQPLGK--WVPETPVPGSDLQV-----CLP-----
NP_001158091.1    ---QRLQPLGK--WVPETPVP-----CLP-----
NP_001158089.1    ---QRLQPLGK--WVPETPVPGSDLQV-----CLP-----
AAH35972.1        ---QRLQPLGK--WVPETPVPGSDLQV-----CLP-----
NP_004475.1        ---QRLQPLGK--WVPETPVPGSDLQV-----CLP-----
                        ** ** : : * : :

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gi|60219551      FLYPPHSCCEEAGLADKPLFRADLALVGTNDADLVDESTFESPYS---PENDSIYSTQASILS
NP_001158090.1    ---KGPTCC--SRKMEEKYQLTARLNM-----EQLLQSA-----KAFEIVV
NP_001158091.1    -----EAFEIVV
NP_001158089.1    ---KGPTCC--SRKMEEKYQLTARLNM-----EQLLQSASMEKFLIIQNAAVFQEAFAEIVV
AAH35972.1        ---KGPTCC--SRKMEEKYQLTARLNM-----EQLLQSASMEKFLIIQNAAVFQEAFAEIVV
NP_004475.1        ---KGPTCC--SRKMEEKYQLTARLNM-----EQLLQSASMEKFLIIQNAAVFQEAFAEIVV
                        . * :

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gi|60219551      LHGSSCGDGLNYGSSLPSPPKDVIENSCDPVLDLHMSLEELYTQNLQRQDENTPSVDI
NP_001158090.1    RHAKN-----YTNA-----
NP_001158091.1    RHAKN-----YTNA-----
NP_001158089.1    RHAKN-----YTNA-----
AAH35972.1        RHAKN-----YTNA-----
NP_004475.1        RHAKN-----YTNA-----
                        * . .      ** :

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gi|60219551      SMGPASGFTINDYTPANAIEQQYECENTIVWTESHLPSEVISSAELPSVLPDSAGKGSEY
NP_001158090.1    -----MFKNNYPSLTPQAFEFVGEF
NP_001158091.1    -----MFKNNYPSLTPQAFEFVGEF
NP_001158089.1    -----MFKNNYPSLTPQAFEFVGEF
AAH35972.1        -----MFKNNYPSLTPQAFEFVGEF
NP_004475.1        -----MFKNNYPSLTPQAFEFVGEF
                        : . : ** : * : : * :

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gi|60219551      LLEQSSSLACNAECVMLQNVSKESFERTINIAGNSSLGMTVSANKDGLGMIVRSIIHGGA
NP_001158090.1    FTDVSLYILG-SDINVDDMVNELFDSLFP-----VIYTQLMNP--
NP_001158091.1    FTDVSLYILG-SDINVDDMVNELFDSLFP-----VIYTQLMNP--
NP_001158089.1    FTDVSLYILG-SDINVDDMVNELFDSLFP-----VIYTQLMNP--
AAH35972.1        FTDVSLYILG-SDINVDDMVNELFDSLFP-----VIYTQLMNP--
NP_004475.1        FTDVSLYILG-SDINVDDMVNELFDSLFP-----VIYTQLMNP--
                        : : * . . : : : : * * : : * : : : *

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gi|60219551      ISRDGRIAIGDCILSINEESTIS-----VTNAQARAMLRRLHSLIGPDIKITYVPAEHL
NP_001158090.1    --LPDSALDINECLRGARRDLKVFGNFPKLIMTQVSKSLQVTRIFLQALNLGIEVINTTD--
NP_001158091.1    --LPDSALDINECLRGARRDLKVFGNFPKLIMTQVSKSLQVTRIFLQALNLGIEVINTTD--
NP_001158089.1    --LPDSALDINECLRGARRDLKVFGNFPKLIMTQVSKSLQVTRIFLQALNLGIEVINTTD--
AAH35972.1        --LPDSALDINECLRGARRDLKVFGNFPKLIMTQVSKSLQVTRIFLQALNLGIEVINTTD--
NP_004475.1        --LPDSALDINECLRGARRDLKVFGNFPKLIMTQVSKSLQVTRIFLQALNLGIEVINTTD--
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gi|60219551      EEFKISLGQQSGRVMALDIFSSYTGRDIPELPEREEGEGEESELQNTAYSNNWNQPRRVEL
NP_001158090.1  ----HLKFSKDCGRMLTRMWYCSYQCGLMMVKPCGG--YCNVVMQGC-M---AGVVEIDKY
NP_001158091.1  ----HLKFSKDCGRMLTRMWYCSYQCGLMMVKPCGG--YCNVVMQGC-M---AGVVEIDKY
NP_001158089.1  ----HLKFSKDCGRMLTRMWYCSYQCGLMMVKPCGG--YCNVVMQGC-M---AGVVEIDKY
AAH35972.1      ----HLKFSKDCGRMLTRMWYCSYQCGLMMVKPCGG--YCNVVMQGC-M---AGVVEIDKY
NP_004475.1      ----HLKFSKDCGRMLTRMWYCSYQCGLMMVKPCGG--YCNVVMQGC-M---AGVVEIDKY
                  : : . : . : . : . : . : . : . : . : . : . : . : . : . : . :
                  : : . : . : . : . : . : . : . : . : . : . : . : . : . :

gi|60219551      WREPSKSLG-----
NP_001158090.1  WREYILSLEELVNGMYRIYDMENVLLGLFSTIHDSIQYVQKNAGKLT-----
NP_001158091.1  WREYILSLEELVNGMYRIYDMENVLLGLFSTIHDSIQYVQKNAGKLT-----
NP_001158089.1  WREYILSLEELVNGMYRIYDMENVLLGLFSTIHDSIQYVQKNAGKLT-----
AAH35972.1      WREYILSLEELVNGMYRIYDMENVLLGLFSTIHDSIQYVQKNAGKLT-----
NP_004475.1      WREYILSLEELVNGMYRIYDMENVLLGLFSTIHDSIQYVQKNAGKLT-----
                  ***      **

gi|60219551      -----ISIVGGRGM----GSRLSNGEVMRGIFIKH-VLEDSPAGKNGTLKPGDRIVE
NP_001158090.1  -----TIGKLCASQQRQYRSAYYPEDLFIDKKVLKVAHVEHEETLSSRRRELI
NP_001158091.1  -----TIGKLCASQQRQYRSAYYPEDLFIDKKVLKVAHVEHEETLSSRRRELI
NP_001158089.1  PIFFLCIGLDLQIGKLCASQQRQYRSAYYPEDLFIDKKVLKVAHVEHEETLSSRRRELI
AAH35972.1      -----TIGKLCASQQRQYRFAYYPEDLFIDKKVLKVAHVEHEETLSSRRRELI
NP_004475.1      -----TIGKLCASQQRQYRSAYYPEDLFIDKKVLKVAHVEHEETLSSRRRELI
                  * :      :      .      . : . : . : . : . : . : . : . :
                  * :      :      .      . : . : . : . : . : . : . : . :

gi|60219551      APSQ-----SESEPEKAPLCSVPPPPSAFAEMGSDHTQSSASKISQDVKEDFGYSWKNI
NP_001158090.1  QKLKSFISFYALPGYICSHSPV-----AENDTLCWNGQEL
NP_001158091.1  QKLKSFISFYALPGYICSHSPV-----AENDTLCWNGQEL
NP_001158089.1  QKLKSFISFYALPGYICSHSPV-----AENDTLCWNGQEL
AAH35972.1      QKLKSFISFYALPGYICSHSPV-----AENDTLCWNGQEL
NP_004475.1      QKLKSFISFYALPGYICSHSPV-----AENDTLCWNGQEL
                  :      *      .      . : . : . : . : . : . : . : . :
                  :      *      .      . : . : . : . : . : . : . : . :

gi|60219551      RERYGTLTGE-----LHMIELEK-GHSG-----LGLSLAGNK
NP_001158090.1  VERYSQKAARNGMKNQFNLHELKMKGPEPVVSQIIDKLKHINQLLRTMSMPKGRVLDKNL
NP_001158091.1  VERYSQKAARNGMKNQFNLHELKMKGPEPVVSQIIDKLKHINQLLRTMSMPKGRVLDKNL
NP_001158089.1  VERYSQKAARNGMKNQFNLHELKMKGPEPVVSQIIDKLKHINQLLRTMSMPKGRVLDKNL
AAH35972.1      VERYSQKAARNGMKNQFNLHELKMKGPEPVVSQIIDKLKHINQLLRTMSMPKGRVLDKNL
NP_004475.1      VERYSQKAARNGMKNQFNLHELKMKGPEPVVSQIIDKLKHINQLLRTMSMPKGRVLDKNL
                  ***      . : . :      : : . : . : . : . :      *      *      *

gi|60219551      DRSRMSVFIVGIDPNGAAGKDGRLQIADELLEINGQILYGRSHQNASSIIKCAPSKVKII
NP_001158090.1  DEEGFESGDCGDDEDECIGGSG-----DGMIVKNQ-----LR
NP_001158091.1  DEEGFESGDCGDDEDECIGGSG-----DGMIVKNQ-----LR
NP_001158089.1  DEEGFESGDCGDDEDECIGGSG-----DGMIVKNQ-----LR
AAH35972.1      DEEGFESGDCGDDEDECIGGSG-----DGMIVKNQ-----LR
NP_004475.1      DEEGFESGDCGDDEDECIGGSG-----DGMIVKNQ-----LR
                  * . . : .      *      *      :      .      *      *      : : : : . :      :

gi|60219551      FIRNKDAVNQMAVCPGNAVEPLPSNSENLNQKETEPTVTTSDAAVDLSSFKNVQHLELPK
NP_001158090.1  FLAELAYDLVDVDDAPGNSQQATPKDN-----EISTFHNLGNVHSPL
NP_001158091.1  FLAELAYDLVDVDDAPGNSQQATPKDN-----EISTFHNLGNVHSPL
NP_001158089.1  FLAELAYDLVDVDDAPGNSQQATPKDN-----EISTFHNLGNVHSPL
AAH35972.1      FLAELAYDLVDVDDAPGNSQQATPKDN-----EISTFHNLGNVHSPL
NP_004475.1      FLAELAYDLVDVDDAPGNSQQATPKDN-----EISTFHNLGNVHSPL
                  * :      :      : . : . : . :      *      *      : : . : . :      *

gi|60219551      DQGGGLGIAISEEDTLSGVVIKSLTEHGVAATDGRKLVGDQILAVDDEIVVGYPPIEFISL
NP_001158090.1  KL-----LTSMA-----IS-----
NP_001158091.1  KL-----LTSMA-----IS-----
NP_001158089.1  KL-----LTSMA-----IS-----
AAH35972.1      KL-----LTSMA-----IS-----
NP_004475.1      KL-----LTSMA-----IS-----
                  .      * : . :

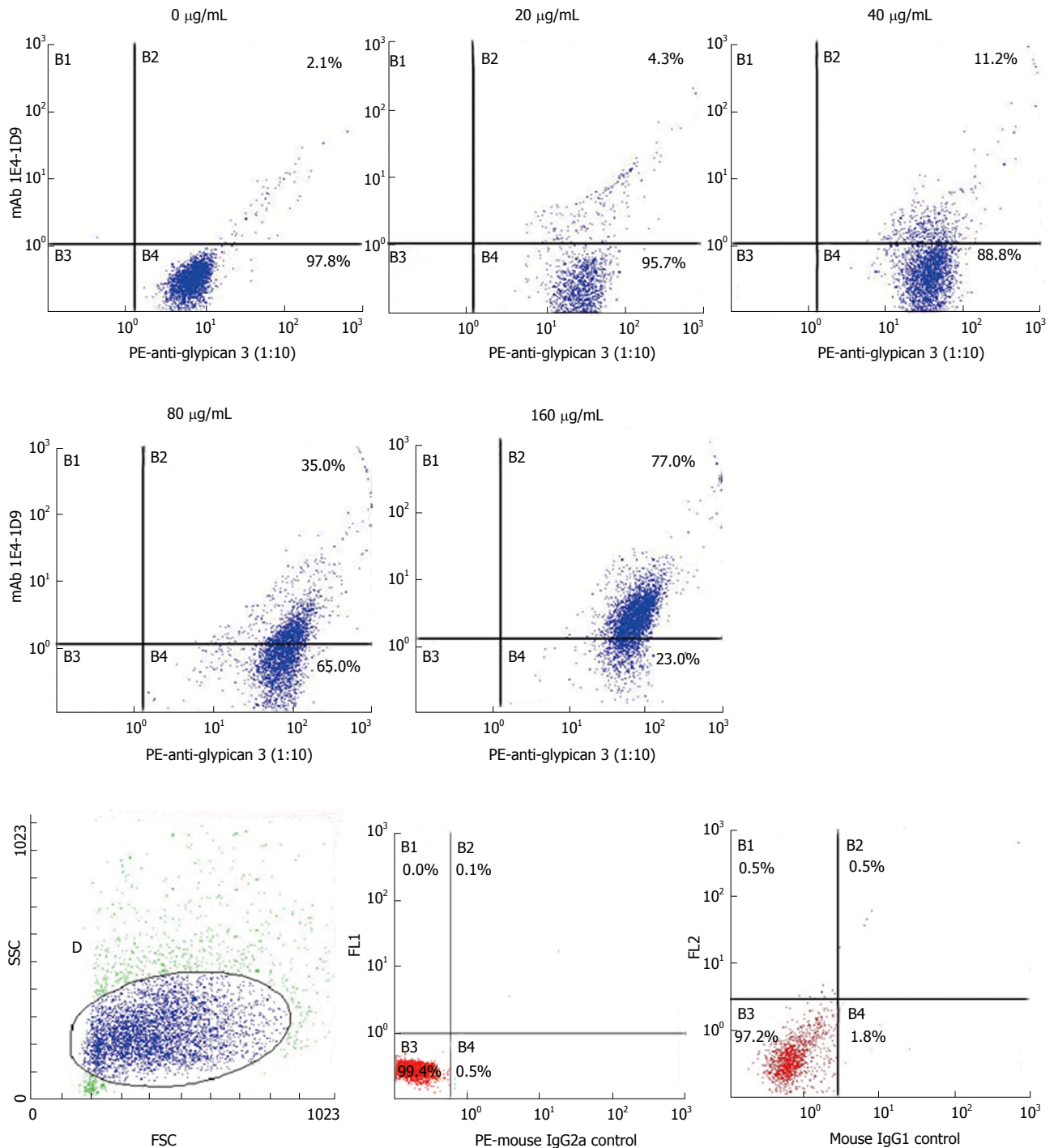
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NP_001158090.1  -----
NP_001158091.1  -----
NP_001158089.1  -----
AAH35972.1      -----
NP_004475.1      -----

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NP_001158089.1	-----
AAH35972.1	-----
NP_004475.1	-----
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NP_001158090.1	-----
NP_001158091.1	-----
NP_001158089.1	-----
AAH35972.1	-----
NP_004475.1	-----
gi 60219551	QKKPGKGLGLSIVGKRNDTGTVFVSDIVKGGIADADGRLMQGDQILMVNGEDVRNATQEAV
NP_001158090.1	-----
NP_001158091.1	-----
NP_001158089.1	-----
AAH35972.1	-----
NP_004475.1	-----
gi 60219551	AALLKCSLGTVTLEVGRKAGPFHSERRPSQSSQVSEGLSSFTFPLSGSSTSESLESSS
NP_001158090.1	-----
NP_001158091.1	-----
NP_001158089.1	-----
AAH35972.1	-----
NP_004475.1	-----
gi 60219551	KKNALASEIQGLRTVEMKKGPTDSLGIAGGVGSPLGDVPIFIAMMHPTGVAAQTQKLR
NP_001158090.1	-----VVCFFFLVH-----
NP_001158091.1	-----VVCFFFLVH-----
NP_001158089.1	-----VVCFFFLVH-----
AAH35972.1	-----VVCFFFLVH-----
NP_004475.1	-----VVCFFFLVH-----
	* * : : *
gi 60219551	VGDRIVTCIGTSTEGMTHTQAVNLLKNASGSIEMQVVAGGDMSVVTGHQQEPASSLSFT
NP_001158090.1	-----
NP_001158091.1	-----
NP_001158089.1	-----
AAH35972.1	-----
NP_004475.1	-----
gi 60219551	GLTSSSIFQDDLGPQCKSITLERGPDGLGFSIVGGYSPHGDLPYVKTVFAKGAASED
NP_001158090.1	-----
NP_001158091.1	-----
NP_001158089.1	-----
AAH35972.1	-----
NP_004475.1	-----
gi 60219551	GRLKRGDQIIAVNGQSLEGVTHEEAVAILKRTKGTVTLMVLS
NP_001158090.1	-----
NP_001158091.1	-----
NP_001158089.1	-----
AAH35972.1	-----
NP_004475.1	-----

Ref. program: <https://www.ebi.ac.uk/Tools/msa/clustalo/>

**Figure 4** Band of approximately 135 kD precipitated by mAb 1E4-1D9 was analyzed by LC-MS. A: Prediction of glycosylation sites and transmembrane region in hypothetical protein sequence gi60219551 was predicted by TMHMM software; B: Number of cysteine was determined from all 1378 amino acid sequence, yellow highlight are N-glycosylation sites, green letter are cysteine, blue letter are glycosylation sites; C: Sequence of gi60219551 was aligned with glypican-3 based on the reliable program on website: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

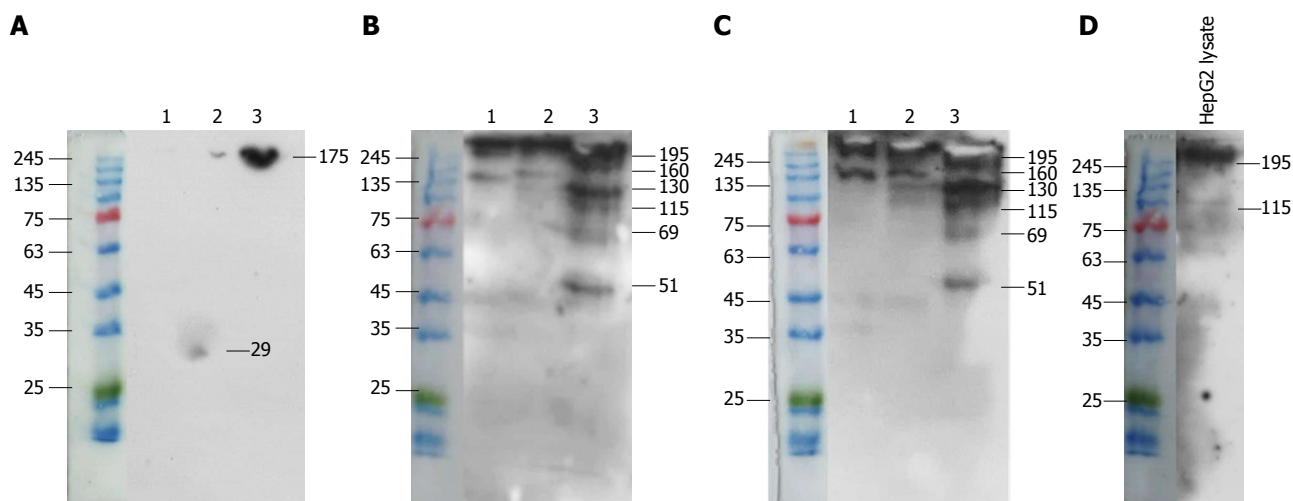


**Figure 5** HepG2 cells ( $4 \times 10^5$  cells/mL) were reacted with various final concentrations of mAb 1E4-1D9 (0-160  $\mu$ g/mL) for 30 min on ice. Mouse IgG1 was used as isotype control. After washing, cells were stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse Igs (1:20) for 30 min on ice and washed. PE-conjugated anti-glypican-3 (1:10) was added and reaction was incubated for another 30 min on ice. Phycoerythrin-conjugated mouse IgG2a was used as isotype control in this step. After washing, cells were suspended with 300  $\mu$ L of 0.5% paraformaldehyde in phosphate buffered saline, pH 7.2 and analyzed by flow cytometry.

amino acid). Both sequences had no transmembrane domain indicating that they might be either intracellular or external proteins. The first sequence with 19 cysteines, gi30722350 was FYVE containing protein and found 99.7% matched to FYCO1. This is very surprising since there have been no reports of a relationship between FYCO1 and glypican-3. FYCO1 is FYVE (Fab1, YOYB,

Vac1, EEA1) and coiled-coil domain containing<sup>[52]</sup> FYCO1, an endogenous protein with MW of 150 kD resides on perinuclear cytosolic vesicles. However, during a starvation period, FYCO1 redistributes to the cell periphery in microtubule-dependent manner<sup>[53]</sup>. It functions as an adapter mediating autophagosome to microtubule plus-end-directed molecular motors<sup>[54]</sup>. FYCO1 can be dimerized





**Figure 6** Specific antigen was precipitated from HepG2 lysate by mouse IgG1 (1), or anti-glypican-3 (2), or mAb 1E4-1D9 (3). The antigen was electrophoresed in 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis at 200V for 45 min in non-reduced condition and blotted onto PVDF membrane. The antigen was probed with mouse IgG1, isotype control (A), or anti-glypican-3 (B), or mAb 1E4-1D9 (C), compared to HepG2 lysate probed with anti-glypican-3 (D). The reaction was detected by horseradish peroxidase-conjugated rabbit anti-mouse Igs and visualized by SuperSignal™ West Pico Chemiluminescent Substrate. PVDF: Polyvinylidene difluoride.

and recruited to the phosphatidylinositol-3-phosphate, PtdIns(3)P. Findings in the study demonstrate a protein band of 160 kD co-precipitated with a band of 69 kD by anti-glypican-3 itself or mAb 1E4-1D9. This band of 160 kD was identified FYCO1. Additional studies, are required to better understand the biological function of this relationship.

Amino acid sequence analysis revealed that the second sequence, gi60219551 with 1378 amino acid was a PDZ containing protein and 85.7% matched to glypican-3. More information confirmed the structure since there are 15 cysteines and 11 putative glycosylation sites with 6 predicted N-glycosylation sites. A band of 69 kD was precipitated with mAb 1E4-1D9 as was with anti-glypican-3. However, there were two bands of 115 and 130 kD with higher MW observed which might correspond to GAG-remaining attached protein. Indirect immunofluorescence staining of various concentrations of mAb 1E4-1D9 on HepG2 cells following with the PE-conjugated anti-glypican-3 was performed to confirm the glypican-3 specificity of mAb 1E4-1D9. It was revealed that increasing amount of mAb 1E4-1D9 showed the higher number cells in upper right quadrant. This demonstrates that HepG2 can react with both antibodies through the same antigen. PE-conjugated anti-glypican-3 used in the experiment was raised against recombinant human glypican-3 at amino acid 25-558 (available information from datasheet). According to the competition experiments, we suggest that antigenic sites of mAb 1E4-1D9 are at or closed to N-terminus. This hypothesis was confirmed by co-precipitation of specific antigen from HepG2 lysate that the same protein bands were precipitated and visualized by cross-reaction between two antibodies. The protein band precipitated by mAb 1E4-1D9 was also detected by anti-glypican-3.

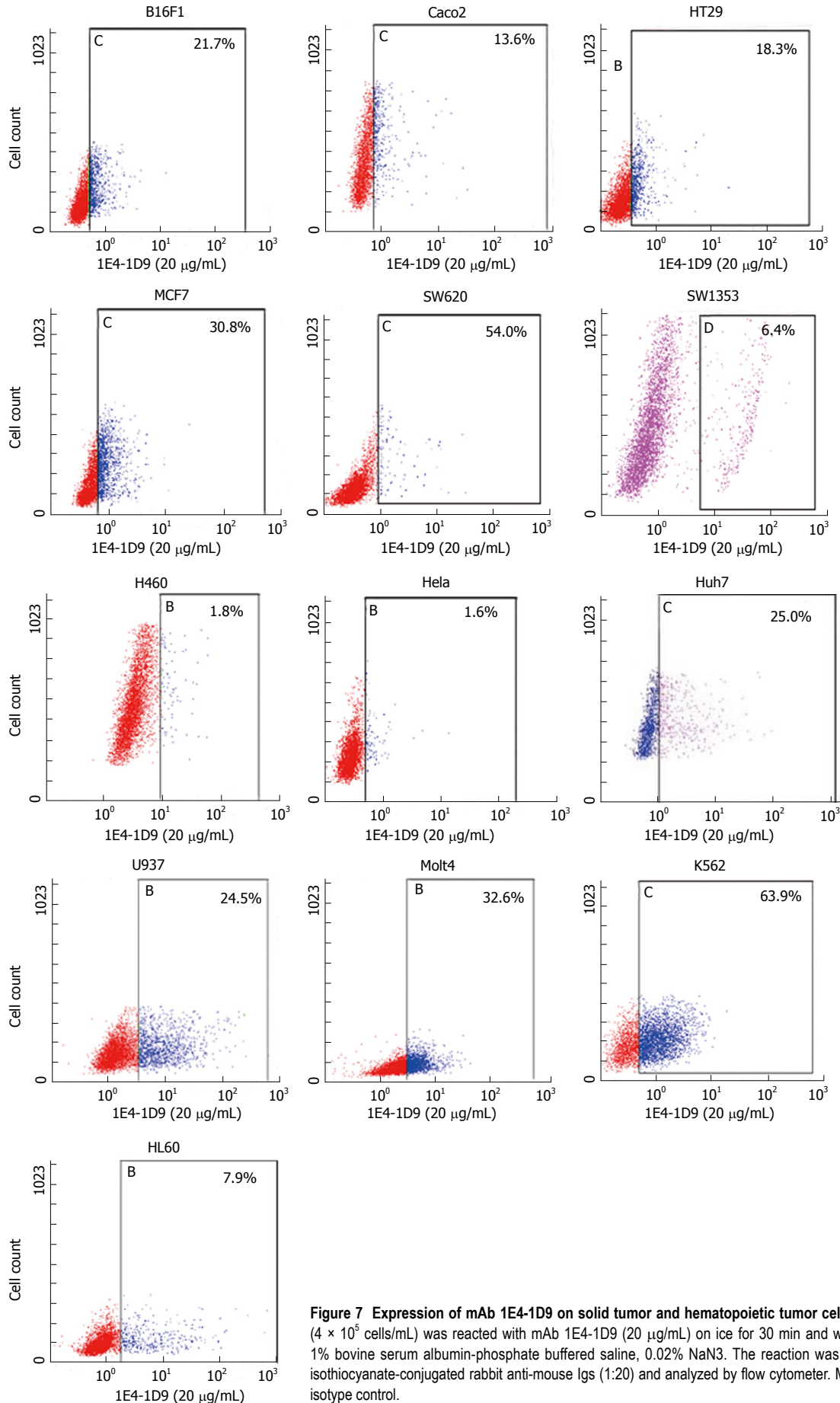
Moreover, since glypican-3 is expressed on a variety of malignant cell lines, indirect immunofluorescence

technique was performed. We found that mAb 1E4-1D9 strongly reacted with an antigen on malignant cell lines including B16F1, Caco2, HT29, MCF7 and SW620. A strongly positive signal was observed when staining hematopoietic cell lines including K562, U937 and Molt4. In some cell lines, mAb 1E4-1D9 was weakly reacted (SW1353 and HL60) and in some no reaction was observed (H460 and Hela). These results are consistent with previous reports<sup>[55-59]</sup>.

Glypican-3 is highly expressed on HCC and plays roles in cellular bioactivities, thus, it is the attractive molecule for developing a therapeutic antibody for HCC treatment. In addition, effect of anti-glypican-3 on proliferation inhibition is dependent on functional epitope of antibody<sup>[15]</sup>. Taken together, these findings support that mAb 1E4-1D9 raised against human liver HSPG is specific for glypican-3. This antibody is specific to HepG2 and glypican-3 expressing malignant cells. The effect of antibody on cell proliferation needs to be studied to understand whether it can be used as a tool for anti-cancer drug development. However, based on its specificity, it should be an excellent candidate monoclonal antibody for applications in tumor investigation as well as for tumor-targeted immunotherapy. Interestingly, the present study also discovers FYCO1 as a possible partner molecule of glypican-3. The findings merit further investigation, which may be applicable and beneficial for immuno- or gene-therapy in clinical settings for the treatment of HCC.

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**Figure 7** Expression of mAb 1E4-1D9 on solid tumor and hematopoietic tumor cell lines. Various cell lines ( $4 \times 10^5$  cells/mL) was reacted with mAb 1E4-1D9 (20 µg/mL) on ice for 30 min and washed 3 times with cold 1% bovine serum albumin-phosphate buffered saline, 0.02% NaN<sub>3</sub>. The reaction was detected by fluorescein isothiocyanate-conjugated rabbit anti-mouse Igs (1:20) and analyzed by flow cytometer. Mouse IgG1 was used as isotype control.

## COMMENTS

## Background

Among most malignant tumors worldwide hepatocellular carcinoma (HCC) is ranked in the fifth most common malignancy and the third leading cause of death. Patients with HCC have a very poor prognosis and the 5-year survival rate of less than 5%-10%. The reasons are that clinical diagnosis usually occurs at a late stage and there are limitations in drug- and surgery-based treatment. Therefore, new strategies and effective treatment as well as early detection using tumor specific monoclonal antibodies are needed. Glypican-3, a glycosylphosphatidylinositol-linked cell surface heparan sulfate proteoglycan (HSPG) is highly expressed in HCC. In some particular conditions, glypican-3 can be cleaved and released into serum and used as a biomarker for HCC. Glypican-3 is involved in growth signalling through Wnts, hedgehogs, fibroblast growth factor, and bone morphogenetic proteins. Based on its function in tumor growth regulation, an antibody specific to glypican-3, would be important for the development of tumor-targeted drug delivery and immunotherapy. Previously, HSPG was isolated from human liver. Biochemical characterization revealed that liver HSPG consisted of heparan sulfate chain with a high level of sulfation. Preliminary result showed that liver HSPG could react with anti-rat glypican. A monoclonal antibody against liver HSPG was raised and mAb 1E4-1D9 obtained was studied to determine whether it recognized glypican-3.

## Research frontiers

Important fields related to this study using mAb 1E4-1D9 as a tool include: (1) tumor detection and investigation such as developing of serological detection system and other clinical applications; (2) tumor-targeted drug delivery and drug design both in immunotherapy and gene therapy; and (3) understanding the role of glypican-3 in regulation of intracellular signalling in many cell types.

## Innovations and breakthroughs

Glypican-3 is upregulated in HCC and many tumor cell types where it enhances cell growth in particular growth-signalling pathways. Research focusing on the production of monoclonal antibodies specific to glypican-3 are important to explore new diagnostic and therapeutic candidates. Findings from present study based on HepG2 cells indicates that specific antigen of a new monoclonal antibody, 1E4-1D9 is glypican-3. In addition, this is the first report showing FYCO1 as a potential partner molecule for glypican-3. The findings merit further investigation, which may be applicable and beneficial for immune- or gene therapy in clinical setting for the treatment of HCC.

## Applications

Glypican-3 specific monoclonal antibody, 1E4-1D9, can be a tool for development of laboratory investigation for HCC and other glypican-3 expressed tumors. In addition, it will be a good candidate for tumor-targeted drug development, immunotherapy and gene therapy.

## Peer-review

Early detection of HCC is very important to study, the glypican-3 is a good point to research, so topic of paper is novel and design of experiment is precise.

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Case Control Study

# Risk factors for hepatocellular carcinoma in cirrhosis due to nonalcoholic fatty liver disease: A multicenter, case-control study

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## Abstract

### AIM

To identify risk factors associated with hepatocellular

carcinoma (HCC), describe tumor characteristics and treatments pursued for a cohort of individuals with nonalcoholic steatohepatitis (NASH) cirrhosis.

## METHODS

We conducted a retrospective case-control study of a well-characterized cohort of patients among five liver transplant centers with NASH cirrhosis with (cases) and without HCC (controls).

## RESULTS

Ninety-four cases and 150 controls were included. Cases were significantly more likely to be male than controls (67% *vs* 45%,  $P < 0.001$ ) and of older age (61.9 years *vs* 58 years,  $P = 0.002$ ). In addition, cases were more likely to have had complications of end stage liver disease (83% *vs* 71%,  $P = 0.032$ ). On multivariate analysis, the strongest association with the presence of HCC were male gender (OR 4.3, 95%CI: 1.83-10.3,  $P = 0.001$ ) and age (OR = 1.082, 95%CI: 1.03-1.13,  $P = 0.001$ ). Hispanic ethnicity was associated with a decreased prevalence of HCC (OR = 0.3, 95%CI: 0.09-0.994,  $P = 0.048$ ). HCC was predominantly in the form of a single lesion with regional lymph node(s) and distant metastasis in only 2.6% and 6.3%, respectively. Fifty-nine point three percent of individuals with HCC underwent locoregional therapy and 61.5% underwent liver transplantation for HCC.

## CONCLUSION

Male gender, increased age and non-Hispanic ethnicity are associated with HCC in NASH cirrhosis. NASH cirrhosis associated HCC in this cohort was characterized by early stage disease at diagnosis and treatment with locoregional therapy and transplant.

**Key words:** Hepatocellular carcinoma; Nonalcoholic fatty liver disease; Cirrhosis; Gender; Ethnicity

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**Core tip:** The present paper identifies male gender, increased age and non-Hispanic ethnicity as factors associated with hepatocellular carcinoma (HCC) in nonalcoholic steatohepatitis cirrhosis. In this series, HCC in nonalcoholic fatty liver disease cirrhosis was diagnosed at an early stage and treated predominantly with locoregional therapy and liver transplantation.

Corey KE, Gawrieh S, deLemos AS, Zheng H, Scanga AE, Haglund JW, Sanchez J, Danford CJ, Comerford M, Bossi K, Munir S, Chalasani N, Wattacheril J. Risk factors for hepatocellular carcinoma in cirrhosis due to nonalcoholic fatty liver disease: A multicenter, case-control study. *World J Hepatol* 2017; 9(7): 385-390 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i7/385.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i7.385>

## INTRODUCTION

The burden of nonalcoholic fatty liver disease (NAFLD) is substantial. Estimates suggest 75-100 million people in the United States have NAFLD, and alarmingly, many of these patients are not aware of or evaluated for this condition<sup>[1,2]</sup>. A subset of individuals with NAFLD will develop nonalcoholic steatohepatitis (NASH), the inflammatory phenotype of NAFLD. Hepatic fibrosis and eventual cirrhosis is a consequence of NASH progression, particularly in genetically predisposed individuals. NASH cirrhosis is projected to be the leading indication for liver transplantation in the next 10-20 years<sup>[3]</sup>.

Hepatocellular carcinoma (HCC), like NAFLD, is also underrecognized. In fact, a recent retrospective study suggested that only 20% of patients received appropriate surveillance before their HCC diagnosis<sup>[4]</sup>. Inadequate screening is a serious concern for patients with cirrhosis of any type. However, recent data suggests that a deficiency in screening may be particularly problematic for patients with NAFLD HCC who present at a later tumor stage, have shorter survival times, and lower rates of liver transplantation<sup>[5]</sup>.

Thus, the convergence of NAFLD and HCC uniquely focuses the narrative for providers caring for these patients to enhance the screening and diagnosis of both diseases. Simultaneously, identifying risk factors for HCC development in patients with underlying NASH cirrhosis is critically important to improve screening and treatment. We have conducted a retrospective case-control study of a well-characterized cohort of patients with NASH cirrhosis with and without HCC in order to identify risk factors associated with HCC. We also provide tumor characteristics and survival data for this cohort. Our data, derived from five academic liver transplant centers, highlights patient characteristics associated with HCC and enhances the growing body of evidence on HCC in the setting NAFLD.

## MATERIALS AND METHODS

We conducted a case-control study of individuals with NAFLD cirrhosis with and without HCC from five academic medical centers in the United States. NAFLD was diagnosed between 1991-2015 and all HCC cases were diagnosed between 2004-2015. This study was approved by the Institutional Review Boards at the respective institutions.

A diagnosis of NAFLD cirrhosis was made either (1) by histology; or (2) clinically. Clinical NAFLD was defined by the exclusion of other causes of chronic liver disease and the presence of one or more risk factors for NAFLD including diabetes, obesity or  $\geq 1$  component of the metabolic syndrome. The diagnosis of cirrhosis was made either by histology or by imaging suggestive of cirrhosis (nodular liver, splenomegaly, ascites or varices) in combination with laboratory values suggesting portal hypertension or impaired synthetic function (platelet count

< 150000/ $\mu$ L, albumin < 3.5 g/dL) or complications of end-stage liver disease. Characteristic liver histology for NASH served as one diagnostic modality; NAFLD Activity Score values were not available for all subjects.

### Definition of cases

Cases were individuals with NAFLD cirrhosis and well-characterized HCC. HCC was defined by histology or imaging consistent with Organ Procurement and Transplantation Network criteria<sup>[6]</sup>.

### Definition of controls

Controls were defined as individuals meeting criteria for NASH cirrhosis but without evidence of HCC on imaging within one year following the diagnosis of cirrhosis. For each case, depending on the availability, we enrolled 1-3 controls from the same institution. Cases and controls were matched for the year of enrollment, *i.e.*, ascertainment of absence of HCC by imaging in the controls was in the same year as the HCC diagnosis in the cases.

### Data collection

Charts were reviewed for weight, height, body mass index (BMI) and co-morbid disease including diabetes mellitus, hypertension, dyslipidemia, cardiovascular disease, obstructive sleep apnea, polycystic ovary syndrome and obesity. Complications of liver disease were also recorded including the presence of ascites, spontaneous bacterial peritonitis, hepatorenal syndrome, hepatic encephalopathy and gastroesophageal varices. These complications were combined in to a composite cirrhosis complication variable. Use of medications including metformin, pioglitazone, vitamin E, HMG-CoA reductase inhibitors ("statins") was also collected. Laboratory values for platelet count, INR, fasting insulin, fasting glucose, creatinine, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase, total bilirubin, albumin, total cholesterol, low-density lipoprotein level, high-density lipoprotein level, triglycerides, glycosylated hemoglobin (A1C), ferritin, alpha-fetoprotein, and model for end-stage liver disease (MELD) score. MELD score was calculated according to the published formula<sup>[7]</sup>.

Pathology reports were reviewed for the presence of HCC as well as TMN classification of malignant tumor status, differentiation status, vascular and/or perineural invasion and lymph node involvement. Imaging including ultrasound, computerized tomography scan or magnetic resonance imaging was reviewed for tumor number, size and location.

### Statistical analysis

All statistical analyses were performed using SAS software, version V.9.2 (SAS Institute, Cary, NC). Continuous variables were analyzed using a Student's *t*-test for normally distributed variables and a Wilcoxon rank sum test for variables that were not normally distributed. Categorical variables were analyzed using a  $\chi^2$  test or Fisher's exact test as appropriate. Nominal, two-sided

*P* values were used and were considered statistically significant if *P* < 0.05. The final model was selected by combining clinical judgment and statistical assessment. We included variables with *P* < 0.1 in univariate analysis and variables that are considered as known confounders. All analyses were carried out using SAS 9.4 (SAS Institute, Cary, NC) and Stata 13.1 (Stata Corp., College Station, TX).

## RESULTS

### Baseline characteristics

Two hundred and forty-four individuals (94 cases and 150 controls) were included. Individuals were predominantly male (54.7%), and Caucasian (81.8%) with a mean age of 59 years. Diabetes (69.5%), dyslipidemia (47.9%) and hypertension (60.1%) were frequent. Mean BMI was 33.5 kg/m<sup>2</sup> and mean MELD score was 12.

Seventy-five point four percent had a complication of cirrhosis with the most frequent being gastroesophageal varices (58.0%), ascites (48.6%) and encephalopathy (39.6%). Hepatorenal syndrome and spontaneous bacterial peritonitis were infrequent (3.3% and 4.2%, respectively).

### Characteristics of cases and controls: Univariate analysis

Ninety-four cases and 150 controls were included in the present study. Cases were significantly more likely to be male than controls (67% vs 45%, *P* < 0.001) and be of older age (mean, 61.9  $\pm$  9.4 vs 58.0  $\pm$  9.9, *P* = 0.002). In addition, cases were more likely to have had complications of end-stage liver disease including ascites, SBP, HRS, gastroesophageal varices or encephalopathy (composite 83% vs 71%, *P* = 0.032). There was no difference between cases and controls by comorbidities, medication use including statins or vitamin E, or biochemical markers such as ALT or MELD score (Table 1).

### Characteristics of cases and controls: Multivariate analysis

On multivariate analysis, after adjustment for the relevant confounders, the strongest association with the presence of HCC among those with NASH cirrhosis was male gender (OR = 4.3, 95%CI: 1.83-10.3, *P* = 0.001). In addition, age (OR = 1.082, 95%CI: 1.03-1.13, *P* = 0.001) was associated with HCC. Hispanic ethnicity was associated with a decreased prevalence of HCC (OR = 0.3, 95%CI: 0.09-0.994, *P* = 0.048) (Table 2).

### Characteristics of HCC in NAFLD cirrhosis

HCC diagnosed in this cohort of individuals with NASH cirrhosis was predominantly in the form of a single lesion (median 1.0, IQR 1.0) with a median size of 2.7 cm (IQR 2.5) (Table 3). Regional lymph node and distant metastasis were recorded in only 2.6% and 6.3%, respectively. Vascular or perineural invasion was documented in



**Table 1** Characteristics of cases and controls

Characteristic		Case ( <i>n</i> = 94)	Control ( <i>n</i> = 150)	<i>P</i> value
Age, yr (mean ± SD)		61.9 ± 9.4	58.0 ± 9.9	0.002
Gender	Female	33%	55%	< 0.001
	Male	67%	45%	
Race	White	85%	80.0%	0.605
	Black	1%	17%	
	Other	14%	3%	
Ethnicity	Not Hispanic	82%	90%	0.149
	Hispanic	18%	10%	
Diabetes mellitus	Yes	74%	67%	0.237
	No	26%	33%	
Hypertension	Yes	61%	60%	0.888
	No	39%	40%	
Dyslipidemia	Yes	50%	47%	0.609
	No	50%	53%	
Cardiovascular disease	Yes	28%	19%	0.093
	No	72%	81%	
Metformin use	Yes	40%	37%	0.660
	No	60%	63%	
Statin use	Yes	25%	21%	0.401
	No	75%	79%	
Vitamin E use	Yes	11%	9%	0.620
	No	89%	91%	
Ascites	Yes	51%	47%	0.628
	No	49%	53%	
Gastroesophageal varices	Yes	66%	53%	0.072
	No	34%	47%	
Hepatic encephalopathy	Yes	40%	40%	0.995
	No	60%	60%	
Complications of cirrhosis	Yes	83%	71%	0.032
	No	17%	29%	
BMI (kg/m <sup>2</sup> )		32.8 ± 5.8	33.9 ± 7.3	0.222
ALT (IU/L) (mean ± SD)		43.3 ± 25.4	42.18 ± 37.3	0.819
MELD score (mean ± SD)		11.6 ± 4.4	12.39 ± 4.8	0.382

BMI: Body mass index; ALT: Alanine aminotransferase; MELD: Model for end-stage liver disease.

13.9% and 1.3%, respectively. Fifty-five percent of tumors involved a single lobe of the liver, 25.6% were bilobar, while the lobar distribution was unknown in 32.4%.

### Treatment for HCC

In this cohort, 59.3% of individuals with HCC underwent either locoregional therapy with radiofrequency ablation, transarterial chemoembolization or radiation. In addition, 61.5% of the entire cohort underwent liver transplantation for HCC. Resection was infrequent and took place in only 10% of the HCC cohort. Sorafenib and/or palliative care was administered in 10% of patients.

## DISCUSSION

NASH cirrhosis is projected to become the leading indication for liver transplantation by 2020, surpassing alcohol and chronic hepatitis C infection<sup>[3]</sup>. Despite its public health impact, however, relatively little is known about the risk factors for HCC development in NASH cirrhosis. The present case-control study sought to address this gap by evaluating individuals with NASH

**Table 2** Variables associated with presence of hepatocellular carcinoma on multivariate analysis<sup>1</sup>

Variable	Univariate <i>P</i> value	Multivariate OR 95%CI	Multivariate <i>P</i> value
Age	0.002	1.08 (1.032-1.13)	0.001
Gender	< 0.001	4.34 (1.83-10.31)	< 0.001
BMI	0.22	0.96 (0.90-1.02)	0.20
Ethnicity	0.15	0.300 (0.090-0.994)	0.045
Platelet count	0.14	1.004 (1.00-1.01)	0.14
CVD	0.09	1.21 (0.61-2.41)	0.58
Gastroesophageal varices	0.07	1.43 (0.63-3.21)	0.39
Complications of cirrhosis	0.03	1.15 (0.43-3.02)	0.78

<sup>1</sup>The final model was selected by combining clinical judgment and statistical assessment. We included variables with *P* < 0.1 in univariate analysis and variables that are considered as known confounders. CVD: Cardiovascular disease; BMI: Body mass index.

cirrhosis with and without HCC.

We found that HCC was associated with male gender and older age. There was no difference between cases and controls with regards to comorbidities, prescription medications, vitamin E use, or biochemical markers such as ALT or MELD score. Surprisingly, the Hispanic ethnicity conferred a decreased risk of HCC.

The observed differences in sex and age are consistent with prior studies. Ascha *et al.*<sup>[8]</sup> compared patients with HCC secondary to NASH cirrhosis to those with HCV cirrhosis and HCC. Compared to those with HCV, individuals with NASH and HCC were significantly older and had a trend toward an increased risk of HCC in men. Bugianesi *et al.*<sup>[9]</sup> also evaluated risk factors for HCC in a cohort of 641 individuals with chronic liver disease of varying etiologies. Six point nine percent of the cohort had cryptogenic cirrhosis largely attributed to NASH. HCC in cryptogenic cirrhosis was associated with older age although no difference in gender was seen. These studies also found that HCC in NASH cirrhosis/cryptogenic cirrhosis was associated with BMI, obesity and diabetes mellitus. The present study did not find associations between diabetes, obesity, BMI or insulin resistance. Our use of NASH cirrhosis controls with high prevalence of diabetes and obesity may account for this difference as prior studies have compared NASH patients who are often characterized by diabetes and obesity to those with other forms of chronic liver disease among whom these comorbidities are less frequent.

Metabolic stress including development of the metabolic syndrome is not only associated with increased risk of cancer in general, but with risk for HCC. Presumably, most NAFLD patients meet criteria for diagnosis of the metabolic syndrome, yet a great proportion of these patients do not develop HCC. The present study did not find a significant difference in comorbidities between cases and controls. Just as only a subset of NAFLD patients progress to NASH, this lends further support to a genetic determinant for development of HCC within NAFLD. Investigation of genetic alterations in insulin signaling including the PI3K-AKT-PTEN pathway and other factors

**Table 3 Tumor characteristics of hepatocellular carcinoma**

Characteristics of HCC	n (%)
Primary tumor (T)	
1	27 (42.86)
2	28 (44.44)
3	8 (12.70)
Regional lymph nodes (N)	
Yes	2 (2.63)
No	43 (56.58)
Unknown	31 (40.79)
Distant metastasis (M)	
Yes	5 (6.33)
No	45 (56.96)
Unknown	29 (36.71)
Tumor size, median (IQR)	2.7 (2.5)
Number of lesions, median (IQR)	1.0 (1.0)
Vascular invasion	
Yes	11 (13.92)
No	56 (70.89)
Unknown	12 (15.19)
Perineural invasion	
Yes	1 (1.30)
No	51 (66.23)
Unknown	25 (32.47)
Bilobar involvement of tumor	
Yes	21 (25.61)
No	45 (54.88)
Unknown	16 (19.51)

HCC: Hepatocellular carcinoma.

in inflammatory pathways including NF-KB may be promising<sup>[10-12]</sup> and possible with a prospective study in a similar cohort of subjects.

Genetic variation may explain reported racial/ethnic disparities. Racial/ethnic disparities have been reported both in NAFLD and HCC: Hispanics tend to have a more progressive course in NAFLD; and have lower rates of curative therapies for HCC<sup>[13]</sup>. Our finding that Hispanic ethnicity was associated with a decreased risk of development of HCC within NAFLD is surprising and needs confirmation with a larger cohort of individuals with NASH and other etiologies of chronic liver disease. Indeed among other causes of chronic liver disease, specifically hepatitis C, Hispanics are more likely to progress to cirrhosis and HCC<sup>[14]</sup>. The present study is limited by a small number of Hispanic patients among both cases and controls and further evaluation of this relationship between ethnicity and HCC among those with NASH cirrhosis is needed.

The tumors observed in our study were typically a single lesion, confined to a single lobe and without any invasion to adjacent structures. This is in contrast with a recent study by Piscaglia *et al*<sup>[15]</sup> who found that NAFLD-HCC tended to be more advanced when compared to HCC in the background of HCV cirrhosis (HCV-HCC). The authors concluded that this was a result of delayed diagnosis of NAFLD and subsequent lack of screening in advanced fibrosis. There was no significant difference in mortality when propensity score analysis was performed. Certainly, detection of early stage HCC centers around

appropriate screening. Our patients were established in our respective clinics and routinely followed. Resection was infrequent and the majority of our patients (61.5%) underwent orthotopic liver transplantation. The earlier stage observed in our study may be a product of referral and/or selection bias, as this cohort was selected from tertiary care and transplant medical center populations.

The limitations of our study include its retrospective nature; only cirrhotic patients were included in the study by design, thus limiting our ability to add to the body of data of HCC in the absence of advanced fibrosis. Similarly, we did not include HCC arising within other etiologies of cirrhosis, and therefore, cannot report that our findings are unique to NAFLD but that these characteristics play a role in the development of HCC in the context of NAFLD cirrhosis. The duration of cirrhosis is not known in this cohort given the case-control design and absence of longitudinal data.

In conclusion, the present study found that male gender and advanced age were associated with increased risk for the development of HCC among individuals with NASH cirrhosis whereas Hispanic ethnicity was associated with lower risk. Larger cohorts of individuals with HCC, from NASH and other etiologies are needed to further explore these associations. Additionally, prospective studies will help address these factors as predictors of HCC development and to risk stratify patients with NAFLD at increased risk for HCC who may benefit from more intense surveillance for HCC.

## COMMENTS

### Background

Both nonalcoholic steatohepatitis (NASH) and hepatocellular carcinoma (HCC) are rising in prevalence worldwide. Recent data suggest that HCC surveillance rates are poor in those with nonalcoholic fatty liver disease (NAFLD) cirrhosis.

### Research frontiers

The authors sought to identify risk factors for HCC in NAFLD cirrhosis to identify individuals at highest risk for HCC.

### Innovations and breakthroughs

Male gender, increased age and non-Hispanic ethnicity are associated with HCC in NASH cirrhosis. NASH cirrhosis associated HCC in this cohort was characterized by early stage disease at diagnosis and treatment with locoregional therapy and transplant.

### Applications

The present study suggests that among those with NAFLD cirrhosis, men with increased age and of non-Hispanic ethnicity are at highest risk of HCC and should be targeted for screening.

### Terminology

NAFLD is a chronic liver disease characterized by hepatic steatosis and can lead to the development of cirrhosis in a subset of patients.

### Peer-review

Kathleen *et al* found male gender, increased age, and non-Hispanic ethnicity are associated with HCC in NASH cirrhosis, and suggested that these parameters may be useful for diagnosis and treatment of NASH cirrhosis associated HCC.

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Retrospective Study

## Features of hepatocellular carcinoma in Hispanics differ from African Americans and non-Hispanic Whites

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**Informed consent statement:** This study was approved under expedited category 5 (Protocol 2005-0283). As such, it was granted a waiver of informed consent and HIPAA authorization.

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## Abstract

### AIM

To compare features of hepatocellular carcinoma (HCC) in Hispanics to those of African Americans and Whites.

### METHODS

Patients treated for HCC at an urban tertiary medical center from 2005 to 2011 were identified from a tumor registry. Data were collected retrospectively, including demographics, comorbidities, liver disease characteristics, tumor parameters, treatment, and survival (OS) outcomes. OS analyses were performed using Kaplan-Meier



method.

## RESULTS

One hundred and ninety-five patients with HCC were identified: 80.5% were male, and 22% were age 65 or older. Mean age at HCC diagnosis was  $59.7 \pm 9.8$  years. Sixty-one point five percent of patients had Medicare or Medicaid; 4.1% were uninsured. Compared to African American (31.2%) and White (46.2%) patients, Hispanic patients (22.6%) were more likely to have diabetes ( $P = 0.0019$ ), hyperlipidemia ( $P = 0.0001$ ), nonalcoholic steatohepatitis (NASH) ( $P = 0.0021$ ), end stage renal disease ( $P = 0.0057$ ), and less likely to have hepatitis C virus ( $P < 0.0001$ ) or a smoking history ( $P < 0.0001$ ). Compared to African Americans, Hispanics were more likely to meet criteria for metabolic syndrome ( $P = 0.0491$ ), had higher median MELD scores ( $P = 0.0159$ ), ascites ( $P = 0.008$ ), and encephalopathy ( $P = 0.0087$ ). Hispanic patients with HCC had shorter OS than the other racial groups ( $P = 0.020$ ), despite similarities in HCC parameters and treatment.

## CONCLUSION

In conclusion, Hispanic patients with HCC have higher incidence of modifiable metabolic risk factors including NASH, and shorter OS than African American and White patients.

**Key words:** Hepatocellular carcinoma; Epidemiology; Treatment pattern; Survival; Hispanics

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**Core tip:** This is a retrospective study evaluating features of hepatocellular carcinoma (HCC) in Hispanics compared to those of African Americans and Whites. This large single institution study found that Hispanic patients with HCC presented with more modifiable risk factors, more advanced liver disease, and shorter survival compared to African American and White patients with HCC. Early identification and intervention upon modifiable risk factors may ameliorate HCC development and HCC morbidity in Hispanic patients.

Venepalli NK, Modayil MV, Berg SA, Nair TD, Parepally M, Rajaram P, Gaba RC, Bui JT, Huang Y, Cotler SJ. Features of hepatocellular carcinoma in Hispanics differ from African Americans and non-Hispanic Whites. *World J Hepatol* 2017; 9(7): 391-400 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i7/391.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i7.391>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide and the third leading cause of cancer related mortality<sup>[1,2]</sup>. While the highest prevalence rates of HCC are in Asia and Africa accounting for 85%

of cases in 2008, the incidence of HCC has increased steadily in the United States among most racial and ethnic groups with a greater rate of growth observed in non-White populations<sup>[3,4]</sup>. Recent SEER analyses reported higher incidence rates for Hispanics (2.5 times) compared to non-Hispanics, and Asian-Pacific Islanders (4 times) and African Americans (1.7 times) compared to Whites<sup>[3]</sup>.

HCC in Hispanics Americans will continue to increase as Hispanics are the most rapidly growing immigrant population in the United States, and projected to comprise 30% of the total population in 2050<sup>[5]</sup>. Recent studies report differences in HCC presentation in Hispanics compared to non-Hispanics, including younger age at diagnosis and greater prevalence of metabolic risk factors for Hispanic patients compared to non-Hispanic Whites<sup>[6,7]</sup>, and higher incidence of HCC in Hispanic women compared to Hispanic men<sup>[6]</sup>. Notably, the incidence of liver cancer in Hispanic men has doubled between 1992 and 2012, and is double that of non-Hispanic men<sup>[8]</sup>. While national HCC incidence is highest in Asians<sup>[3,6]</sup>, Hispanic patients continue to have poorer 5 year survival in comparison to their White and Asian counterparts (respectively; 15% vs 18% vs 23%), and higher overall mortality rates<sup>[9,10]</sup>. Age adjusted HCC-related mortality rates were reported as more than double in native Hispanic men vs immigrant Hispanic men, suggesting that synergy between biologic, environmental, and acquired risk factors contributes to HCC development in Hispanics in the United States<sup>[6]</sup>. Despite disproportionately higher incidence and mortality rates of HCC in Hispanics, there is a paucity of information about HCC presentation and features in Hispanics compared to non-Hispanics.

Identifying the role of modifiable risk factors associated with HCC in Hispanics will be critical to begin to address racial disparities in HCC incidence rates and outcomes. The aim of the current study was to evaluate HCC risk factors with specific emphasis on modifiable risk factors, disease characteristics, and treatment outcomes in Hispanic patients seen in an academic tertiary medical center in Chicago, Illinois and to compare HCC presentation and outcomes in Hispanics to African American and White patients.

## MATERIALS AND METHODS

### Patient populations

All adult patients  $\geq 18$  years of age with HCC treated at the University of Illinois at Chicago (UIC) from January 2005 to December 2011 were identified from the UIC tumor registry. HCC diagnosis was confirmed by histopathology or according to the American Association of the Study of Liver Diseases non-invasive diagnostic criteria<sup>[11]</sup>. Hispanic, African American, and White patients were included in the study population; other racial groups were excluded. Patient charts were reviewed for relevant demographic data including comorbidities, liver disease etiology and characteristics, tumor parameters, treatment patterns, and length of survival from presentation. The protocol for this study was approved by

the Institutional Review Board at UIC as an expedited review under expedited category 5 (Protocol 2005-0283), and was granted a waiver of informed consent and HIPAA authorization.

### Variable selection

The primary category of interest was patient identified race/ethnicity. The primary outcome of interest was patient survival. Demographic factors included race, age at diagnosis, gender, insurance status, body mass index (BMI), and metabolic syndrome criteria per the adult treatment panel III guidelines<sup>[12]</sup>. Comorbidities included diabetes, hyperlipidemia, end stage renal disease requiring dialysis (ESRD), and history of smoking and alcohol use. Assessment of smoking and alcohol consumption was based on patient self-reporting per chart review. Cirrhosis was confirmed by liver biopsy or *via* characteristic clinical and radiologic features. Liver disease etiology was characterized as hepatitis B virus, hepatitis C virus (HCV), alcoholic liver disease, nonalcoholic steatohepatitis (NASH), and other non-viral, non-NASH etiologies including autoimmune, hemochromatosis, and cryptogenic (other). Liver disease characteristics included MELD score calculated based on baseline laboratory values rather than tumor exception points, baseline AFP level, presence of hepatic encephalopathy, and presence of ascites.

Tumor parameters were categorized by size of the largest tumor, stage at diagnosis, portal vein involvement, tumor grade (when tissue was available), and whether HCC was within Milan criteria. Stage at diagnosis was defined as unifocal, multifocal, or metastatic. Assessment was performed regarding whether patients were diagnosed during active HCC surveillance.

Type of treatment was recorded including loco regional therapy, resection, transplantation, chemotherapy, or observation. Cause of death was categorized as attributable to HCC (evidence of radiographic progression in the last 3 mo of life), decompensated cirrhosis (evidence of liver failure or complications of portal hypertension), other, or unknown based on review of outpatient notes within 1 mo of death, discharge summary, and hospice documentation. Two physicians independently reviewed cause of death categorization to ensure criteria standardization.

### Statistical analysis

Patient characteristics were first summarized using mean  $\pm$  SD for normally distributed continuous variables, median and interquartile range for non-normally distributed continuous variables, and percentages for categorical variables. Analysis of variance was used to examine mean differences by race for continuous variables with regards to demographics, comorbidities, liver disease etiology and characteristics, tumor parameters, and treatment patterns. Two-sided  $\chi^2$  tests or Fishers' exact test ( $\leq 5$  patients) were conducted to assess specific pairwise differences by race (between Hispanics, African

Americans, and Whites) for variables that showed significant overall differences by race ( $P < 0.05$ ). Further analysis was not performed for groups including  $\leq 5$  patients.

A Cox proportional hazard regression model was developed to evaluate survival adjusted by demographic and clinical factors, and a stepwise model was used for variable selection. Variables approaching statistical significance in univariate analysis ( $P = 0.10$ ) and clinically meaningful variables were included in a forward stepwise selection. Potential confounders examined included gender, race, insurance, stage at diagnosis, MELD at diagnosis, Milan Criteria, receipt of locoregional therapy, HCV, hepatic encephalopathy, metabolic syndrome, diabetes, ascites, NASH, smoking history, and AFP level. Only variables reaching statistical significance at 0.05  $\alpha$  level were retained in the final multivariable model. Multivariable analysis rather than multivariate analysis was conducted to best assess for multiple independent variables and relationships while adjusting for potential confounders<sup>[13,14]</sup>.

The Kaplan-Meier method was utilized to estimate survival distribution for two overall survival analyses, first with inclusion of all patients, and second with exclusion of liver transplant recipients. Overall survival was defined as the interval between date of HCC diagnosis and date of death due to any cause, or date of data censorship (June 6, 2013) for patients still alive.

All tests were two sided. Analysis was performed *via* SAS 9.3 (SAS Institute, Cary, NC).

## RESULTS

### Patient characteristics

One hundred and ninety-five patients with HCC were identified for analysis, including 44 Hispanics, 61 African Americans, and 90 Whites. Patient characteristics and selected pairwise comparisons between races are summarized in Table 1. Patients were predominantly male (80.5%), White (46.1%), and had a median age of 59.7 years (range, 50.0-69.5) with 22% of patients  $\geq 65$  years old. The majority of patients had Medicare or Medicaid insurance (61.5%) with a small group of uninsured patients (4.1%).

The observed female to male ratio was 1:2.8 in the Hispanic group, 4:5 in the African American group, and 1:2.5 in the White group, showing a higher proportion of women in the Hispanic and African American groups ( $P = 0.022$ ). Among Hispanic patients, women presented at an older age in comparison to men (respectively:  $71.7 \pm 6.5$  years vs  $59.4 \pm 12.6$  years;  $P = 0.0037$ ).

### Comorbidities and modifiable risk factors

Hispanic patients demonstrated a higher prevalence of modifiable metabolic risk factors and comorbidities than African Americans or Whites. In comparison to African American and Whites, Hispanic patients had more frequent diagnoses of diabetes ( $P = 0.0007$ ;  $P =$

**Table 1** Demographics, comorbid conditions and disease characteristics of hepatocellular carcinoma patients, by race

Patient characteristics	Total (n = 195)	Hispanic (n = 44)	African-American (n = 61)	White (n = 90)	P <sup>1</sup>
<b>Demographics</b>					
Age (yr, mean ± SD)	59.7 ± 9.8	62.5 ± 12.5	58.7 ± 10.2	58.9 ± 7.7	
Female (n, %)	38, 19.5	11, 25.0	17, 27.9	10, 11.1	<sup>a</sup> HW, AW
Insurance (n, %)					
Medicare/medicaid	120, 61.5	31, 70.5	36, 59.0	53, 58.9	
Private	67, 34.4	12, 27.3	22, 36.1	33, 36.7	
None	8, 4.1	1, 2.3	3, 4.9	4, 4.4	
BMI > 24.9 (n, %)	137, 70.3	31, 70.5	44, 72.1	62, 68.9	
Metabolic syndrome <sup>3</sup> (n, %)	27, 14.1	8, 18.2	3, 4.9	16, 18.4	<sup>a</sup> HA, AW
<b>Comorbid conditions</b>					
Hyperlipidemia (n, %)	31, 25.4	16, 55.2	5, 13.2	10, 18.2	<sup>b</sup> HW, HA
On dialysis (n, %)	7, 3.6	5, 11.4	0, 0	2, 2.3	<sup>b</sup> HNH <sup>2</sup>
Diabetes mellitus 2 (n, %)	91, 46.7	29, 65.9	19, 31.1	43, 47.8	<sup>b</sup> HA, <sup>a</sup> AW
History smoking (n, %)	126, 65	16, 36.3	43, 70.5	67, 75.2	<sup>b</sup> HW, HA; <sup>a</sup> AW
Current smoker (n, %)	57, 29.4	2, 4.5	28, 45.9	27, 30.3	<sup>b</sup> HW, HA; <sup>a</sup> AW
Triglycerides (median ± SD)	99.5 ± 66.5	101.0 ± 70.2	111.0 ± 64.7	80.5 ± 65.8	
History alcohol use (n, %)	163, 83.6	39, 88.6	50, 82	74, 82.2	
<b>Cirrhosis characteristics</b>					
Etiology <sup>4</sup>					
HCV (n, %)	132, 67.7	18, 40.9	49, 80.3	65, 72.2	<sup>b</sup> HW, HA
HBV (n, %)	14, 8.1	2, 5.0	8, 14.3	4, 5.2	
ETOH (n, %)	55, 28.2	11, 25.0	15, 24.6	29, 32.2	
NASH (n, %)	35, 18.0	15, 34.9	5, 8.2	15, 16.7	<sup>b</sup> HA, <sup>a</sup> HW
Other <sup>5</sup> (n, %)	22, 11.3	11, 25	2, 3.3	9, 11.3	0.056
MELD (median ± SD)	11.0 ± 4.6	11.5 ± 4.4	9.0 ± 3.1	12.0 ± 5.1	<sup>a</sup> HA, <sup>b</sup> AW
<b>AFP</b>					
Median (IQR)	22.4 (6.1-217.2)	19 (5.9-434.85)	82 (11.9-434.8)	12 (5.0-53.2)	
AFP > 200	49, 25.1	12, 27.3	22, 36.1	15, 16.7	<sup>a</sup> HA, <sup>b</sup> AW
Hepatic encephalopathy (n, %)	65, 33.3	16, 36.4	8, 13.1	41, 45.6	<sup>a</sup> HA, <sup>b</sup> AW
Ascites (n, %)	80, 44.5	23, 54.8	14, 26	43, 51.2	<sup>a</sup> HW, HA, AW
HCC surveillance performed (n, %)	113, 61.1	27, 65.9	31, 51.7	55, 65.5	
<b>Tumor parameters</b>					
Tumor size (cm)					
Median (± SD) (IQ range)	3.0 ± 3.7 (2.0-6.0)	3.0 ± 3.9 (2.0-5.0)	3.0 ± 3.6 (2.0-6.0)	3.0 ± 3.7 (2.0-6.0)	
> 5 cm (n, %)	43, 26.9	8, 22.2	14, 28.6	21, 28	
Median > 5 cm (± SD) (IQ range)	8.0 ± 4.0 (6.0-12.0)	13.0 ± 3.4 (7.5-13.0)	9.5 ± 3.1 (6.0-11.0)	7.0 ± 4.7 (6.0-8.0)	
Stage at diagnosis (n, %)	99, 52.1	20, 45.5	30, 50.8	49, 56.3	
Unifocal	77, 40.5	21, 47.7	25, 42.4	31, 35.6	
Multifocal	14, 7.4	3, 6.8	4, 6.8	7, 8.0	
Metastatic	29, 24.2	9, 33.3	9, 23.1	11, 20.4	
Portal vein involvement (n, %)	35, 47.9	6, 54.5	13, 54.2	16, 42.1	
Poorly differentiated within milan criteria (n, %)	121, 62.1	28, 63.6	36, 59.0	57, 63.3	

<sup>1</sup>P values from  $\chi^2$  tests (two-sided) and fisher for overall race effect followed by pairwise comparisons, for  $P < 0.05$ . A significance level of 0.05 was used for the overall race comparisons. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.001$ , P values were not calculated for  $n < 5$ ; <sup>2</sup>Given  $n < 5$  for AA ( $n = 0$ ) and W ( $n = 2$ ), limited statistical tests for Hispanic to non-Hispanic with  $P = 0.0074$ ; <sup>3</sup>Metabolic syndrome: Three of the following five traits per adult treatment panel III guidelines. Abdominal obesity, defined as a waist circumference in men  $\geq 102$  cm (40 in) and in women  $\geq 88$  cm (35 in): (1) serum triglycerides  $\geq 150$  mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides; (2) serum HDL cholesterol  $< 40$  mg/dL (1 mmol/L) in men and  $< 50$  mg/dL (1.3 mmol/L) in women or drug treatment for low HDL-C; (3) blood pressure  $\geq 130/85$  mmHg or drug treatment for elevated blood pressure; (4) fasting plasma glucose (FPG)  $\geq 100$  mg/dL (5.6 mmol/L) or drug treatment for elevated blood glucose; <sup>4</sup>Some patients with more than one listed etiology of cirrhosis; <sup>5</sup>Other: Cryptogenic, hemochromatosis, autoimmune, other not specified. H: Hispanics; A: African Americans; W: Non-Hispanic Caucasians; SD: Standard deviation; IQ: Percentile interquartile range (25%, 75%); HW: Hispanics compared to Whites; HA: Hispanics compared to African Americans; AW: African Americans compared to Whites; HNH: Hispanics compared to non-Hispanics; BMI: Body mass index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Nonalcoholic steatohepatitis; HCC: Hepatocellular carcinoma.

0.0648;  $P = 0.0019$  on initial  $\chi^2$  analysis, see appendix A), hyperlipidemia ( $P = 0.0004$ ;  $P = 0.001$ ), and end stage renal disease requiring dialysis ( $P < 0.0001$ ), and were less likely to have a smoking history ( $P < 0.0001$ ). In comparison to African Americans, Hispanic patients were more likely to meet criteria for metabolic syndrome ( $P = 0.0491$ ). The three racial groups were similar with regards to age at presentation, insurance status, other comorbidities including BMI > 24.9 and history of alcohol

use.

Among Hispanics, women trended towards a higher frequency of metabolic syndrome compared to men (36.4% vs 12.1%,  $P = 0.09$ ).

#### Liver disease characteristics

Etiology and liver disease features in Hispanic patients differed from African Americans and Whites. NASH cirrhosis was significantly more common in Hispanics compared to

**Table 2 Treatment patterns for non-metastatic patients at diagnosis: First line treatment patterns for non-metastatic patients by race**

First line treatment characteristics	Total ( <i>n</i> = 176) <sup>1</sup>	Hispanic ( <i>n</i> = 41) ( <i>n</i> , %)	African American ( <i>n</i> = 55) ( <i>n</i> , %)	White ( <i>n</i> = 80) <sup>1</sup> ( <i>n</i> , %)	<i>P</i> <sup>2</sup>
Surgery	2	0, 0	2, 3.6	0, 0	
Liver directed	154	38, 92.7	48, 87.2	68, 85	
Chemotherapy	6	1, 2.4	2, 3.6	3, 3.8	
Observation	6	0, 0	1, 1.8	4, 5	
Lost to follow-up	7	2, 4.9	2, 3.6	4, 5	

<sup>1</sup>One patient missing information; <sup>2</sup>*P* values from  $\chi^2$  tests (two-sided) and fisher for overall race effect followed by pairwise comparisons, for *P* < 0.05. A significance level of 0.05 was used for the overall race comparisons.

**Table 3 Treatment patterns for non-metastatic patients at diagnosis: Transplantation patterns by race**

Transplantation patterns	Total listed	Hispanic	African American	White	<i>P</i> <sup>2</sup>
Met milan criteria	121	28	36	57	
Transplanted	34 <sup>1</sup> , 1.4%	10, 35.7%	6, 16.7%	18 <sup>1</sup> , 31.6%	
Listed	68	20	17	31	
Tumor exception points	33, 48.5%	10, 50%	6, 35.3%	17, 48.4%	
Transplanted	38 <sup>1</sup> , 55.9%	10, 50%	6, 35.3%	22 <sup>1</sup> , 71% <sup>1</sup>	<sup>a</sup> AW
Removed from list	28, 41.1%	9, 45%	11, 64.7%	8, 25.8%	<sup>b</sup> AW
Death	7	3	0	4	
Progression	10	3	5	2	
Transfer of care	5	2	2	1	
Other	5	1	4	0	

<sup>1</sup>Four patients initially outside of Milan criteria, subsequently listed and transplanted after reassessment and locoregional treatment; <sup>2</sup>*P* values from  $\chi^2$  tests (two-sided) and fisher for overall race effect followed by pairwise comparisons, for *P* < 0.05. A significance level of 0.05 was used for the overall race comparisons. <sup>a</sup>*P* < 0.05; <sup>b</sup>*P* < 0.001. *P* values were not calculated for *n* < 5. AW: African American compared to Whites.

African Americans and Whites (*P* < 0.0001; *P* = 0.026) while HCV cirrhosis was less common in Hispanics (*P* < 0.0001). There was a trend towards more non-viral, non-NASH cirrhosis etiologies in the Hispanic patients compared to other groups (*P* = 0.056).

Hispanic patients with HCC showed more evidence of advanced liver disease. In comparison to African Americans and White, ascites was more common in Hispanics (*P* = 0.006; *P* = 0.042). Hispanic patients presented with higher median MELD scores (*P* = 0.0159) and more hepatic encephalopathy (*P* = 0.0087) than African Americans. Median AFP levels were similar among groups, although Hispanic and African Americans demonstrated more variability in AFP based on inter-quartile range, and Hispanics were more likely to have AFP > 200 IU/mL in comparison to Whites (*P* = 0.035).

Among Hispanics, women had a lower prevalence of alcoholic cirrhosis compared to men (0% vs 37.93%, *P* = 0.0186), while the prevalence of HCV cirrhosis was similar by gender.

### Tumor parameters

The three groups demonstrated similar frequency of HCC diagnosis made during active surveillance, and similar tumor parameters at presentation including stage at diagnosis, tumor size, tumor differentiation, presence of portal vein invasion, and transplant eligibility *via* Milan criteria at diagnosis (Table 1).

### HCC treatment patterns

While median time from HCC diagnosis to time of last

follow-up was similar among groups, median time from HCC diagnosis to time of first treatment was longer for African Americans in comparison to both Hispanics and Whites (median time to first line treatment; Hispanics 25.0 d (IQR 7.0-34.0 d); African Americans 39.0 d (IQR 17.0-70.0 d), Whites 32.5 d (IQR 13.0-64.0 d, *P* = 0.0373).

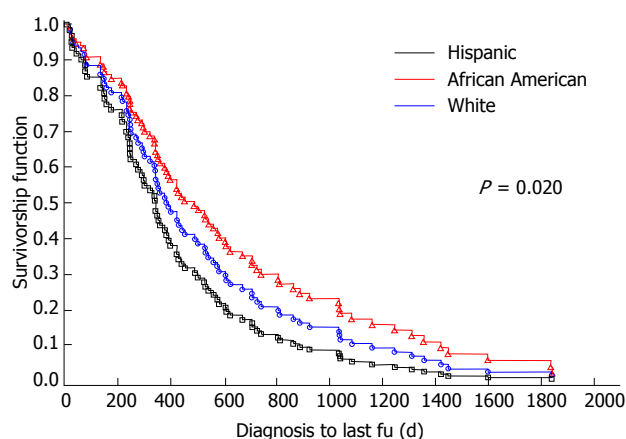
As shown in Table 2, the use of loco regional therapy (chemoembolization and radiofrequency ablation) for non-metastatic HCC was similar among racial groups (*P* = 0.1168). The vast majority of patients (87.5%) received loco regional therapy as their initial treatment, while the remaining 12.5% of patients received other initial treatments including chemotherapy, resection, or observation. *P* values are not reported for the remaining 12.5% due to small numbers of patients per individual group, by race.

There was no difference in HCC presentation within Milan Criteria, listing for transplant, receipt of tumor exception points, or liver transplantation for patients meeting Milan Criteria among the three ethnic/racial groups (Table 3). However, once listed, African Americans were more frequently removed from the transplant list due to HCC progression and death (64.7% vs 25.8%, *P* = 0.0084) and were less likely to receive liver transplantation (35.3% vs 71%, *P* = 0.0165) compared to Whites. Hispanics did not differ significantly from Whites or African Americans with regard to being transplanted once listed, or removed from the list (Table 3).

### Overall survival

Forty-nine of the 195 patients died from all causes during





	Day 0	Day 500	Day 1000	Day 1500	Day 2000
Hispanic	44	16	8	2	0
African American	61	29	12	4	0
White	90	20	7	1	0

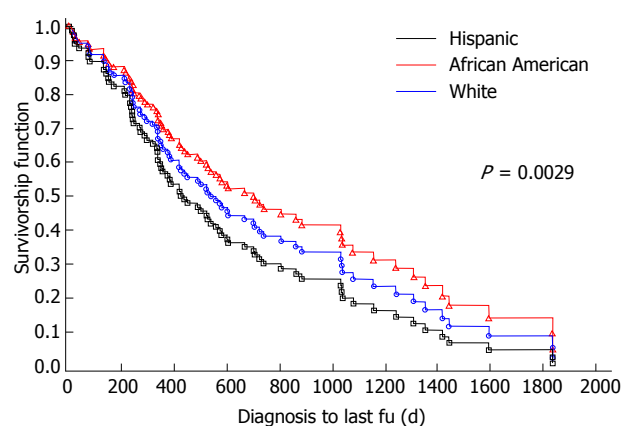
**Figure 1** Unadjusted survival curve stratified in patients with hepatocellular carcinoma by race from time of presentation to time of death or censorship (with numbers of subject at risk). Hispanic ( $n = 44$ ), African American ( $n = 61$ ), Whites ( $n = 90$ ).  $P$ -value was obtained by the log-rank test.

the study period (Hispanic  $n = 9$ ; African American  $n = 15$ ; Whites  $n = 25$ ). The median follow-up for the entire cohort was 563 d and was similar among racial groups. In a multivariable analysis examining possible confounders, three variables were identified as independently related to survival including HCV, metabolic syndrome, and race. However, when all three variables were entered in a stepwise fashion for model building, only race was found to be predictive of survival.

Hispanic patients appeared to have poorer survival compared to both African American and Whites (log-rank test for overall differences by race:  $P = 0.0220$ ) (Figure 1). The hazard ratio for death was 1.52 (95%CI: 0.354, 1.223), for Hispanics in comparison to African Americans and 1.36 (95%CI: 0.739-2.511), for Hispanic in comparison to Whites. After excluding patients who underwent liver transplantation, a second multivariable model adjusting for the factors mentioned above confirmed that Hispanics with HCC had the highest mortality rate (log-rank test for overall differences by race:  $P = 0.0029$ ) (Figure 2). Cause of death was similar for all groups for cases in which the cause of death could be discerned (Figure 3), with similar frequency of death due to HCC ( $n = 11$ ) vs liver cirrhosis ( $n = 19$ ) vs other ( $n = 11$ ) in Hispanics, African Americans, and Whites.

## DISCUSSION

Hispanics with HCC had significantly shorter survival in comparison to both African American and Whites, with race as the only independent predictor of survival in multivariable analysis. This observation is consistent with previous studies showing that Hispanic ethnicity was an independent risk factor for HCC-related mortality, with



	Day 0	Day 500	Day 1000	Day 1500	Day 2000
Hispanic	34	10	3	1	0
African American	55	27	11	4	0
White	69	13	4	0	0

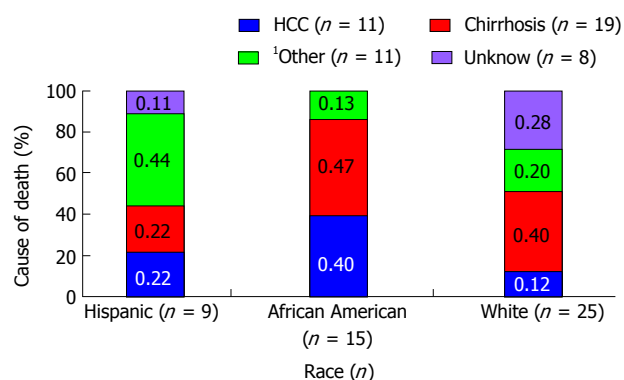
**Figure 2** Overall survival curves by race after exclusion of patients who underwent orthotopic liver transplantation (with numbers of subjects at risk). Hispanic ( $n = 34$ ), African American ( $n = 55$ ), Whites ( $n = 69$ ).  $P$ -value was obtained with the use of the log-rank test.

shorter 5 year survival<sup>[9,10]</sup> in Hispanic patients with HCC compared to White and Asian counterparts, and higher mortality rates in Hispanics aged 50-64<sup>[15]</sup>.

A substantive body of prior research has shown that health disparities, barriers to care, socioeconomic characteristics, and later diagnosis of more advanced malignancy impact on survival in minority groups<sup>[16-18]</sup>. An important contribution of the current study was that we found no evidence that reduced survival in Hispanics with HCC was related to differences in access to care; groups were similar with regard to insurance status, age at diagnosis, HCC diagnoses made during active surveillance, and tumor parameters at presentation including stage and tumor grade at diagnosis.

Little is known about features of patients with HCC that might contribute to disparate outcomes by race. Data from the current study shows important and intriguing differences in HCC presentation and disease characteristics for Hispanics. Characteristics that distinguished Hispanic patients included significantly higher rates of comorbidities and modifiable risk factors for liver disease such as diabetes, hyperlipidemia, metabolic syndrome, as well as a greater prevalence of NASH and ESRD. Hispanics also had evidence of more advanced liver disease with higher rates of ascites than African Americans and Whites and higher MELD scores and more hepatic encephalopathy than African Americans.

The clinical correlates of HCC in Hispanics provide a context to consider potential causes for the shorter overall survival in Hispanics. Patients with HCC are at risk for complications and mortality from cirrhosis, HCC, and other comorbidities. Consistent with prior studies, we found that Hispanic patients had higher rates of comorbidities including metabolic syndrome<sup>[19,20]</sup> and ESRD<sup>[21-23]</sup>. Our data builds on existing literature by



**Figure 3 Distribution of cause of death in patients with hepatocellular carcinoma by race.** There was no difference in HCC ( $P = 0.1051$ ), cirrhosis ( $P = 0.6162$ ), or other ( $P = 0.0581$ ) as cause of death between Hispanics, African Americans, and White. <sup>1</sup>Cause of death other includes: Immediate complications post liver transplant ( $n = 3$ ), sepsis ( $n = 3$ ), complications from second malignancy ( $n = 2$ ), cardiogenic shock ( $n = 1$ ), PEA ( $n = 1$ ), intracerebral hemorrhage ( $n = 1$ ). Of Hispanic patient ( $n = 4$ ), immediate complications post liver transplant ( $n = 2$ ), cardiogenic shock ( $n = 1$ ), complications from a second malignancy ( $n = 1$ ). Fischers pairwise comparison not performed due to  $n < 5$  per group. HCC: Hepatocellular carcinoma.

showing that these differences persist in patients with HCC. Moreover, metabolic disease might contribute to the development of HCC and to poorer outcomes in Hispanics. There is increasing evidence that diabetes and obesity are individually associated with significant risk of HCC development<sup>[24-26]</sup>, and Hispanics appear to demonstrate a stronger association between diabetes and HCC compared to non-Hispanics<sup>[27]</sup>. A longitudinal study reported that diabetic Hispanics had a  $3.3 \times$  higher risk of HCC development compared to non-diabetic Hispanics, and that there was a  $2.17 \times$  higher risk of HCC for diabetic non-Hispanics compared to non-diabetic counterparts<sup>[28]</sup>.

In addition to higher rate of comorbidities and modifiable risk factors, Hispanics had more complications of portal hypertension and compared to African Americans had higher MELD scores at presentation, indicating more advanced liver disease. This is consistent with national data reporting a higher prevalence of chronic liver disease, more advanced disease features at presentation, and higher liver disease related mortality in Hispanics<sup>[29-31]</sup>. Although chronic liver disease is the 6<sup>th</sup> most common cause of death in Hispanic populations in 2010 per the United States National Center for Health Statistics data, it is not within the top ten causes of death for African American or White populations. Mortality rates from chronic liver disease are almost 50% higher in Hispanics than non-Hispanics<sup>[32]</sup>. One potential explanation may be that increased comorbidities in Hispanics could contribute to higher chronic liver disease mortality. Recent SEER data found parallel mortality trends for diabetes, chronic liver disease, and HCC by state; states with high HCC mortality also demonstrated elevated mortality rates for diabetes and liver disease, including cirrhosis<sup>[15]</sup>. Racial/ethnic biologic differences in cirrhosis pathogenesis might also contribute; Hispanics with HCV

appear at significantly higher risk for cirrhosis and HCC development compared to non-Hispanic Whites and African Americans, independently of BMI, diabetes, HCV treatment and genotype<sup>[33]</sup>. Additionally, Hispanics with HCV cirrhosis showed lower median time to cirrhosis at a younger age<sup>[34]</sup>, and higher rates of cirrhosis mortality for Hispanics in both the United States and Mexico<sup>[29-31]</sup>. The finding of higher rates of ESRD in Hispanics in the current study is consistent with prior literature reporting higher incidence of ESRD in Hispanics than non-Hispanics, and a higher risk of kidney failure despite similar prevalence of stage 3 and 4 chronic kidney disease<sup>[22,23,35,36]</sup>. Renal failure is associated with increased risk of mortality in patients with cirrhosis<sup>[37]</sup>. It is intriguing that Hispanics carry a disproportionate burden of ESRD and cirrhosis severity and incidence, although ESRD did not independently predict shorter survival in our study.

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States<sup>[38-40]</sup> and is increasingly being recognized as an important cause of cirrhosis and HCC<sup>[41]</sup> with higher prevalence in Hispanics compared to non-Hispanic<sup>[42]</sup>. Recent data also suggest NAFLD's role in hepatocarcinogenesis in the absence of cirrhosis<sup>[40]</sup>. NASH comprises a subgroup of NAFLD with hepatocyte injury and inflammation, and is considered to be the hepatic manifestation of the metabolic syndrome. In the current series, NASH was the second leading cause of liver disease in Hispanics with HCC, accounting for 34% of cases. Consistent with prior studies, we found Hispanics demonstrated notable differences in cirrhosis etiologies compared to non-Hispanics, including higher rates of NASH<sup>[43,44]</sup> and autoimmune cirrhosis<sup>[45]</sup>, and lower incidence of HCV cirrhosis<sup>[46]</sup>. Additionally, we observed that NASH was particularly prevalent in Hispanic women compared to Hispanic men (72.7% vs 21.9%). Although the risk of HCC development in NAFLD is lower than with HCV<sup>[47]</sup>, NASH is poised to become the primary etiology for cirrhosis and HCC in developed countries over the next decade. One new observation from our study is that Hispanics and Whites with HCC had similar rates of diabetes and metabolic syndrome, although Hispanics had more NASH cirrhosis and hypertriglyceridemia. This suggests that Hispanics may have differences in NAFLD progression, NASH pathogenesis and a greater susceptibility towards cirrhosis. A role for biologic differences in cirrhosis pathogenesis and hepatocarcinogenesis unique to Hispanics has been suggested by prior studies demonstrating that Hispanic patients with NASH, NAFLD, and hepatitis C<sup>[48]</sup> demonstrate more fibrosis and higher rate of aminotransferase abnormalities in comparison to other ethnic groups<sup>[49,50]</sup>. The high prevalence of metabolic disease and NASH in Hispanics with HCC has a critically important implication. Early identification of Hispanics with risk factors for NASH and intervention to modify metabolic risk factors could have a major impact on reducing the development of HCC in Hispanics. Specifically, elimination of diabetes and metabolic syndrome could significantly

decrease HCC incidence across all ethnic groups, but with largest reduction in Hispanics. Additionally, targeted HCC screening for Hispanics with metabolic syndrome, diabetes, and NASH risk factors for NASH could also improve diagnosis, timely treatment, and survival for Hispanics with HCC.

The retrospective design of the current study made it difficult to assess whether reduced survival in Hispanics with HCC was related to increased mortality from complications of cirrhosis, HCC, or comorbid conditions. It is likely that synergy between biologic, genetic, and environmental factors may contribute to racial differences in cirrhosis pathogenesis, HCC development, and survival. Recent proteomic and tissue microarray studies have demonstrated racial and regional differences in molecular pathogenesis of cirrhosis and HCC, including variations of molecular signatures for HCV induced HCC<sup>[51]</sup> unique to African Americans compared to Whites, down-regulation of p53 and MDM2 in Americans compared to South Koreans<sup>[52]</sup>, higher prevalence of PNPLA3 polymorphisms associated with high NAFLD susceptibility and worse survival in Hispanics<sup>[53]</sup> and greater expression of genetic polymorphisms predisposing towards higher NASH severity in Hispanics compared to non-Hispanics<sup>[8]</sup>. Genetic and biologic differences are associated with susceptibility to increased fibrosis and inflammation in NAFLD, NASH and HCV, influencing more aggressive cirrhosis progression and hepatocarcinogenesis<sup>[48-50,54-56]</sup>. Racial and ethnic differences modulating insulin resistance have also been identified; compared to non-Hispanics, Hispanics express a higher frequency of an insulin receptor gene regulator (high-mobility group AT-hook, or HMGA1) associated with higher BMI, lower HDL, and type 2 diabetes<sup>[57]</sup>. While our study did not include biologic correlates, given the paucity of Hispanic specific information, studies comparing Hispanic tumor and cirrhosis samples to other multi-ethnic HCC and cirrhotic cohorts are necessary to better understand ongoing racial disparities in HCC and cirrhosis mortality and progression.

Despite being one of the largest single institution studies of HCC in Hispanics, African Americans and Whites, the major limitation of the present study was the retrospective design. The study identified important clinical factors associated with HCC in Hispanics. However, it was unable to discern the cause of reduced overall survival in Hispanics with HCC. Moreover, single center data might not be applicable to all Hispanic populations. Prospective studies with molecular analyses are needed to determine the relative contributions of co-morbidities, cirrhosis, HCC and biologic correlative information to the reduced overall survival in Hispanics.

In conclusion, the current study provides important new insights into clinical factors distinguishing Hispanics with HCC. Hispanics with HCC present with a higher prevalence of modifiable metabolic risk factors, more advanced liver disease, and shorter survival compared to African Americans and Whites. Increased mortality in Hispanics with HCC may be explained by compounding risk from metabolic comorbidities, NASH cirrhosis, and

unique biologic gene-environment interactions influencing higher susceptibility towards NAFLD development, and more aggressive cirrhosis progression and hepatocarcinogenesis. Further clinical, epidemiologic, and molecular data are necessary to determine the relative contributions of modifiable comorbidities such as diabetes, hyperlipidemia, metabolic syndrome, and NASH to HCC pathogenesis in Hispanics. Development of prospective multi-institutional HCC databases with specimen sharing is essential. There is an additional need for prospective case controlled studies, and therapeutic clinical trials with proportional representation of Hispanics to assess the impact of modifying comorbidities such as metabolic syndrome, hyperlipidemia, ESRD, diabetes, and NASH through lifestyle and medical management upon cirrhosis and HCC progression in Hispanic and non-Hispanics. Identification of clinical factors associated with HCC in Hispanics provides direction for public health efforts at HCC prevention through intervening on modifiable risk factors, targeted HCC screening for high risk ethnic populations, and more timely HCC treatment and management in this population.

## COMMENTS

### Background

There is a dearth of information about hepatocellular carcinoma (HCC) race specific risk factors and disease characteristics in Hispanic patients, compared to African American and White patients, despite higher incidence and mortality rates. This is one of the largest published single institution retrospective studies of Hispanic, African American, and White patients treated for HCC at an urban tertiary academic medical center.

### Research frontiers

The results of this study contribute to new insights and a deeper understanding of racial disparities in HCC incidence, cirrhosis progression and mortality in Hispanic patients, compared to African American and White patients.

### Innovations and breakthroughs

The results of this study demonstrate significant differences in survival and modifiable risk factors for Hispanic patients compared to other racial groups, with Hispanic patients showing lower survival, more advanced liver disease, and higher incidence of modifiable risk factors including metabolic syndrome, nonalcoholic steatohepatitis (NASH), and end stage renal disease. This is consistent with prior data suggesting compounding risks unique to Hispanic patients, including modifiable risk factors, biologic differences in cirrhosis and NASH pathogenesis, and gene-environmental interactions influencing a higher susceptibility towards hepatocarcinogenesis and more aggressive cirrhosis progression.

### Applications

Identification of clinical factors associated with HCC in Hispanics provides direction for public health efforts at HCC prevention through intervening on modifiable risk factors, targeted HCC screening for high risk ethnic populations, and more timely HCC treatment and management in this population.

### Terminology

HCC: Hepatocellular carcinoma; OS: Overall survival; UIC: University of Illinois, Chicago; AASLD: American Association for the Study of Liver Diseases; BMI: Body mass index; ATP: Adult treatment panel; ESRD: End stage renal disease; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease.

### Peer-review

An interesting observation study for the clinical outcome between HCC in



Hispanics to those of African Americans and Whites. A clearly data presentation and manuscript written.

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Prospective Study

## Phase angle obtained by bioelectrical impedance analysis independently predicts mortality in patients with cirrhosis

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## Abstract

### AIM

To evaluate the prognostic value of the phase angle (PA)

obtained from bioelectrical impedance analysis (BIA) for mortality prediction in patients with cirrhosis.

## METHODS

In total, 134 male cirrhotic patients prospectively completed clinical evaluations and nutritional assessment by BIA to obtain PAs during a 36-mo follow-up period. Mortality risk was analyzed by applying the PA cutoff point recently proposed as a malnutrition marker ( $PA \leq 4.9^\circ$ ) in Kaplan-Meier curves and multivariate Cox regression models.

## RESULTS

The patients were divided into two groups according to the PA cutoff value ( $PA > 4.9^\circ$ ,  $n = 73$ ;  $PA \leq 4.9^\circ$ ,  $n = 61$ ). Weight, height, and body mass index were similar in both groups, but patients with  $PA > 4.9^\circ$  were younger and had higher mid-arm muscle circumference, albumin, and handgrip-strength values and lower severe ascites and encephalopathy incidences, interleukin (IL)-6/IL-10 ratios and C-reactive protein levels than did patients with  $PA \leq 4.9^\circ$  ( $P \leq 0.05$ ). Forty-eight (35.80%) patients died due to cirrhosis, with a median of 18 mo (interquartile range, 3.3-25.6 mo) follow-up until death. Thirty-one (64.60%) of these patients were from the  $PA \leq 4.9^\circ$  group.  $PA \leq 4.9^\circ$  significantly and independently affected the mortality model adjusted for Model for End-Stage Liver Disease score and age (hazard ratio = 2.05, 95%CI: 1.11-3.77,  $P = 0.021$ ). In addition, Kaplan-Meier curves showed that patients with  $PA \leq 4.9^\circ$  were significantly more likely to die.

## CONCLUSION

In male patients with cirrhosis, the  $PA \leq 4.9^\circ$  cutoff was associated independently with mortality and identified patients with worse metabolic, nutritional, and disease progression profiles. The PA may be a useful and reliable bedside tool to evaluate prognosis in cirrhosis.

**Key words:** Bioelectrical impedance analysis; Body composition; Phase angle; Nutritional assessment; Liver disease; Cirrhosis; Mortality

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**Core tip:** This article provides original data displaying the good performance of the phase angle (PA) obtained by bioelectrical impedance analysis in the evaluation of mortality prognosis in patients with cirrhosis. The findings suggest that the PA is a safe, practical, and inexpensive tool for the prediction of mortality potentially associated with malnutrition.

Belarmino G, Gonzalez MC, Torrinhas RS, Sala P, Andraus W, D'Albuquerque LAC, Pereira RMR, Caparbo VF, Ravacci GR, Damiani L, Heymsfield SB, Waitzberg DL. Phase angle obtained by bioelectrical impedance analysis independently predicts mortality in patients with cirrhosis. *World J Hepatol* 2017; 9(7): 401-408 Available from: URL: <http://www.wjgnet.com>

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## INTRODUCTION

Liver transplantation (LT) is the best option for patients with advanced cirrhosis, but its clinical application is often limited by the low availability of organ donors, risk of organ rejection, and implied high cost<sup>[1,2]</sup>. Consequently, the control and treatment of cirrhosis-associated complications remains the mainstay for this population. Malnutrition is a major complication often observed in patients with cirrhosis, and it has been associated with more severe disease, the manifestation of other cirrhosis-associated complications, and mortality<sup>[3]</sup>. Early diagnosis of malnutrition in patients with cirrhosis is important for prompt management and to improve quality of life<sup>[4-7]</sup>.

In general, ascites, edema, and other chronic liver disease-associated complications (*i.e.*, altered immuno-competence, decreased protein synthesis, and renal failure) can impair the performance of traditionally applied criteria for nutritional assessment (NA)<sup>[8]</sup>. Consequently, weight loss, anthropometric measurements, the creatinine-height index, nitrogen balance, lymphocyte count, and serum albumin, transferrin, prealbumin, and retinol-bound protein levels should be interpreted with restrictions when assessing the nutritional status of cirrhotic patients<sup>[9]</sup>. In this scenario, a gold standard NA method is required for the proper diagnosis of malnutrition in this patient population<sup>[10-15]</sup>.

The phase angle (PA) obtained from bioimpedance analysis (BIA) has been proposed as a nutritional status marker, with low values associated with malnutrition and nutritional risk at the time of hospital admission<sup>[16]</sup>. The PA reflects the relationship between the resistance component (R), meaning tissue opposition to the passage of electric current, and reactance (Xc), meaning the resistance effect produced by the interface of tissues and cell membranes<sup>[17]</sup>. A main advantage of the use of PA is that it can be applied even under unstable tissue hydration conditions, such as edema and ascites<sup>[18]</sup>.

By potentially reflecting malnutrition, the PA can be a useful prognostic marker in several clinical settings<sup>[16,18-29]</sup>. As with any biological marker, the PA is influenced by the specific characteristics of each clinical population and may vary according to sex and age. Thus, specific PA reference and cutoff values have been proposed to establish prognoses for different diseases<sup>[16,18-26,30-34]</sup>. Recently, the  $4.9^\circ$  PA value was identified as the best cut-off for malnutrition associated to disease severity of patients with liver cirrhosis and shown to have important prognostic value for malnutrition-associated mortality in this patient population<sup>[35]</sup>.

In this study, we aimed to test whether this PA cutoff ( $\leq 4.9^\circ$ ) had prognostic value for mortality in a population of patients with cirrhosis of different ethnicity than used for its initial identification.

## MATERIALS AND METHODS

### Patients

This study included 134 male patients with biopsy-proven cirrhosis who were recruited prospectively from the Digestive Tract Surgery Service at the Hospital das Clínicas of the University of São Paulo Medical School between January 2012 and December 2014. Exclusion criteria were alcohol abuse; human immunodeficiency virus positivity; cancer diagnosis, acute liver failure, or chronic or acute disease of the lung, kidney, or heart; previous LT; orthopedic prosthesis use; and dementia. All patients provided written informed consent before trial participation.

### Protocol design

Our protocol was designed to determine whether the PA has prognostic value for mortality in male patients with cirrhosis, by considering the PA cutoff point proposed by Ruiz-Margáina *et al.*<sup>[35]</sup> ( $PA \leq 4.9^\circ$ ) as a malnutrition marker. All recruited subjects were instructed to refrain from excessive physical activity, diuretic use, and alcohol consumption for 24 h before the assessment, which was performed in a 4-h fasting state<sup>[36]</sup>. Demographic data were recorded for all subjects. Death events were recorded for all patients with cirrhosis during the 36-mo follow-up period. A single trained technician performed all study procedures according to the ethical standards of the Declaration of Helsinki of the World Medical Association. All procedures were approved by the Institutional Ethics Review Board (0646/11) and registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT02421848).

### Demographic and clinical data collection

The following demographic, clinical, inflammatory, and anthropometric data were collected: Age, liver cirrhosis etiology, Child-Pugh and Model for End-Stage Liver Disease (MELD) scores, presence of severe ascites, presence of encephalopathy, interleukin (IL)-6/IL-10 ratio, C-reactive protein (CRP) level, body weight and height, body mass index (BMI), non-dominant handgrip-strength (ND-HGS), and mid-arm muscle circumference (MAMC). Body weight was measured with the participant standing in the center of a single electronic scale platform (ADP; BOD POD™ BC system device; Life Measurement Instruments, Concord, CA, United States) while barefoot and wearing only light clothes<sup>[37]</sup>. Height was measured with a single stadiometer (Sanny, São Paulo, SP, Brazil) with the individual standing barefoot with the heels together, back upright, and arms extended next to the body<sup>[38]</sup>. BMI was calculated as weight divided by height squared ( $\text{kg}/\text{m}^2$ ). ND-HGS was measured using a digital dynamometer (Charder Co. Ltd., Taichung City, Taiwan), as described previously<sup>[39]</sup>. Arm circumference (AC) was measured around the mid-upper arm, between the shoulder and elbow, using a flexible tape. Triceps skinfold thickness (TST) was assessed and MAMC was calculated using the formula:  $MAMC = AC \text{ (cm)} = \pi \times [TST$

(mm)/10].

### Phase angle estimation

The PA was assessed by whole-body BIA<sup>[40]</sup> at 50 kHz (Bodystat 4000 model; Bodystat Ltd., Douglas, Isle of Man, British Isles) with APEX software (version 4.02; Hologic Inc., Bedford, MA, United States). Participants removed all metal objects and other items that might interfere with the scan and were instructed to empty the bladder. Each participant was positioned supine in the center of the scanning table with the palms down and the arms beside the body. His age, height, weight, sex, and ethnicity were entered into the computer. The PA value was calculated as  $PA = \arctan(Xc/R \times 180/\pi)$ . Patients were grouped according to PA value ( $PA > 4.9^\circ$ ,  $PA \leq 4.9^\circ$ )<sup>[35]</sup>.

### Survival

Death events were assessed by telephone calls at the end of the study period. Only deaths related directly to cirrhosis complications were counted. The prognostic value of the PA for mortality prediction was evaluated in mortality models adjusted for variables potentially impacting nutritional status and/or cirrhosis severity (age, Child-Pugh and MELD score)<sup>[35,41,42]</sup>. A longitudinal analysis of mortality was used to assess the prognostic value of malnutrition.

### Sample size

The sample size required to analyze the prognostic value of the PA for mortality was calculated using the G Power software package (version 3.1.9.2; Heinrich Heine University, Dusseldorf, Germany). A sample size of 134 patients was obtained from a Cox proportional-hazards regression model, considering a significance level of 5% and rate of 36% at 36 mo of follow-up, with 80% power to detect a hazard ratio (HR) of 2.50 for mortality prediction.

### Statistical analysis

Survival probabilities were estimated by the Kaplan-Meier method, compared using the log-rank test, and estimated in terms of the failure rate according to independent and multiple models of Cox proportional hazards. The mortality models included PA values and were adjusted for MELD score and age. Data were expressed as means  $\pm$  SDs, medians, interquartile ranges (IQRs; 25<sup>th</sup>-75<sup>th</sup> percentile), or percentages, depending on the normality of distribution and type of variable. Data were analyzed using the R software package (version 3.1.3, 2015; R Core Team, Vienna, Austria) and a significance level of 5%.

## RESULTS

### Patient characteristics

A total of 134 patients (mean age,  $54.30 \pm 10.10$  years) with cirrhosis of different etiologies (59.80% alcoholic,



**Table 1** Baseline characteristics and body composition of patients with cirrhosis

Variable	PA > 4.9° (n = 73)	PA ≤ 4.9° (n = 61)	Total (n = 134)	P value <sup>a</sup>
Age (yr)	52.10 ± 9.80	56.90 ± 9.80	54.30 ± 10.10	0.005 <sup>1</sup>
Weight (kg)	76.60 ± 13.10	76.40 ± 15.30	76.50 ± 14.10	0.919 <sup>1</sup>
Height (m)	1.70 ± 0.10	1.70 ± 0.10	1.70 ± 0.10	0.536 <sup>1</sup>
Child Pugh A (%)	25	10	18	
Child Pugh B (%)	45	65	55	
Child Pugh C (%)	30	25	27	0.031 <sup>3</sup>
Model for end-stage liver disease score	13.41 ± 5.11	14.95 ± 4.65	14.11 ± 4.95	0.073 <sup>3</sup>
Severe ascites (%)	10.00	29.00	18.20	0.016 <sup>3</sup>
Encephalopathy (%)	40.00	60.00	50.00	0.044 <sup>3</sup>
Body mass index (kg/m <sup>2</sup> )	26.70 ± 4.10	26.40 ± 5.00	26.60 ± 4.50	0.683 <sup>1</sup>
Mid-arm muscle circumference (cm)	25.80 ± 3.20	23.20 ± 3.10	24.70 ± 3.40	< 0.001 <sup>1</sup>
Handgrip strength (kg)	31.80 ± 7.00	24.40 ± 8.90	28.60 ± 8.70	< 0.001 <sup>1</sup>
IL-6/IL-10 ratio (pg/mL)	1.10 (0.51; 2.35)	1.29 (0.71; 4.68)	1.17 (0.58; 2.68)	0.086 <sup>2</sup>
C-reactive protein (mg/dL)	0.88 (0.42; 1.96)	1.20 (0.60; 4.72)	1.09 (0.54; 2.62)	0.030 <sup>2</sup>
Albumin (g/dL)	3.90 (3.40; 4.30)	3.50 (2.90; 3.80)	3.60 (3.20; 4.20)	0.002 <sup>2</sup>

<sup>a</sup>PA > 4.9° vs PA ≤ 4.9°; <sup>1</sup>Student's *t* test; <sup>2</sup>Mann-Whitney test; <sup>3</sup>χ<sup>2</sup> test. Data are presented as mean ± SD (confidence interval), or percentage. PA: Phase angle; IL: Interleukin.

**Table 2** Mortality estimates for patients with cirrhosis from a multiple Cox regression model

Variable	HR (95%CI)	P value
Age (yr)	1.03 (1.00, 1.06)	0.042
MELD score	1.10 (1.05, 1.16)	0.001
Phase angle 50 kHz (< 4.9°)	2.05 (1.11, 3.77)	0.021

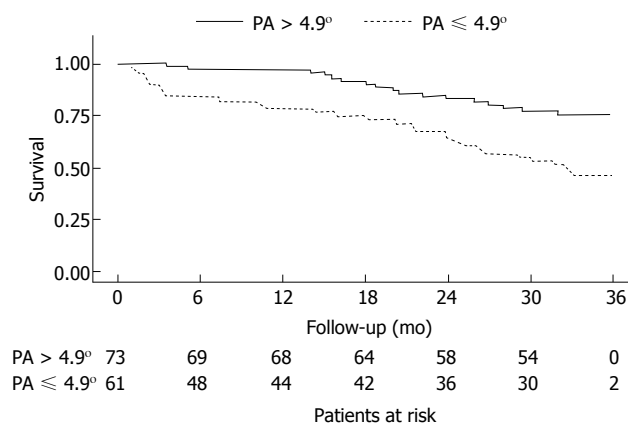
P values for independent Cox regression models refer to three models explained by age, MELD score, and phase angle. HR: Hazard ratio; MELD: Model for end-stage liver disease.

20.10% viral, 10.40% cryptogenic, and 9.70% other), presenting as 17.90% Child A, 54.50% Child B, and 27.60% Child C and with a mean MELD score of 14.11 ± 4.95, were enrolled in the study. Of these patients, 73 (54.48%) were assigned to the PA > 4.9° group and 61 (45.52%) were assigned to the PA ≤ 4.9° group. Weight, height, and BMI were similar in both groups, but patients from the PA > 4.9° group were younger and had higher MAMC, albumin, and ND-HGS values and lower severe ascites and encephalopathy incidences, IL-6/IL-10 ratios, and CRP levels than did patients from the PA ≤ 4.9° group (Table 1).

### Prognostic value of malnutrition, identified by the phase angle

The mean follow-up duration was 25 mo (median, 32.1 mo). Of the 134 patients included in the mortality prediction analysis, 48 (35.80%) died due to cirrhosis, with a median of 18 mo (IQR, 3.3; 25.6 mo) of follow-up until death. Thirty-one (64.60%) patients who died were from the PA ≤ 4.9° group.

The Child-Pugh score had no significant effect in the initial mortality model and was not included in the final model (Table 2). PA values ≤ 4.9° significantly affected the mortality model adjusted for MELD score and age (HR = 2.05, 95%CI: 1.11-3.77, *P* = 0.021). In addition,



**Figure 1** Kaplan-Meier survival curves for 134 patients with cirrhosis, obtained using cutoff scores based on phase angle obtained by bioelectrical impedance analysis (PA < 4.9°, *n* = 61; PA > 4.9°, *n* = 73). PA: Phase angle.

the mortality prediction was not influenced by MELD or age. Patients from the PA ≤ 4.9° group were significantly more likely to die, as demonstrated by Kaplan-Meier curves (Figure 1). In the median follow-up period of 18 mo, the incidence ratios of death were 27.10% for patients from the PA ≤ 4.9° group and 9.90% for those from the PA > 4.9° group.

## DISCUSSION

Although malnutrition implies a poor prognosis for patients with cirrhosis, its diagnosis has been masked in this population due to the unavailability of a clinically accessible method that is not affected by edema and/or ascites<sup>[18]</sup>. The PA is not affected by hydric changes and was recently proposed as a good tool for malnutrition diagnosis in patients with cirrhosis, with a cutoff value of ≤ 4.9°<sup>[35]</sup>. Here, we showed that PA ≤ 4.9° predicted mortality in male cirrhotic patients, in a model adjusted for age and MELD score.

We identified four studies evaluating the prognostic value of the PA in Brazilian ( $n = 2$ ), German, and, more recently, Mexican patients with cirrhosis. These studies showed that PA cutoff values of  $5.18^\circ$ ,  $5.44^\circ$ ,  $5.4^\circ$  and  $4.9^\circ$ , respectively, were related to disease severity and even mortality, when controlling for age and other nutritional variables<sup>[14,18,35,43]</sup>. Here, we applied the PA cutoff value proposed recently by Ruiz-Margáin *et al.*<sup>[35]</sup> ( $\leq 4.9^\circ$ ), which was further used to establish malnutrition with good prognostic value for mortality in a cohort of Mexican cirrhotic patients.

In our study, the prognostic value of this PA cutoff was tested in mortality models adjusted for age and MELD score, as the main markers of PA performance and disease severity, respectively. Age has been proposed as the main indicator for PA determination in women and men, and the MELD score has been considered a good predictor of short-term mortality in patients with cirrhosis<sup>[35,41,42]</sup>.

The Child-Pugh score was added to our initial mortality model because it may reflect the progression of liver damage and indirectly detect metabolic changes that may influence the prognosis of the disease<sup>[42]</sup>. However, it had no significant effect on mortality prediction. Notably, the MELD score has been validated as a good predictor of the survival of adult patients on the LT list, and has been found to better predict short-term results than does the CP score<sup>[44]</sup>. This difference in performance may explain the significant value of the MELD score, and not the CP score, for mortality prediction in our initial model. Data from the final mortality model support the prognostic value of  $PA \leq 4.9^\circ$ , as it was associated independently with mortality. Furthermore, our HR for mortality was similar to that reported by Ruiz-Margáin *et al.*<sup>[35]</sup>.

Results from some studies suggest that malnutrition is related strongly to mortality and cirrhosis-related complications<sup>[14,18,27,35,43,44]</sup>. Despite evidence suggesting the utility of the PA as a nutritional marker, its validity has been questioned. According to our data, the  $PA \leq 4.9^\circ$  cutoff was able to identify patients with significant changes in inflammatory and nutritional markers highly indicative of catabolism and malnutrition (*i.e.*, increased IL-6/IL-10 ratio and CRP level and decreased albumin level and HGS, a relevant marker of muscle loss associated largely with poor prognosis in cirrhosis). The notably increased mortality rate observed in our patients with  $PA \leq 4.9^\circ$  may be associated closely with the deleterious effects of malnutrition.

PA values change in response to nutritional interventions, with greater sensitivity than achieved with other nutritional markers<sup>[45]</sup>. Thus, even if the PA cannot actually represent the nutritional status of a patient, it seems to adequately reflect minimal changes in this clinical variable. In this scenario, the PA could be applied for nutritional monitoring of patients for whom the risk of malnutrition could significantly influence clinical outcomes. For instance, the incidences of severe ascites and

encephalopathy complications were significantly higher among patients with  $PA \leq 4.9^\circ$  than among those with  $PA$ s above this cutoff in our study, in response to the metabolic consequences of the disease.

Patients with cirrhosis often display circulatory dysfunction with portal hypertension, leading to vasodilatation of splanchnic vessels and favoring decreased peripheral resistance and effective central blood volume, with consequent arterial hypotension and hyperdynamic circulation. These abnormalities result in the activation of vasoconstrictor systems through the renin-angiotensin-aldosterone system and of the sympathetic nervous system, with increased levels of antidiuretic hormone and renal vasoconstriction that culminate in ascites and/or edema<sup>[46]</sup>. These altered physiological states limit the application of available methods to evaluate nutritional status<sup>[47]</sup>.

Indeed, as a result of ascites and/or edema, anthropometric measures such as BMI usually overestimate lean mass in patients with end-stage liver disease who require LT<sup>[3]</sup>. Consequently, although easier, traditional NA may underestimate the prevalence and severity of malnutrition in patients with cirrhosis<sup>[13]</sup>. Moreover, the presence of body fluid changes, mainly ascites, may explain the marked discrepancies in malnutrition frequencies (ranging from 5.4% to 68.2%) among NA methods in patients with cirrhosis<sup>[12,47-54]</sup>. As PA values are not influenced by unstable hydration, we suggest that this tool is useful for nutritional monitoring of patients with cirrhosis, and that the PA cutoff value proposed by Ruiz-Margáin *et al.*<sup>[35]</sup> can identify those at high risk of death if not nutritionally treated.

One limitation of our study was the inclusion of solely male patients. We assessed only male patients to make our sample as uniform as possible, as liver cirrhosis *per se* is a progressive disease and hepatic damage may differ, even slightly, among patients. In addition, cirrhosis is more common in men and malnutrition seems to have greater prognostic value for disease progression in men than in women. The prognostic ability of the studied cutoff value for phase angle is associated directly with malnutrition. Thus, by evaluating only men, we were able to access not only a more uniform sample, but also the population most susceptible to the studied disease and its associated nutritional complications. Ruiz-Margáin *et al.*<sup>[35]</sup> did not specify the sex of the cirrhotic patients with which the studied PA cutoff value was developed. Thus, we cannot confirm whether this value performs similarly in the prediction of malnutrition-associated mortality in women. We can conclude that the  $PA \leq 4.9^\circ$  cutoff was associated independently with mortality in male patients with cirrhosis, potentially associated to malnutrition. The PA may be a useful and reliable bedside tool to evaluate prognosis in cirrhosis.

## ACKNOWLEDGMENTS

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participated in the study.

## COMMENTS

### Background

Liver transplantation is the best option for patients with advanced cirrhosis, but its clinical application is often limited. Malnutrition is a major complication often observed in patients with cirrhosis. Early diagnosis of malnutrition in patients with cirrhosis is important. In general, ascites, edema, and other chronic liver disease-associated complications can impair the performance of traditionally applied criteria for nutritional assessment (NA). Consequently, weight loss, anthropometric measurements, the creatinine-height index, nitrogen balance, lymphocyte count, and serum albumin, transferrin, prealbumin, and retinol-bound protein levels should be interpreted with restrictions when assessing the nutritional status of cirrhotic patients. In this scenario, a gold standard NA method is required for the proper diagnosis of malnutrition in this patient population.

### Research frontiers

The phase angle (PA) obtained from bioimpedance analysis has been proposed as a nutritional status marker, with low values associated with malnutrition and nutritional risk at the time of hospital admission. The PA reflects the relationship between the resistance component, meaning tissue opposition to the passage of electric current, and reactance, meaning the resistance effect produced by the interface of tissues and cell membranes. A main advantage of the use of PA is that it can be applied even under unstable tissue hydration conditions, such as edema and ascites.

### Innovations and breakthroughs

This article provides original data displaying the good performance of the PA obtained by bioelectrical impedance analysis in the evaluation of mortality prognosis in patients with cirrhosis.

### Applications

The findings suggest that the PA is a safe, practical, and inexpensive tool for the prediction of mortality potentially associated with malnutrition.

### Peer-review

The authors aim to explore the potential value of PA in cirrhosis. In general, the topic is interesting, and the design is sound.

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## Limitations and opportunities of non-invasive liver stiffness measurement in children

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**Author contributions:** Engelmann G performed most of the writing of the manuscript; Quader J created the figures and tables and performed the statistical analyses; Teufel U coordinated the writing and performed data analyses; Schenk JP wrote the sections on real-time tissue elastography and magnetic resonance elastography.

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### Abstract

Changes in liver structure are an important issue in chronic hepatopathies. Until the end of the 20<sup>th</sup> century, these changes could only be determined by histological analyses of a liver specimen obtained *via* biopsy. The well-known limitations of this technique (*i.e.*, pain, bleeding and the need for sedation) have precluded its routine use in follow-up of patients with liver diseases. However, the introduction of non-invasive technologies, such as ultrasound and magnetic resonance imaging, for measurement of liver stiffness as an indirect marker of fibrosis has changed this situation. Today, several non-invasive tools are available to physicians to estimate the degree of liver fibrosis by analysing liver stiffness. This review describes the currently available tools for liver stiffness determination that are applicable to follow-up of liver fibrosis/cirrhosis with established clinical use in children, and discusses their features in comparison to the "historical" tools.

**Key words:** Children; Transient elastography; Liver fibrosis; Liver biopsy

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**Core tip:** Non-invasive liver stiffness measurement is a new and helpful tool for assessing liver fibrosis in children, but it cannot yet replace liver biopsy.

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## INTRODUCTION

Until the end of the 20<sup>th</sup> century structural changes of the liver could only be determined by histological analyses of a liver specimen obtained by percutaneous liver biopsy. The well-known limitations of this technique (*i.e.*, pain, bleeding and the need for sedation), however, precluded its routine use in follow-up of patients with liver diseases, and it has only been used routinely in studies<sup>[1]</sup>. The introduction of non-invasive imaging technologies, such as ultrasound and magnetic resonance imaging, has changed this situation, allowing for measurement of liver stiffness as an indirect marker of fibroses. Today, several non-invasive tools are available to physicians to estimate the degree of liver fibrosis by analysing liver stiffness.

This review will describe the currently available tools for liver stiffness determination that are applicable to follow-up of liver fibrosis/cirrhosis with established clinical use in paediatric patients (children between 0 and 18-year-old), and discusses their features in comparison to the “historical” tools.

Liver fibrosis is a dynamic reaction of the healthy liver towards chronic cell injury<sup>[2]</sup>. It is frequently observed in patients with chronic liver disease, regardless of aetiology<sup>[3]</sup> and patient age. Structural changes of liver architecture usually appear slowly, within years or decades, and accompanied by a continual development from low-grade fibrosis to liver cirrhosis. Liver cirrhosis, itself, represents the end-stage of fibrotic liver diseases.

Development of fibrosis leads to an increase in liver stiffness, detectable by non-invasive methods. Progression from liver fibrosis to cirrhosis may be preventable, if the fibrosis is detected early in the course. Examples of preventable fibrosing liver diseases are hepatitis B or hepatitis C infections<sup>[4,5]</sup>, liver transplantation<sup>[6]</sup> or Wilson’s disease. For other fibrosis aetiologies, a close follow-up is recommended to detect changes in liver structure in a timely manner and to determine the disease course. This holds true for post-liver transplant patients and patients with autoimmune liver diseases. Today, histology is the gold standard for the diagnosis of liver fibrosis.

### Liver biopsy

Liver biopsy remains the method of choice for clarification of the aetiology of hepatopathies. It has the advantage of obtaining direct information, not only on the degree of fibrosis but also on the presence of inflammation, necrosis, steatosis, and iron or copper deposits. However, the histopathologic examination of a liver specimen also has limitations. Studies have clearly indicated that liver biopsy is prone to sampling errors and may underestimate the amount of liver fibrosis. As such, cirrhosis could be missed on a percutaneous liver biopsy, reportedly affecting an estimated 30% of cases<sup>[7,8]</sup>. Liver biopsy has further technical limitations. There is a small risk of clinically relevant bleeding (0.3%) and mortality due to the intervention, shown to affect 0.04%-0.07%

in a large case series<sup>[9]</sup>. In a paediatric series, major complications occurred in 1.5% and minor complications in 25% of 275 liver biopsies<sup>[10]</sup>. Another drawback of this method is the size of the specimen obtained<sup>[8]</sup>. A single liver biopsy reportedly has a 20%-30% chance of missing the relevant area of interest, thereby underestimating liver diseases<sup>[11]</sup>. Paediatric patients have an additional risk due to the need of sedation for the biopsy procedure. Therefore, in clinical practice liver histology is almost exclusively used for diagnoses and only in certain settings, such as liver transplantation, and for therapy control<sup>[1,12]</sup>.

On the other hand, liver biopsy has some clear advantages. A recent study of a cohort of patients with either histologically-proven non-alcoholic steatohepatitis (NASH) or non-alcoholic fatty liver disease (NAFLD) showed that outcome (*i.e.*, death, liver transplantation or severe liver disease) was directly dependent upon the degree of fibroses<sup>[13]</sup>. Another recent study by Mann *et al.*<sup>[14]</sup> demonstrated an association of portal inflammation, metabolic syndrome and fibrosis in 430 obese children. These findings support the current tenet that portal inflammation and exact degree of fibrosis are best determined by liver biopsy.

### Histological assessment of liver biopsy

The liver biopsy specimen is recommended to have length of at least 10 mm and width of at least 1 mm (obtained with > 18 gauge needle)<sup>[15]</sup>. Several histological scoring systems have been established for grading (necroinflammatory activity) and staging (fibrosis) of structural liver damage in patients<sup>[16]</sup>. The Desmet score<sup>[17]</sup> is used to evaluate adult hepatitis C patients, and the METAVIR<sup>[18,19]</sup> and Ishak score<sup>[20]</sup> are used in cases of chronic viral hepatitis (B and C). The SSS-score of Chevallier<sup>[21]</sup> was developed to quantify fibroses irrespective of the underlying disease. Some of these scores have been evaluated in children (Table 1), and a detailed break-down of each (in children and adults) is provided below: (1) the METAVIR score<sup>[18]</sup> assesses fibrosis qualitatively on a 0-4 scale, with F0 indicating absence of fibrosis, F1 indicating portal fibrosis without septa, F2 indicating portal fibrosis with a few septa, F3 indicating architectural distortion with numerous septa without cirrhosis, and F4 indicating cirrhosis. This score has been used to evaluate adult patients with hepatitis B and C<sup>[19]</sup> and paediatric patients after liver transplantation<sup>[22]</sup>, biliary atresia<sup>[23]</sup>, intestinal failure<sup>[24]</sup> and total parenteral nutrition<sup>[25]</sup>; (2) the grading score of Ishak *et al.*<sup>[20]</sup> assesses fibrosis qualitatively on a 0-6 scale. The Ishak score has been used in paediatric populations with various liver diseases, and including children after liver transplantation<sup>[26]</sup> or cardiovascular surgery<sup>[27]</sup>; (3) the grading score of Desmet *et al.*<sup>[17]</sup> assesses fibrosis qualitatively on a 0-4 scale, with F0 indicating absence of fibrosis, F1 indicating portal fibrosis, F2 indicating fibrosis with septa without distortion of the liver architecture, F3 indicating septal fibrosis with severe

**Table 1 Comparison of the 4 main histological scoring systems used in the evaluation of fibrosis in paediatric liver diseases today**

Scoring system	Staging	Evaluated in adults with	Evaluated in children with
METAVIR	F0-F4	Hepatitis B and C	Biliary atresia, intestinal failure, total parenteral nutrition and post-liver transplantation
Ishak	F0-F6	Hepatitis B and C	Post-liver transplantation and after cardiovascular surgery
Desmet	F0-F4	Hepatitis C	No
SSS-score	0- > 15	Hepatitis B and C	Hepatitis B

distortion of the liver architecture, and F4 indicating cirrhosis. It has been used to evaluate adult patients with chronic hepatitis C<sup>[28]</sup>; and (4) the semi-quantitative severity score of Chevalier *et al.*<sup>[21]</sup> has been used in children<sup>[29]</sup> and adults with hepatitis B<sup>[30]</sup> and C<sup>[31]</sup>.

### Aminotransferases

Numerous attempts have been made to determine liver fibrosis by non-invasive means. One of the oldest is measurement of serum aminotransferases, which remains the most widely used, and convenient, tool to measure liver cell integrity. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are inexpensive laboratory values. They can be easily obtained from a patient and are stable in serum specimen. ALT, especially, is highly liver specific.

Unfortunately, aminotransferases poorly reflect the stage of liver fibrosis or cirrhosis. If they are elevated, a more detailed examination of the liver is obligate. But, ALT and AST may even be normal or only slightly elevated in fibrotic or cirrhotic liver diseases. The positive predictive value of aminotransferases for NAFLD or NASH is low. In a series of 222 patients with histologically-proven NAFLD, 37% of the patients with advanced fibrosis or NASH presented with normal ALT levels. This phenomenon was also recently demonstrated in children, in a study of paediatric cases of NAFLD conducted by Molleston *et al.*<sup>[32]</sup>.

Aminotransferases may serve as a first screening tool for detection of fibrosis, but even normal levels of aminotransferases do not exclude severe liver disease with changes in liver structure. Some of the techniques that have been developed to identify NAFLD in adult patients have been tested in children, including the AST to platelet ratio index (APRI) score, the NAFLD fibrosis score<sup>[33]</sup> and the Fibrosis-4 index score. Yet, recent data have indicated that only the APRI score and the paediatric NAFLD fibrosis score reliably reflect fibrotic changes of the liver. Alkhoury *et al.*<sup>[34]</sup> have developed and published a new paediatric NAFLD fibrosis score based on a model using ALT, alkaline phosphatase, platelet counts and gamma-glutamyl transferase, and demonstrated its predictive ability of fibrosis as good.

Collectively, these tests are reliable in detecting severe fibrosis or cirrhosis (grade 2 or greater for the Desmet score). Thus, while they can reliably show if the patient suffers from a change in liver structure they cannot reliably predict the exact degree of fibrosis.

## SONOELASTOGRAPHY

### Transient elastography

Transient elastography (TE) is a technique based on the measurement of the velocity of a shear wave that is induced to the liver by a mechanical impulse. To apply that impulse to the liver, the probe has to be pressed onto the skin with a certain force, and the thoracic wall prevents the liver from being compressed by the probe. Therefore, TE can only be measured reliably in the right lobe of the liver and not in other organs or in other parts of the liver.

The velocity of the shear wave is directly proportional to the stiffness of the liver. Stiffness mainly depends on the amount of fibrotic material in the liver. Therefore, liver elasticity is measured in kilopascal (kPa) and liver stiffness increases with liver fibrosis. The probe is placed in the 7<sup>th</sup> or 8<sup>th</sup> intercostal space in the right ventral axillary line. The patient lies in supine position, with the right arm in maximal abduction. This technique has been described in detail elsewhere<sup>[35]</sup>. A mechanical impulse of 50 Hz induces an elastic shear wave that passes through the liver tissue. The speed of this wave is measured *via* ultrasound. For more detailed information on the basic physical principle, the Young Modules, see Frulio *et al.*<sup>[36]</sup>.

TE reliably detects liver fibrosis, as demonstrated in numerous studies and meta-analyses comparing the technology to liver biopsy<sup>[35-42]</sup>. The median liver stiffness in adults varies between 4.4 and 5.5<sup>[43,44]</sup>. In addition, there is evidence that stiffness is greater in males, increases with body mass index in adult patients, and tends to increase with age but not to a statistically significant extent<sup>[44]</sup>. In children, the median liver stiffness significantly rises with age, starting with 4.4 in preschool children and rising to 5.1 in pubertal children. Liver stiffness in children has also been shown to differ according to sex, with girls showing significantly less (4.7) than boys (5.6)<sup>[45]</sup>. In split liver transplants of left liver, which is the main transplantation technique used in infants, toddlers and preschool children, liver stiffness measurement cannot be used because it is technically performable only in the right liver lobe (as detailed above). A clinical example of TE use in a paediatric patient is presented in Figure 1.

Introduction of the small TE-probe that is also suitable for use with infants and very young children has made TE possible for every age group. But liver stiffness measurement can only be performed in a patient that is laying calmly in supine position. This is usually not an





**Figure 1** Transient elastography findings for a 10-year-old female suffering from Wilson's disease. The patient's brother had previously developed acute liver failure, which triggered routine monitoring of the patient thereafter. The patient was clinically completely healthy. The transient elastography shows 9.3 kPa, which is above the 6.5 kPa upper limit of normal. Histology findings for the patient showed the liver to be cirrhotic.

attainable state in toddlers without sedation. Therefore, the problem of invalid liver stiffness measurement due to moving and crying of the patients makes this method questionable in infants.

Another general drawback of this method is the price. The technique is reliant on hardware that ultrasound machines do not come equipped with normally. Therefore, an extra-device is required to accompany the ultrasound machine and this produces extra-costs of more than 50000 Euros. Finally, the capacity for integrated measurement in B-mode ultrasound images is not yet available.

Findings from a recent Cochrane analysis of adult patients with alcoholic liver disease led to the recommendation of TE as a useful tool to exclude fibroses and, in cases of liver stiffness measurement above 12.5 kPa, to suggest cirrhosis. These data, however, still have to be confirmed in further studies<sup>[46]</sup>, especially for their applicability to the paediatric age group. It is well accepted that TE enables the investigator to clearly exclude severe changes in liver architecture, but it remains a matter of debate whether TE can also enable clear staging of fibrosis. As such, TE is routinely used to assess liver fibrosis in adult patients with chronic hepatitis C, and this use is confirmed in the EASL Clinical Practice Guidelines 2011<sup>[47]</sup>. With the increasing application of TE in children with viral hepatitis, however, TE has the capability to gain more relevance for detection of liver fibrosis.

### Acoustic radiation force impulse

Acoustic radiation force impulse (ARFI) is a point shear wave elastography that measures tissue elasticity independent of an external mechanical impulse to the

tissue. Therefore, this method is not only useful for liver stiffness measurement but also for determination of changes in stiffness of the spleen<sup>[48]</sup>, testis<sup>[49]</sup>, thyroid<sup>[50]</sup>, breast<sup>[51]</sup>, placenta<sup>[52]</sup>, pancreas in chronic pancreatitis<sup>[53]</sup> and transplanted kidney<sup>[54]</sup>. The technique is based on an acoustic impulse and measurement of the speed of the shear wave induced by it; results are displayed in m/s. The stiffer the organ, the faster the shear wave.

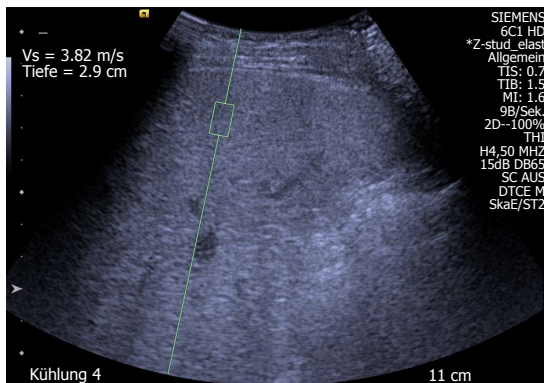
The ARFI method has two advantages. First, it can be performed by an additional technical tool for a high-end ultrasound system, providing integrated B-mode images. Second, the tissue is not compressed by the probe, as in TE. Compression itself causes changes in stiffness, and this feature of ARFI enables measurement of stiffness in numerous tissues. Many studies have shown the reliability and reproducibility of this technique in adult patients<sup>[55]</sup> and in children<sup>[56]</sup>. The correlation of ARFI and fibroses is in a good range<sup>[57]</sup>, comparable to that of TE<sup>[58]</sup>, and control-values have been established for children<sup>[56,59]</sup> and adults<sup>[36]</sup> (Table 2). Moreover, ARFI was demonstrated as effective in paediatric patient groups with biliary atresia or severe fibrosis<sup>[60,61]</sup> and in follow-up after liver transplantation<sup>[62]</sup>. A clinical example of ARFI use in a paediatric patient is presented in Figure 2.

Children with biliary atresia could gain particular benefit from non-invasive examinations for assessment of timing of liver transplantation after kasai-portoenterostomy<sup>[63,64]</sup>. According to METAVIR or SSS-score, ARFI shows overlap of shear wave velocity values in different fibrosis stages, as shown in the study by Hanquinet *et al.*<sup>[65]</sup>. ARFI might offer diagnostic advantages over B-mode imaging in terms of combining stiffness measurement with sonomorphological parameters as the qualitative sonomorphological aspect becomes

**Table 2** Control and normal values of non-invasive liver stiffness measurement

	Normal values (ULN is defined as mean + 1.64 SD)		Impulse generation
	Children	Adults	
TE	ULN: 6.47 kPa <sup>[40]</sup>	8.3/7.83 (m/f) <sup>[39]</sup>	Mechanical
RTE	Median: 106 a.u. <sup>[67]</sup>	127 a.u. <sup>[78]</sup>	Aortal pulsing
MRE	Mean: 2.71 kPa <sup>[79]</sup> -2.93 kPa <sup>[71]</sup>	3.45 kPa <sup>[80]</sup>	Acoustic
ARFI	ULN: 1.39 m/s (mean + 1.64 SD) <sup>[59,81]</sup>	1.35 m/s <sup>[36]</sup>	Ultrasound

Normal values are defined as mean + 1.64 times SD, while control values are expressed as mean. ARFI: Acoustic radiation force impulse; MRE: Magnetic resonance elastography; RTE: Real-time tissue elastography; TE: Transient elastography; ULN: Upper limit of normal.



**Figure 2** Acoustic radiation force impulse measurement of the liver in a 16-year-old female patient with cystic fibrosis. Hyperechoic liver parenchyma with irregular liver surface in fibrotic liver parenchyma was revealed. The shear wave velocity was 2.3-3.82 m/s in multiple measurements, significantly above normal values. The same patient had undergone a Fibroscan and the results showed a stiffness of  $21.3 \pm 2.5$  kPa. Six months previously, another Fibroscan had shown a value of  $20.4 \pm 2.8$  kPa.

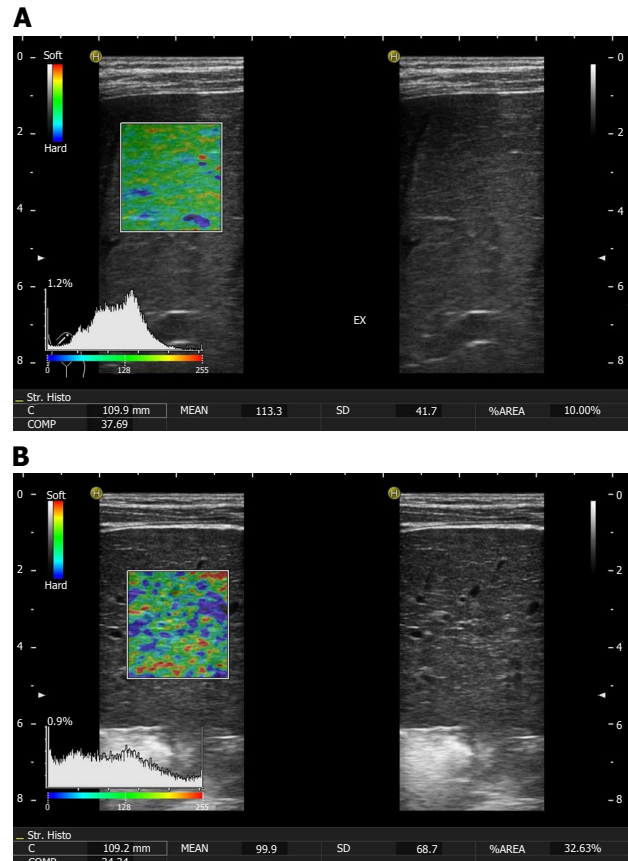
quantitative<sup>[61]</sup>. This makes comparison in patients easier.

Similar to TE, increased application of ARFI in children could lead to an implementation of this type of measurement in the routine clinical work flow, especially for patients with specific paediatric diseases, such as cystic fibrosis or biliary atresia.

### Real-time tissue elastography

Real-time tissue elastography (RTE) examinations can be performed with an ultrasound device and a standard linear transducer<sup>[66]</sup>. The RTE software captures images of tissue motions caused by heartbeats or respiration. These images are then transferred into colour-coded plane and the system calculates a histogram of strain elasticity values of the matrix in arbitrary units (a.u.), ranging from 0 to 255<sup>[67]</sup>. The method can be performed without extra-hardware, but data on the value of this method in children are scarce. Morikawa *et al.*<sup>[67]</sup> analysed RTE in 101 adult patients with hepatitis c and found a good correlation of the RTE values with the histologic grading of fibroses. In contrast, data obtained from children in another study<sup>[68]</sup> showed only a moderate correlation, and it was concluded that RTE could not be recommended for a clear differentiation of fibrosis stages while the difference between stage IV fibrosis and normal liver tissue or stage I fibrosis was significant.

Other studies of adult patients<sup>[69]</sup> have concentrated



**Figure 3** Real-time tissue elastography in a normal and cirrhotic liver. A: RTE with a normal strain histogram (mean: 113.3 a.u.; %AREA: 10%) in a 8-year-old female patient with cystic fibrosis and nearly normal liver structure; B: RTE with pathological strain histogram in a 6-year-old female patient with tyrosinemia type 1 and liver cirrhosis with small nodules. The mean value was 99.9, and the peak of histogram shifted to the left to lower values of the mean. The percentage of stiffer areas (colour-coded in blue; %AREA) increased up to 23.6%. This histogram is more flattened in comparison to the normal strain histogram. RTE: Real-time tissue elastography.

on the elastic or fibrosis index values, which have not been adequately studied in the paediatric age group. In a meta-analysis of RTE conducted by Kobayashi *et al.*<sup>[70]</sup>, the authors concluded that RTE has low accuracy for detecting any stage of fibrosis. Today, we would not recommend the use of single statistical parameters as the mean elasticity value of strain histogram or %AREA in children alone to predict the histological fibrosis stage. Differentiation of high fibrosis stages to normal tissue is possible, but application in young infants can be

difficult. Clinical examples of RTE use in two paediatric patients are presented in Figure 3.

Further studies on the use of the elastic index in paediatric patients should be conducted. High fibrosis stages can be differentiated from low fibrosis stages, but no clinical recommendations exist as of yet.

### MR-elastography

MR-elastography (MRE) is an elastography technique using an acoustic impulse to produce a shear wave. The impulse is produced by an audio subwoofer and subsequently transmitted to the liver *via* a connecting-tube that is placed on the skin of the patient. Then, the shear wave induced by this acoustic impulse is measured and stiffness is calculated in kPa<sup>[71]</sup>. Studies of MRE in adult patients with hepatitis C have shown good relation of MRE-measured liver stiffness, as compared to Child-Pugh score<sup>[72]</sup>. In another study of adult patients with cystic fibrosis<sup>[73]</sup> the liver stiffness measurement was shown to correlate well with serum levels of aminotransferases and also with ultrasound findings, but there were insufficient data to make any conclusions regarding histopathologic changes.

A new and promising application of MRE involves the differentiation of NASH from NAFLD. Both diseases can occur in obese patients, but there is yet no non-invasive method capable of distinguishing between the two. Patients with NASH develop cirrhosis in 10% of cases, while patients with NAFLD do not. Neither aminotransferases<sup>[32]</sup> nor ultrasound can differentiate these two diseases. Recent studies have suggested that MRE might be able to reliably determine the presence of NASH in an obese patient<sup>[74]</sup>. Future studies may prove that MRE, therefore, is useful, even in clinical analysis of obese patients, for defining relevant end-points.

## DISCUSSION

ARFI does not replace liver biopsy for staging of liver fibrosis or cirrhosis, neither do TE, RTE or MRE<sup>[75,76]</sup>. The limitations of these non-invasive techniques are low specificity and high cost, the latter being especially relevant for TE.

Liver structure changes can be excluded by each of these non-invasive techniques, with an acceptable sensitivity but an unacceptable low specificity. TE, ARFI and MRE have the potential to exclude severe liver structure changes. For RTE, however, the data are conflicting and do not support a recommendation; certainly, further studies are necessary. For diagnosing liver disease, none of these non-invasive techniques is useful. But, in many patients, the etiology is quite clear due to readily assessable clinical or laboratory aspects, such as the presence of obesity, chronic viral hepatitis or alpha-1 antitrypsin deficiency. In cases of the patient being post-liver transplantation or with an already-obtained liver biopsy, the analysis of liver structure changes is of greater importance.

A possible diagnostic approach to patients with liver disease in 2016 is to first perform clinical examinations to obtain anthropometric data, ultrasound images and standard laboratory measures. If then there is evidence for liver disease, ARFI or TE should be performed. If those findings then suggest liver structure changes, a biopsy should be obtained in any case. If the findings suggest normal liver structure, the biopsy may be delayed and further laboratory studies may be performed first. If there is no change in aminotransferase levels after 6 mo, a liver biopsy should be performed. Non-invasive liver stiffness measurement can be used for follow-up after liver biopsy if the stage of fibrosis has been determined based on histopathological criteria<sup>[77]</sup>.

In patients with obesity, MRE possibly offers a new approach by which to define patients at risk for NASH or even to diagnose NASH in obese patients. Therefore, in the setting of an obese patient, MRE presents a real advantage over the classical methods of hepatology and future studies will show if this promising technique is suited to becoming part of the routine diagnostic workup in obese patients early in their clinical course and also in follow-up.

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Basic Study

## Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver

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## Abstract

### AIM

To evaluate the effects of chronic exposure to ethanol in the liver and the expression of inflammatory genes

in zebrafish.

## METHODS

Zebrafish ( $n = 104$ ), wild type, adult, male and female, were divided into two groups: Control and ethanol (0.05 v/v). The ethanol was directly added into water; tanks water were changed every two days and the ethanol replaced. The animals were fed twice a day with fish food until satiety. After two and four weeks of trial, livers were dissected, histological analysis (hematoxylin-eosin and Oil Red staining) and gene expression assessment of adiponectin, adiponectin receptor 2 (*adipor2*), sirtuin-1 (*sirt-1*), tumor necrosis factor- $\alpha$  (*tnf-a*), interleukin-1b (*il-1b*) and interleukin-10 (*il-10*) were performed. Ultrastructural evaluations were conducted at fourth week.

## RESULTS

Exposing zebrafish to 0.5% ethanol developed intense liver steatosis after four weeks, as demonstrated by oil red staining. In ethanol-treated animals, the main ultrastructural changes were related to cytoplasmic lipid particles and droplets, increased number of rough endoplasmic reticulum cisterns and glycogen particles. Between two and four weeks, hepatic mRNA expression of *il-1b*, *sirt-1* and *adipor2* were upregulated, indicating that ethanol triggered signaling molecules which are key elements in both hepatic inflammatory and protective responses. *Adiponectin* was not detected in the liver of animals exposed and not exposed to ethanol, and *il-10* did not show significant difference.

## CONCLUSION

Data suggest that inflammatory signaling and ultrastructural alterations play a significant role during hepatic steatosis in zebrafish chronically exposed to ethanol.

**Key words:** Ethanol; Hepatic steatosis; Inflammation; Zebrafish; Alcoholic fatty liver

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**Core tip:** Excessive alcohol consumption remains one of the most important causes of liver disease worldwide. Alcoholic steatosis results from the deposition of fat in liver cells and is the earliest stage of alcohol-related liver disease. Usually inflammation is associated with steatohepatitis, however our results demonstrate that chronic ethanol exposure increased the expression of the inflammatory gene interleukin-1b. Paradoxically the expression of adiponectin receptor-2 and sirtuin-1 also increased for attenuating the liver injury. Ultrastructural abnormalities were observed showing early alterations in liver cells. Knowledge of alcohol injury mechanisms will contribute to the development of novel therapies in the treatment of alcoholic liver disease.

Schneider ACR, Gregório C, Uribe-Cruz C, Guizzo R, Malysz T, Faccioni-Heuser MC, Longo L, da Silveira TR. Chronic exposure to ethanol causes steatosis and inflammation in zebrafish liver.

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## INTRODUCTION

Alcoholic liver disease (ALD) encompasses a wide spectrum of injury, ranging from simple steatosis, leading to steatohepatitis, fibrosis and finally to cirrhosis<sup>[1,2]</sup>. Hepatic steatosis is the first and most common consequence of alcohol abuse, develops in about 90%-95% of individuals who drink heavily, is usually asymptomatic and self limited; but may also occur in individuals who drink moderately<sup>[2]</sup>. Several studies have suggested that progression to more severe liver disease occurs in about 5%-20% of alcohol consumers<sup>[1]</sup>. As a consequence, it is important to better understand the pathogenesis of hepatic steatosis and the relationship between steatosis and liver injury.

Excessive accumulation of triglycerides in hepatocytes is the hallmark of hepatic steatosis. The source of triglyceride in the liver of ethanol consumers may be originated from disturbances of fatty acid oxidation mechanisms, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, alterations in lipogenic and lipolytic pathways and immune responses to ethanol<sup>[3-7]</sup>. A number of molecular mediator pathways regulating the synthesis, export, and oxidation of lipids have been discovered to be altered by ethanol: Sterol regulatory element-binding proteins, peroxisome proliferator-activated receptors  $\alpha$  and  $\gamma$ , adiponectin, sirtuins and others<sup>[8]</sup>.

Zebrafish is increasingly used as an *in vivo* model system for translational research, since zebrafish have a high degree of genetic conservation and their morphological and molecular basis of tissue and organ development is either identical or similar to other vertebrates including humans<sup>[9,10]</sup>. In previous studies regarding to hepatic diseases related to ethanol, zebrafish proved to be a valuable strategy for identifying lipogenic mechanisms, genes and pathways that influence hepatic steatosis<sup>[11-14]</sup>. Studies focused in inflammatory pathways in steatosis are scarce and the issue is not completely elucidated. Chronic ethanol consumption results in the activation of innate immunity and an inflammatory state, which contributes to the pathogenesis of ethanol-induced liver injury. The expression of tumor necrosis factor -  $\alpha$  (*tnf-a*), interleukin-1b (*il-1b*), interleukin-10 (*il-10*), adiponectin, adiponectin receptor 2 (*adipor2*) and sirtuin-1 (*sirt-1*) were evaluated and histological and ultrastructural evaluations were performed in liver of adult zebrafish after chronic ethanol exposure.

## MATERIALS AND METHODS

### Animal care and use statement

Wild-type, adult zebrafish (*Danio rerio*), male and female, were purchased from a commercial distributor (Fish Flower, Porto Alegre, RS). The animals were of



**Table 1** Primers and probes identification assays

Gene	Assay ID
<i>adiponectin</i>	F: 5'-AGG CTT AGA CTG TGA ACG GTG GGA C-3' R: 5'-AGC AGG TGT GTC CAG ATG TTT CCA G-3'
<i>adipor2</i>	dr0342657
<i>sirt-1</i>	ENSDART00000098209
<i>tnf-a</i>	dr03126848
<i>il-1b</i>	dr03114368
<i>il-10</i>	dr03103209
<i>ef-1a</i>	dr03432748

*tnf-a*: Tumor necrosis factor-alpha; *il*: Interleukin; *adipor2*: Adiponectin receptor 2; *sirt-1*: Sirtuin-1; *ef-1a*: Elongation factor- $\alpha$  gene.

heterogeneous wild type stock from the standard short-fin phenotype and were housed for 2 wk before the experiments in order to acclimate to the laboratory facility. The animal protocol was designed to minimize pain or discomfort to the animals. Fish were maintained in aerated water at  $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , 6.8-7 pH, on a 12/12 light/dark photoperiod cycle (lights on at 7:00 am). Biochemical parameters and quality of the water were monitored regularly: pH, presence of nitrates and nitrites, oxygen and ammonia levels. The animals were fed twice a day with fish food until satiety. Experiments were performed using a total of 104 animals. All fish used in this study were healthy and free of any signs of disease.

After acclimation period, the fish were randomly allocated into experimental tanks, density of 1 fish per liter of water. The following groups were performed ( $n = 52/\text{group}$ ): Control (C) and ethanol (E). E group received 0.5% (v/v) of ethanol (Merck KGaA, Germany) directly added into water; tank water was changed every two days and the ethanol replaced<sup>[15]</sup>. This ethanol dose was chosen due to the liver damage observed by Schneider and coworkers in zebrafish exposed to 0.5% of ethanol<sup>[15]</sup>. The tank water of C group was also changed in same days of E group. At 2 and 4 wk, fish were euthanized by hypothermal shock<sup>[16]</sup> and livers were completely removed for molecular and histological analysis.

The protocols were approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre, Brazil (No. 10.0327), and conducted in accordance with international guidelines for the care and use of laboratory animals.

### Histological analysis

Livers of zebrafish dissected at 2<sup>nd</sup> and 4<sup>th</sup> weeks were stained with hematoxylin and eosin ( $n = 5/\text{group}$ ) or Oil Red ( $n = 5/\text{group}$ ). Livers were fixed in 10% formalin, embedded in paraffin wax, sectioned ( $5\text{ }\mu\text{m}$ ), and slices were stained with hematoxylin and eosin. Livers embedded in Tissue-Tek OCT Compound (Sakura Finetek, United States) were cryosectioned ( $8\text{ }\mu\text{m}$  thick) and stained with Oil Red (Sigma-Aldrich, United States) to assess fatty droplet accumulation.

For ultrastructural evaluation, livers of 2 animals (male) of each group (C and E) were fixed in 2.5% glutaraldehyde diluted by 0.12 mol/L phosphate buffer (pH 7.2-7.4) for 3 h at  $4^{\circ}\text{C}$ . The material was washed three times in the same buffer at 30-min intervals and then post-fixed for 30 min in 1% buffered osmium tetroxide followed by a phosphate buffer (0.1 mol/L) wash three times at 15-min intervals. Livers were dehydrated using ascending grades of alcohols and embedded in an epon-araldite mixture. Ultrathin sections (90 nm) were stained with 2% uranyl acetate and 1% lead citrate<sup>[17]</sup>. The ultrathin sections were examined under JEM 1200 FX II transmission electron microscope.

### Gene expression assessment

The liver samples (pool = 3 livers;  $n = 5/\text{group/period}$ ) were immediately immersed in liquid nitrogen and stored in ultrafreezer ( $-80^{\circ}\text{C}$ ). Total RNA was extracted using the TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol and the concentrations were quantified by Nanodrop (Thermo Fisher Scientific, United States) at 260 nm. RNA purity was verified by a 260/280 nm ratio of 1.8 or greater. First-strand cDNA was synthesized from 3  $\mu\text{g}$  of total RNA using the Superscript<sup>TM</sup> II RT system (Invitrogen, United States). Gene expression analysis of *adiponectin*, *adipor2*, *sirt-1*, *tnf-a*, *il-1b* and *il-10* were performed from 5  $\mu\text{L}$  of cDNA and run in duplicate using TaqMan Gene Expression Assays (Life Technologies, United States) (Table 1).

PCR amplifications were run on a Step One<sup>TM</sup> Real time PCR System (Applied Biosystems, United States) and performed starting with a 2 min denaturation step at  $50^{\circ}\text{C}$ , 10 min at  $95^{\circ}\text{C}$  followed by 40 cycles with 15 s at  $95^{\circ}\text{C}$  and 1 min at  $60^{\circ}\text{C}$ .

Gene expression was quantified using the  $2^{-\Delta\Delta\text{Ct}}$  (threshold cycle) method and normalization was done using the *elongation factor- $\alpha$  gene* (*ef-1a*).

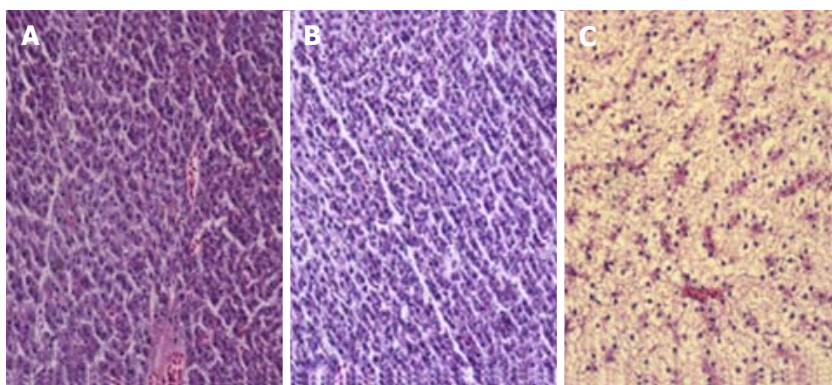
### Statistical analysis

Log-transformed data was tested with Kruskal-Wallis test and Dunn as *post hoc* test for multiple comparisons. Results with  $P < 0.05$  were considered statistically significant. All analysis were performed using the Statistical Package for the Social Sciences (SPSS 18.0) software.

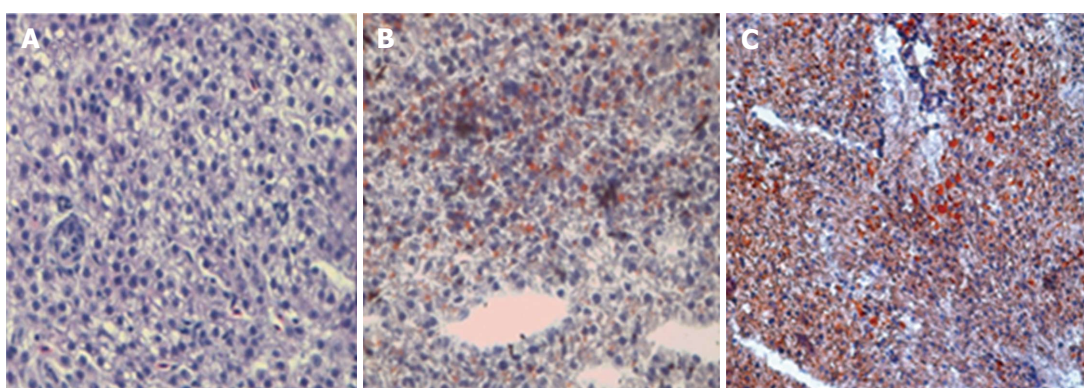
## RESULTS

### Ethanol effects on zebrafish liver histology

Sections of livers from control animals stained with hematoxylin-eosin showed well-preserved liver cells without signs of fat deposits (Figure 1A). After 2 wk of ethanol exposure, the liver appearance of animals from E group were similar to the C group (Figure 1B), however at 4 wk, the hepatocytes of animals from E group showed an expressive enlargement and presented nuclei displaced to the periphery of the cytoplasm due to fatty infiltration (Figure 1C). Livers of the control animals stained with



**Figure 1** Hematoxylin-eosin staining of liver sections from zebrafish. A: C group (2 wk), the hepatocytes are aligned in cords, absence of fat droplets; B: E group (2 wk), without apparent changes compared with the C group; C: E group (4 wk), enlarged hepatocytes due to fatty infiltration. Magnification: 400  $\times$ .



**Figure 2** Oil red staining sections of zebrafish liver. A: C group (4 wk), absence of lipid droplets; B: E group (2 wk), mild presence of lipid droplets; C: E group (4 wk), intense lipid accumulation induced by ethanol in hepatocytes. Magnification: 400  $\times$ .

Oil Red did not present any lipid droplets (Figure 2A). However, ethanol-treated animals presented a light steatosis at 2<sup>nd</sup> week (Figure 2B) which increased severely in the 4<sup>th</sup> week (Figure 2C).

The supplemental file contains the results of ultra-structural evaluations. Control group showed hepatocytes with hexagonal shape, evident nucleoli of moderate size and located in the centre of the spherically shaped nuclei (Figure 3A), intracellular duct with microvilli (Figure 3C), rough endoplasmic reticulum (RER) contained few cisternae and were closely associated with mitochondria (Figure 3E). Compared to control group, hepatocytes of ethanol-treated fish showed a large amount of glycogen associated with numerous lipid droplets (Figure 3B); the intracellular canaliculi often showed signs of degeneration with aspects of myelin figures therein (Figure 3D); and augmented number of RER cisterns (Figure 3F).

### Gene expression assessment

At 2<sup>nd</sup> week the genes evaluated did not present statistical difference in mRNA expression between E and C groups, except for *tnf-a*, which was decreased. An increase of expression of *tnf-a*, *il-1b*, *adipor2* and *sirt-1* was observed between two and four weeks in E group, demonstrating that time to ethanol exposure had influence on expression of these genes. The *il-10* expression did not

reach significant statistical difference between groups at any period (data not shown). *Adiponectin* mRNA was not detected in liver of animals from C and E groups.

### The effect of ethanol on the expression of cytokines mRNA

The hepatic *tnf-a* expression in E group was lower than in C group at 2 wk ( $P = 0.018$ ). The *il1-b* expression was significantly increased between C and E groups at 4<sup>th</sup> week ( $P = 0.024$ ) (Figure 4).

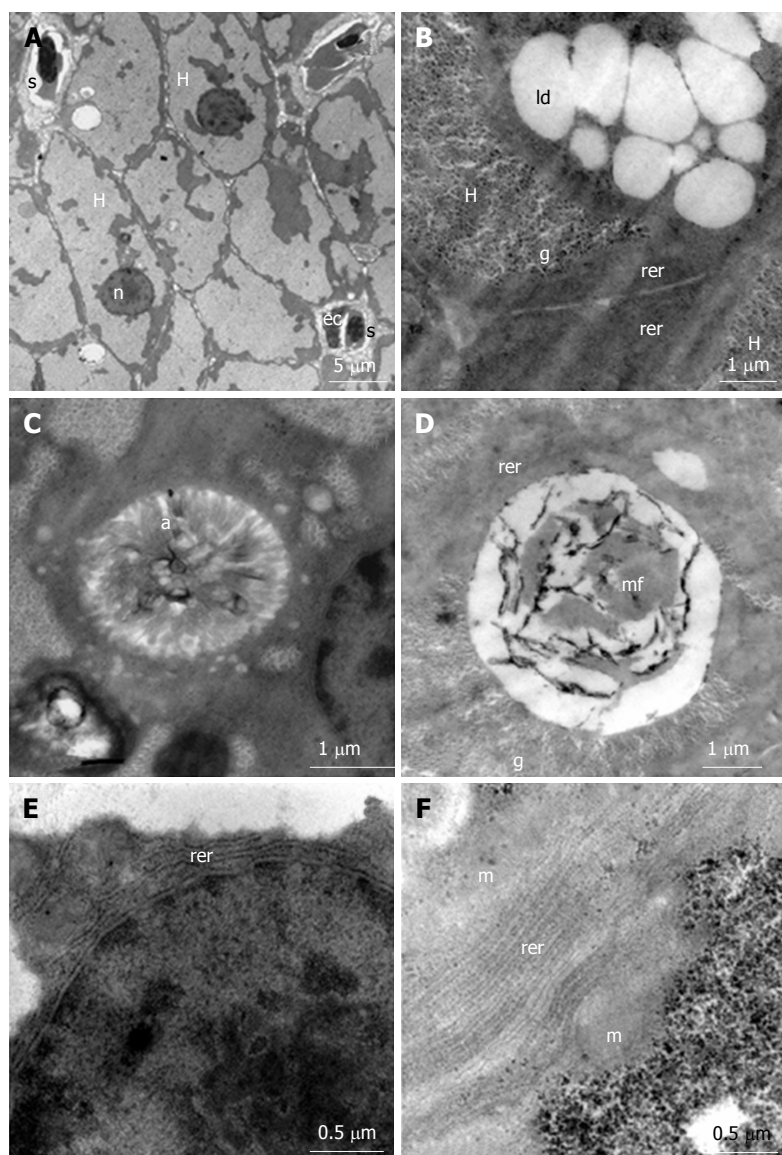
### Ethanol effects on mRNA expression of *adipor2* and *sirt-1*

The expression of *adipor2* increased in E group between 2 and 4 wk ( $P < 0.0001$ ) and was higher in E compared to C group ( $P = 0.006$ ) at 4<sup>th</sup> week (Figure 5). *Sirt-1* showed an increased expression in E group along time until the 4<sup>th</sup> week ( $P = 0.001$ ) (Figure 5).

## DISCUSSION

Hepatic metabolic derangements are key components in the development of steatosis, considered the first hit for development of ALD. Until recently, the role of inflammation was linked to the presence of steato-hepatitis, and scarce evidences have shown the precocity





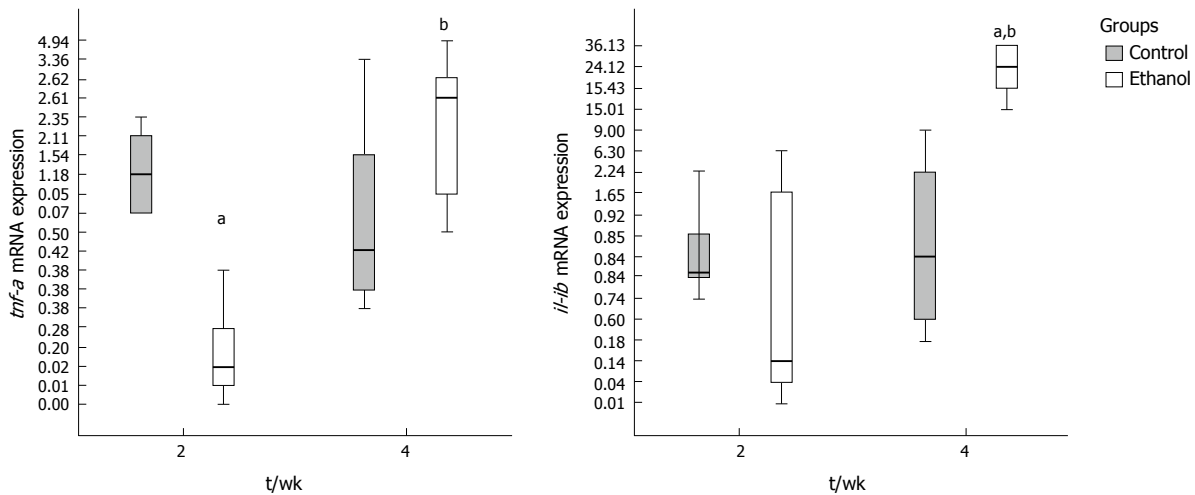
**Figure 3** Electron micrographs of liver sections of control (A, C and E) and ethanol exposed groups (B, D and F). A: Polygonal hepatocytes (H), spherical nucleus (n) sinusoid (s), endothelial cell (ec); B: Presence of large amount of glycogen (g) and lipid droplets (ld) in the hepatocytes cytoplasm; C: Intracellular canaliculus with large number of microvilli (a) within; E: It is noted the parallel arrangement of rough endoplasmic reticulum (rer) around the core; D: Myelin figure (mf) inside an intracellular canaliculus; F: Rough endoplasmic reticulum (rer) composed by 8-12 parallel cisterns; H: Hepatocytes; ec: Endothelial cell.

of inflammatory signaling during steatosis<sup>[18]</sup>.

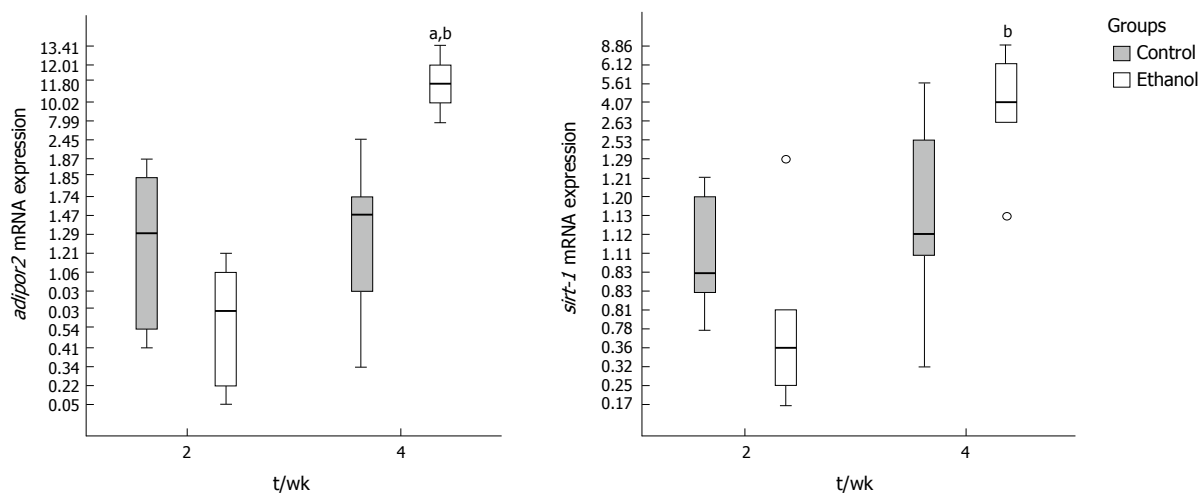
Important histological and ultrastructural abnormalities in liver of chronic ethanol-exposed zebrafish were seen at 4<sup>th</sup> week in this study. A light steatosis was detected by oil red staining at 2<sup>nd</sup> week, which increased severely at 4<sup>th</sup> week. Electron transmission microscopy revealed concurrent marked accumulation of glycogen and lipid droplets in cytoplasm, committed intracellular canaliculi, and increased RER cisterns. Our findings were similar to those described in alcoholic humans: Increased glycogen and fat deposits in the cytoplasm, abnormalities in endoplasmic reticulum<sup>[19]</sup>. Howarth *et al.*<sup>[13]</sup> observed abnormalities of endoplasmic reticulum and biliary canaliculi in acutely ethanol treated zebrafish larvae. Mitochondrial and ER abnormalities were seen in a model of non-alcoholic fatty liver disease (NAFLD) induced by

fructose in zebrafish<sup>[20]</sup>.

In accordance to histological findings, there were changes in hepatic mRNA expression of *il-1b*, *tnf-a*, *sirt-1* and *adipor2*. At fourth week, in the presence of more advanced steatosis, *il-1b* showed an expressive increase. Growing evidence indicates that increased pro-inflammatory cytokines are involved in the progression of alcohol-induced liver injury<sup>[21,22]</sup>. The activation of innate immunity also stimulates the release of hepatoprotective and anti-inflammatory cytokines, which play a compensatory role against liver damage and inflammation<sup>[22]</sup>. In our study the *il-10* expression was not different between C and E groups. The *il-10* is produced by macrophages, lymphocytes, and Kupffer cells, and the liver is considered to be the main source of *il-10* production in response to lipopolysaccharides (LPS) stimulation<sup>[23]</sup>.



**Figure 4** Effect of ethanol on mRNA liver expression of tumor necrosis factor-alpha and interleukin-1b. *tnf-a* was decreased significantly in E group compared to C at 2<sup>nd</sup> week ( $P = 0.018$ ) and increased along time up 4<sup>th</sup> week ( $P < 0.001$ ), reaching C group levels. *il-1b* expression increased between 2 and 4 wk ( $P = 0.001$ ) and at 4<sup>th</sup> week there was a significant difference between C and E groups ( $P = 0.024$ ). Statistical data were determined by the Kruskal-Wallis test and Dunn as post hoc test. Values significantly different where indicated: <sup>a</sup>Significant statistical difference between 2 and 4 wk; <sup>b</sup>Significant statistical difference between C and E groups.  $P < 0.05$  was considered. *tnf-a*: Tumor necrosis factor-alpha; *il*: Interleukin.



**Figure 5** Effect of ethanol on mRNA liver expression of adiponectin receptor 2 and sirtuin-1 of zebrafish. *adipor2* and *sirt-1* expression increased in E group between 2 and 4 wk;  $P < 0.0001$  and  $P = 0.001$ , respectively. At 4<sup>th</sup> week *adipor2* of E group was increased compared to C,  $P = 0.006$ . <sup>a</sup>Significant statistical difference between C and E groups; <sup>b</sup>Significant statistical difference between 2 and 4 wk.  $P < 0.05$  was considered significant. *adipor2*: Adiponectin receptor 2; *sirt-1*: Sirtuin-1.

Sepulcre *et al.*<sup>[24]</sup> demonstrated that zebrafish responds to LPS with much lower sensitivity than mammals, what can explain the absence of difference in hepatic expression of *il-10*, between C and E groups.

Elevated circulating levels of *TNF-α* and *IL-1b* have been observed in human patients and animal models of ethanol-induced liver injury<sup>[25,26]</sup>. The expression levels of these cytokines correlate well with the progression of the disease. In our study the *tnf-a* liver expression was initially decreased in E group compared to C at 2<sup>nd</sup> week and increased significantly along 2<sup>nd</sup> and 4<sup>th</sup> week, reaching the C expression levels at 4<sup>th</sup> week. Liu *et al.*<sup>[27]</sup> demonstrated that only zebrafish with previous intestinal inflammation presented elevated *tnf-a* expression in liver compared to healthy animals after LPS exposure. Zebrafish are indeed able to respond to LPS, however

with much lower sensitivity than mammals and *via* a *tlr4/myd88*-independent signaling pathway<sup>[24,28]</sup>. Among the few studies that evaluated *tnf-a* expression in the liver of zebrafish, Sapp observed an elevation of *tnf-a* in fructose-treated larvae and Hammes in thiocetamide-treated fish<sup>[20,29]</sup>. Although there was no direct evidence in our study, these cited findings conducted us to the following conclusions: *tnf-a* is not promptly induced by LPS in zebrafish exposed to ethanol as occurring in mammals and its activation mechanism seems to be associated to more aggressive hepatotoxicants.

Differently, the hepatic expression of *il-1b* increased significantly over the period considered and at 4<sup>th</sup> week it was significantly higher in E group compared to C. *Interleukin-1*, the "gatekeeper" of inflammation, is the apical cytokine in a signaling cascade that drives the



early responses to injury or infection<sup>[30]</sup>. *Il-1b* production requires caspase-1 activation by inflammasomes-multiprotein complexes that are assembled in response to danger signals. Vojtech *et al.*<sup>[31]</sup> have described the cleavage of zebrafish *il-1b* by the caspase-1 homologues caspase-A and caspase-B, implying that the basic facets of the inflammasome platform of immune activation are conserved in zebrafish. The induction of *il-1b* demonstrated an early response to inflammatory stimuli in the present study. The up regulation of *il-1b* did not occur synergistically with *tnf-a* expression, as seen in mammals with ALD<sup>[26]</sup>. This result may suggest that *il-1b* is up regulated during chronic alcohol induced steatosis in zebrafish in a LPS independent pathway.

Adiponectin is a hormone that is secreted exclusively by adipocytes and has anti-inflammatory and hepatoprotective activities<sup>[32]</sup>. Circulating adiponectin is decreased in mammals with alcoholic disease<sup>[32,33]</sup>. In our study, *adiponectin* mRNA did not amplify in the livers of animals of both groups. Amali and collaborators observed elevated expression of *adiponectin* in liver of zebrafish treated with thioacetamide, but not in control animals<sup>[34]</sup>.

In this study, the *adipor2*, a receptor of adiponectin, was over expressed in liver of animals exposed to ethanol, during the period that hepatic steatosis became more severe. To date, very few data are available regarding the effect of chronic ethanol exposure on hepatic *adipor2*. Hammes *et al.*<sup>[29]</sup> observed decreased mRNA expression of hepatic *adipor2* and *sirt-1* and increased *tnf-a* in a model of NAFLD induced by thioacetamide in zebrafish. Possibly, thioacetamide, a more aggressive liver toxicant, contributed to down regulate *adipor2*. In humans, it was observed by Kaser that in presence of nonalcoholic steatohepatitis, *adiponectin receptor 2* expression was decreased compared to simple steatosis<sup>[35]</sup>. Neumeier *et al.*<sup>[36]</sup> showed that animals (rodents) with liver steatosis presented elevated liver expression of *adipor2*. The increased expression of *adipor2* may be related to hepatic protection during steatosis.

*SIRT-1* is a NAD<sup>+</sup>-dependent class III protein deacetylase that regulates lipid metabolism by deacetylation of modified lysine residues on histones, and targets a number of transcription factors involved in the regulation of gluconeogenesis, mitochondrial biogenesis, resistance to oxidative stress, adipogenesis and lipolysis, glycolysis, inflammation, apoptosis, cell differentiation, and angiogenesis<sup>[37]</sup>. To date, little is known about the function of *SIRT-1* in innate immunity and host defense. Studies in mammals have indicated that *SIRT-1* suppresses innate inflammatory responses<sup>[38]</sup>. Other authors have shown an expressive increase of *sirt-1* in liver of zebrafish chronically exposed to ethanol (0.5% vv)<sup>[39,40]</sup>. In our study occurred a significant increase of *sirt-1* between second and fourth weeks in fish treated with ethanol. We can speculate that the *sirt-1* hepatoprotective role might be involved in this process.

Ethanol effectively induced hepatic lipid accumulation and ultrastructural abnormalities in liver of zebrafish. Augmented expression of *il-1b* suggests that inflammatory signaling plays a significant role in hepatic

steatosis and *adipor2* and *sirt-1* increased expression appears to represent compensatory efforts to alleviate consequences of ethanol liver injury, probably, indicating a hepatoprotective reaction. Hepatic steatosis is considered the first hit of chronic progressive ALD. The investigation of earliest events linked to ALD requires multiple strategies to reverse the damage effects of ethanol to the liver and to contribute to development of new therapies.

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## COMMENTS

### Background

Alcohol abuse is an acute health problem throughout the world and alcohol consumption is related to the occurrence of chronic liver disease. Hepatic steatosis is the first step of liver damage, and in spite of being considered a benign event, may progress to alcoholic steatohepatitis and more severe liver disease. The zebrafish has been proposed for the study of the effects of ethanol on several organs and has been helpful to unravel the pathways of liver damage by alcohol.

### Research frontiers

The zebrafish (*Danio rerio*) is increasingly recognized as an important model system for studying liver development and human liver disease. Despite differences in the anatomical architecture of the zebrafish liver from mammals, alcoholic liver damages are similar to those of human beings, including alcoholic steatosis. This animal model will likely be a useful tool to further elucidate the pathogenesis and related disorders of alcoholic liver disease, as well as to discover new treatments.

### Innovations and breakthroughs

Proinflammatory cytokines were frequently linked to steatohepatitis, however, this study describes early ultrastructural alterations in hepatocytes and cytokines increase in the onset of ethanol-induced liver damage.

### Applications

The major advantage of zebrafish as a model system for hepatic processes is the ability to perform screening (genetic or chemical) in a vertebrate organism. The investigation of earliest events linked to alcoholic liver disease can contribute to the development of new strategies to prevent the advance of such disease.

### Terminology

Hepatic steatosis: Or fatty liver. It is caused by an excessive fat deposition in the liver; Steatohepatitis: It is a type of fatty liver disease characterized by the presence of inflammation; Fibrosis: Scars produced in a reparative or reactive process in the liver; Ultrastructure: The detailed structure of a biological specimen, such as a cell, that can be observed by electron microscopy; Histology: The study of the microscopic anatomy of tissues. The cell of the tissue can be observed under a light microscope.

### Peer-review

The paper is well-written.

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Retrospective Cohort Study

## Factors associated with long-term survival after liver transplantation: A retrospective cohort study

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**Informed consent statement:** For this retrospective, observational study neither informed consent nor approval of the ethics committee was needed according to the Professional Code of the German Medical Association (article B.III. § 15.1) and to the recommendations of our local ethical committee (Ethikkommission der Ärztekammer Hamburg).

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## Abstract

### AIM

To identify predictive factors associated with long-term patient and graft survival (> 15 years) in liver transplant recipients.

### METHODS

Medical charts of all *de novo* adult liver transplant recipients ( $n = 140$ ) who were transplanted in Hamburg between 1997 and 1999 were retrospectively reviewed. In total, 155 transplantations were identified in this time period (15 re-transplantations). Twenty-six orthotopic liver transplant (OLT) recipients were early lost to follow-up due to moving to other places within 1 year after transplantation. All remaining 114 patients were included in the analysis. The following recipient factors were analysed: Age, sex, underlying liver disease, pre-OLT body mass index (BMI), and levels of alanine aminotransferase (ALT), bilirubin, creatinine and gamma-glutamyltransferase (gamma-GT), as well as warm and cold ischemia times. Furthermore, the following donor factors were assessed: Age, BMI, cold ischemia time and warm ischemia time. All surviving patients were followed until December 2014. We divided patients into groups according to their underlying diagnosis: (1) hepatocellular



carcinoma ( $n = 5$ , 4%); (2) alcohol toxic liver disease ( $n = 25$ , 22.0%); (3) primary sclerosing cholangitis ( $n = 6$ , 5%); (4) autoimmune liver diseases ( $n = 7$ , 6%); (5) hepatitis C virus cirrhosis ( $n = 15$ , 13%); (6) hepatitis B virus cirrhosis ( $n = 21$ , 19%); and (7) other ( $n = 35$ , 31%). The group "other" included rare diagnoses, such as acute liver failure, unknown liver failure, stenosis and thrombosis of the arteria hepatica, polycystic liver disease, Morbus Osler and Caroli disease.

## RESULTS

The majority of patients were male ( $n = 70$ , 61%). Age and BMI at the time point of transplantation ranged from 16 years to 69 years (median: 53 years) and from 15 kg/m<sup>2</sup> to 33 kg/m<sup>2</sup> (median: 24), respectively. Sixty-six OLT recipients (58%) experienced a follow-up of 15 years after transplantation. Recipient's age ( $P = 0.009$ ) and BMI ( $P = 0.029$ ) were identified as risk factors for death by  $\chi^2$ -test. Kaplan-Meier analysis confirmed BMI or age above the median as predictors of decreased long-term survival ( $P = 0.008$  and  $P = 0.020$ ). Hepatitis B as underlying disease showed a trend for improved long-term survival ( $P = 0.049$ ,  $\chi^2$ -test,  $P = 0.055$ ; Kaplan-Meier analysis, Log rank). Pre-transplant bilirubin, creatinine, ALT and gamma-GT levels were not associated with survival in these patients of the pre-era of the model of end stage liver disease.

## CONCLUSION

The recipients' age and BMI were predictors of long-term survival after OLT, as well as hepatitis B as underlying disease. In contrast, donors' age and BMI were not associated with decreased survival. These findings indicate that recipient factors especially have a high impact on long-term outcome after liver transplantation.

**Key words:** Liver transplantation; Age; Body mass index; Long-term survival; Hepatitis B

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**Core tip:** Due to organ shortage and epidemiological developments, the number of older potential orthotopic liver transplant (OLT) recipients increased greatly over the last decades. In order to identify predictors for long-term survival after liver transplantation, we analysed all adult, first OLTs performed at the University Medical Center Hamburg-Eppendorf between 1997 and 1999 and compared these findings with the Eurotransplant database. Our study shows that recipient's age and body mass index as well as hepatitis B as underlying disease are predictors of long-term survival after OLT.

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[com/1948-5182/full/v9/i8/427.htm](http://dx.doi.org/10.4254/wjh.v9.i8.427) DOI: <http://dx.doi.org/10.4254/wjh.v9.i8.427>

## INTRODUCTION

Survival after liver transplantation has strongly improved in the last decades, but factors associated with long-term survival have not been well defined yet. Research on the factors associated with best long-term outcome is, therefore, essential for an optimal use of the donated organs. This is even more relevant since age of donors and recipients is increasing. This development is mostly due to the organ shortage as well as epidemiological developments.

The majority of deaths after older potential orthotopic liver transplant (OLT) occur within the first months after transplantation. This is predominantly caused by pulmonary infections, sepsis or multiple organ failure<sup>[1]</sup>. An analysis of a large cohort from the Eurotransplant database included more than 90000 patients that were liver transplanted between 1968 and 2009<sup>[1]</sup>. Within this cohort the early mortality was 6%, 9% and 12% for 1-, 3- and 6-mo mortality in patients who were liver transplanted after the year 2000<sup>[1]</sup>.

Although several transplant centres worldwide now have more than 20 years of clinical experience in the field of liver transplantation, only few studies have analysed the long-term outcomes in OLT recipients<sup>[2,3]</sup>.

Several donor and recipient factors, including age and body mass index (BMI), are well-known to influence short-term survival<sup>[4]</sup>. Their relevance for long-term outcome has not been studied in detail yet. However, the negative influence of obesity on survival in non-transplant recipients is a well-known fact, since the Framingham study of the 1990s<sup>[5]</sup>. The World Health Organization has defined obesity as a condition of excessive accumulation of body fat, causing severe damage to health (<http://www.who.org>). In fact, the prevalence of obesity is increasing worldwide and is a major threat to liver transplant recipients as well as the health of the general population. Common co-morbidities associated with obesity are hypertension, coronary heart disease, heart failure, stroke, hyperuricemia, dyslipidemia, insulin resistance and glucose intolerance. In addition, within the Framingham study, it was shown that fluctuations in body weight in non-transplant patients were associated with an increased mortality, independent of obesity and the trend of body weight over time<sup>[5]</sup>.

In contrast to the general population, the role of bodyweight in liver transplant recipients is less clear. Werneck *et al*<sup>[6]</sup> demonstrated in a study including 136 liver transplant recipients that there was no significant difference between obese and normal weight patients regarding length of stay in the Intensive Care Unit or in 2-year survival. On the other hand, Sawyer *et al*<sup>[4]</sup> demonstrated a decreased short-term survival in obese

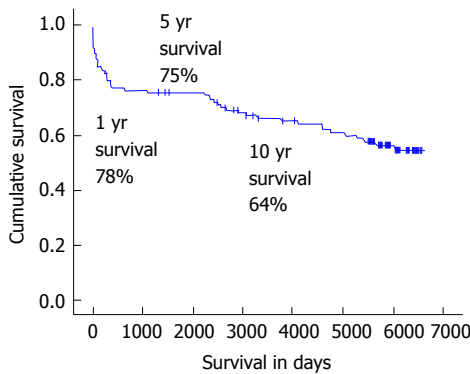


Figure 1 Overall survival of liver transplant recipients, monitored for 15 years.

patients in comparison to normal weight liver transplant recipients.

In addition to BMI, ages of donor and recipient have been discussed controversially within the last years<sup>[7]</sup>. Recipients' age is also known to have an influence on the outcome of liver transplantation. Schoening *et al.*<sup>[2]</sup> studied the 20-year survival rate of 313 liver transplant recipients. Those authors divided their cohort into three sub-groups: Patients below the age of 30, between 30 years and 55 years, and patients above 55 years. Patients below the age of 30 lived significantly longer after transplantation, as compared to the other two groups. However, no analysis was performed in which the patients were divided according to the median age in that study. Furthermore, the long-time survival of transplant recipients was compared with a "virtual control group", based on the life expectancy in the general population. While patients younger than 55 years showed a decreased survival, as compared to the general population, there was no difference in life expectancy between patients older than 55 years and the general population.

The aim of the present study was to identify factors associated with long-term patient and graft survival (> 15 years) in liver transplant recipients and compare these to the Eurotransplant database. This study focused specifically on recipient's age and BMI, as the influence of these factors is still not well defined.

## MATERIALS AND METHODS

This study was performed at the University Medical Center Hamburg-Eppendorf, a tertiary centre in North Germany. Since the first liver transplantation in Hamburg was performed in 1984, more than 2000 liver transplantations have been performed at this centre.

Medical charts of all *de novo* adult liver transplant recipients ( $n = 140$ ), who were transplanted in Hamburg between 1997 and 1999, were retrospectively reviewed (Figure 1). In total, 155 transplantations were identified in this time period (15 re-transplantations). Twenty-six OLT recipients were early lost to follow-up due to moving to other places within 1 year after transplantation (Figure 1). All remaining 114 patients were included in the

analysis. The following recipient factors were analysed: Age, sex, underlying liver disease, pre-OLT BMI, and levels of alanine aminotransferase (ALT), bilirubin, creatinine and gamma-glutamyltransferase (gamma-GT), as well as warm and cold ischemia times. Furthermore, the following donor factors were assessed: Age, BMI, cold ischemia time and warm ischemia time. All surviving patients were followed-up until December 2014. We divided patients into groups according to their underlying condition (Table 1): (1) hepatocellular carcinoma (HCC) ( $n = 5$ , 4%); (2) alcohol toxic liver disease ( $n = 25$ , 22.0%); (3) primary sclerosing cholangitis ( $n = 6$ , 5%); (4) autoimmune liver diseases ( $n = 7$ , 6%); (5) hepatitis C virus (HCV) cirrhosis ( $n = 15$ , 13%); (6) hepatitis B virus (HBV) cirrhosis ( $n = 21$ , 19%); and (7) other ( $n = 35$ , 31%). The group "other" included rare diagnoses, such as acute liver failure, unknown liver failure, stenosis and thrombosis of the arteria hepatica, polycystic liver disease, Morbus Osler and Caroli disease.

In addition to patient survival, the graft survival was also analysed. By definition, graft loss resulted in re-transplantation or death. The factors that were significantly associated with graft survival in our cohort were then compared with a large cohort of 2971 patients from Eurotransplant, which had been transplanted within the same period (1997-1999).

### Statistical analysis

Categorical variables were compared using  $\chi^2$  test. Metric data were compared using the non-parametric Mann-Whitney test. Survival analysis was performed utilizing Kaplan-Meier analysis. All investigated factors were tested utilizing univariate and multivariate models.

As metric values did not fulfil the criteria for a normal distribution (Kolmogorov Smirnov test  $P < 0.01$ ), median values instead of mean values were depicted. All statistical analyses were performed utilizing SPSS (version 13.0) and  $P$ -values < 0.05 were considered to be statistically significant.

For this retrospective, observational study neither informed consent nor approval of the ethics committee was needed according to the Professional Code of the German Medical Association (article B.III. § 15.1) and to the recommendations of our local ethical committee (Ethikkommission der Ärztekammer Hamburg).

### Control cohort

To discuss the survival of transplant patients with an age below and above the median of age (53 years) we constructed an imaginary control cohort. Therefore, we analysed the survival of historical data (<https://www.destatis.de>) of an age-matched cohort of the healthy German population.

In addition, to improve reliability of data, we compared our results with data from a cross-sectional Eurotransplant cohort including 2971 patients who underwent liver transplantation between 1997 and 1999. Eurotransplant kindly supported us with de-personalized

**Table 1 Patient characteristics directly before transplantation**

	Patients who survived ( <i>n</i> = 68)	Patients who died ( <i>n</i> = 46)	<i>P</i> -value ( $\chi^2$ test)
Male	39 (57%)	31 (67%)	NS
Age, yr (median, SD)	16-65 (50.5, 13)	17-69 (56.0, 12)	0.009
BMI, range kg/m <sup>2</sup> (median, SD)	18-33 (23.1, 3)	15-29 (25.9, 4)	0.029
Pre-LTx creatinine, mg/dL (median, SD)	0.4-3.5 (1.0, 0.5)	0.3-2.9 (1.1, 0.6)	NS
GFR, mL/min (median, SD)	15.3-230.2 (73.3, 38.1)	22.6- 240.4 (62.5, 48.0)	NS
ALT, U/L (median, SD)	4-2610 (35.5, 449.5)	6-1566 (19.5, 339.0)	NS
Gamma-GT, U/L (median, SD)	7-374 (47.0, 8)	13-184 (43.0, 45)	NS
Bilirubin, mg/dL (median, SD)	0.4-28.1 (2.4, 5.7)	0.4-28.3 (2.4, 5.8)	NS
Warm ischemia time, min (median, SD)	25-100 (50.0, 18)	22-75 (54.0, 15)	NS
Cold ischemia time, min (median, SD)	242-940 (542.5, 157)	174-825 (521.0, 146)	NS
Donor age, yr (median, SD)	12-70 (36.5, 16)	13-75 (41.0, 1)	NS
Donor BMI, kg/m <sup>2</sup> (median, SD)	17-30 (23.5, 3)	18-31 (24.2, 2)	NS
Underlying diagnosis <i>n</i> (%)			
HCC	2 (3)	3 (6)	NS
Alcohol toxic liver cirrhosis	12 (18)	13 (27)	NS
PSC	4 (6)	2 (4)	NS
Autoimmune	5 (8)	2 (4)	NS
HCV cirrhosis	9 (14)	6 (13)	NS
HBV infection	16 (24)	5 (10)	0.049
Other	18 (27)	17 (35)	NS

ALT: Alanine aminotransferase; BMI: Body mass index; OLT: Orthotopic liver transplantation; HCV: Hepatitis C virus; HBV: Hepatitis B virus; NS: No statistically significant difference; GFR: Glomerular filtration rate; HCC: Hepatocellular carcinoma; PSC: Primary sclerosing cholangitis.

data that were already arranged and categorized according to our median values of age and BMI and to status of HBV positivity. To compare this cohort with our own cohort, survival of these patients was analysed up to the same time point (until December 2014).

## RESULTS

### Patient characteristics

Overall, 114 OLT recipients were included in the study (Table 1). The majority of the patients were male (*n* = 70, 61%). The age and BMI at the time of transplantation ranged from 16 years to 69 years (median: 53 years) and from 15.1 kg/m<sup>2</sup> to 33.3 kg/m<sup>2</sup> (median: 24 kg/m<sup>2</sup>), respectively. See Table 1 for an overview of the overall investigated factors. The median follow-up was 5139 d. Sixty-six (58%) OLT recipients experienced a follow-up of 15 years after OLT (Figure 1). The 1-, 5- and 10-year patient survival rates were 78%, 74% and 64% (Figure 1).

### Follow-up and graft survival

Graft survival 15 years post-OLT was 53%. Fifty-three patients experienced a graft loss either by death (34%) or re-transplantation (13%). Characteristics of patients with graft survival and those with graft loss are depicted in Table 1.

### Association between patient survival and recipient's age

During the observational period, the mortality rate was significantly higher in patients with an age above the median (53 years) at transplantation as compared to patients younger than the median (*P* = 0.009). The Kaplan-Meier analysis confirmed that older patients had a decreased patient survival rate (*P* = 0.008; Figure 2).

Furthermore, the median age at the time of transplantation was higher in patients who deceased within 15 years of follow-up in comparison with patients who were still alive at the end of the study period (*P* = 0.006, Mann-Whitney test; Figure 3). These findings were confirmed in the cross-sectional Eurotransplant cohort (*n* = 2973) transplanted in the same period, with a follow-up of 15-17 years. In this cohort, 625/1145 (55%) patients with an age above 53 years died within the 15-year to 17-year follow-up period, while only 653/1809 (36%) patients with an age below 53 years died (*P* < 0.001; Table 2).

In a sub-analysis, we defined age above 60 years as "old" and analysed the groups of transplant younger (*n* = 89) and older (*n* = 25) than this threshold, separately. In patients older than 60 years, the patient survival rate was significantly lower as compared to younger patients ( $\chi^2$  test *P* = 0.007, Kaplan-Meier analysis *P* = 0.002). Donor age (12-75 years, median: 40) was not significantly correlated with patient survival. A multivariate analysis confirmed age as an independent factor associated with graft survival (*P* < 0.01).

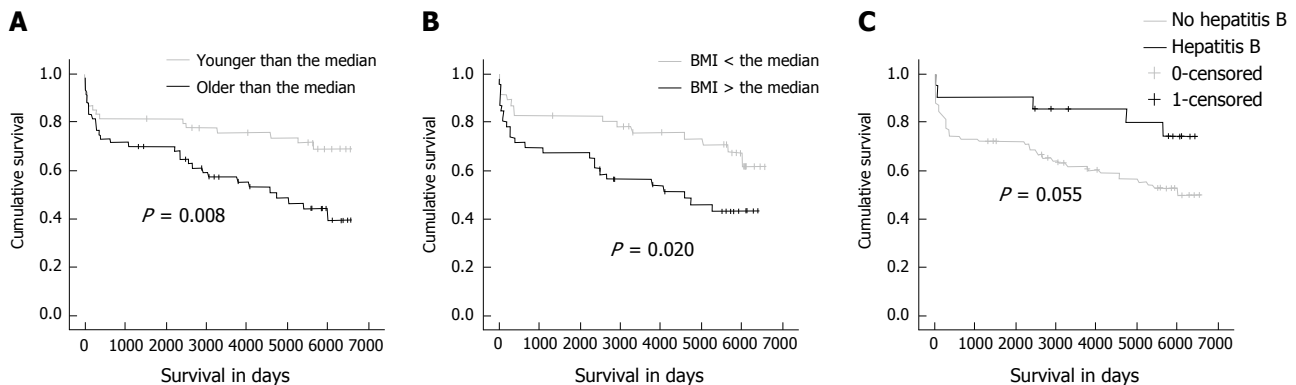
### Association between graft survival and age

Patients with an older age at the time of transplantation had a significantly worse graft survival, compared to patients younger than the median (Figure 4). This was confirmed by  $\chi^2$  test (*P* = 0.017) and Mann-Whitney test (*P* = 0.017). Looking at the subgroup of patients older than 60 years, there was a significantly lower graft survival according to Kaplan-Meier survival analysis (*P* = 0.05) but not according to the  $\chi^2$ -test. Donor's age was not related to graft survival in this study (*P* = ns). There was no significant association between patients

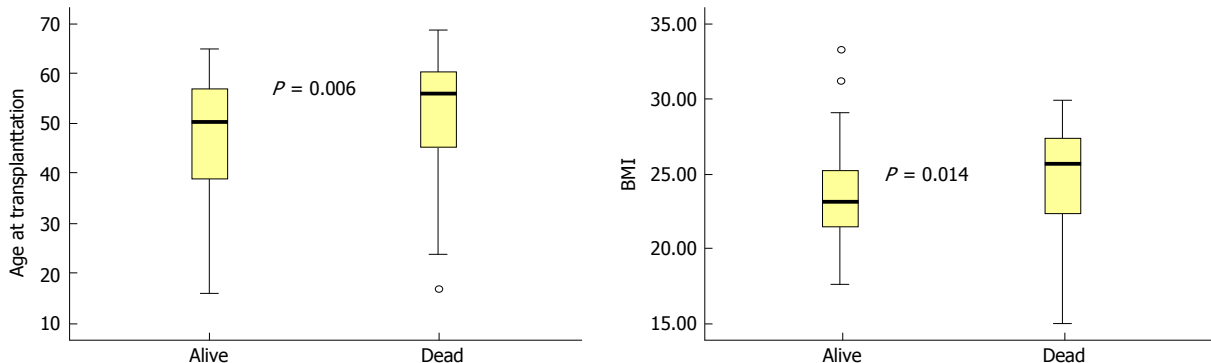
**Table 2** Comparison of survival according to age, body mass index and hepatitis B virus status in a Eurotransplant control cohort ( $n = 2973$ )<sup>1</sup>

	Patients who survived $n$ (%)	Patients who died $n$ (%)	$P$ -value ( $\chi^2$ test)
Age below 53 yr ( $n = 1809$ )	1156 (64)	653 (36)	
Age above 53 yr ( $n = 1145$ )	520 (45)	625 (55)	$< 0.001$
BMI below 24 kg/m <sup>2</sup> ( $n = 1454$ )	880 (61)	574 (39)	
BMI above 24 kg/m <sup>2</sup> ( $n = 1493$ )	796 (53)	697 (47)	$< 0.001$
Hepatitis B as underlying disease ( $n = 255$ )	170 (67)	85 (33)	
Non-hepatitis B patients ( $n = 1705$ )	946 (55)	759 (45)	$< 0.001$

<sup>1</sup>Data for age, BMI and hepatitis B virus status were not available for the total cohort. BMI: Body mass index.



**Figure 2** Kaplan-Meier survival analysis reveals increased survival for patients younger than the median (53 years) (A), with body mass index lower than the median (24 kg/m<sup>2</sup>) (B) and hepatitis B as underlying disease (C).



**Figure 3** Age and body mass index at the time point of transplantation were higher in deceased patients in comparison to patients who survived.

who survived more than 1 year and had age above the median ( $\chi^2$  test  $P = 0.498$ ).

#### Association of patient survival and BMI

Patients with a BMI above the median (24 kg/m<sup>2</sup>) displayed a higher mortality than patients with a BMI below the median ( $P = 0.029$ ). This reduced survival rate was confirmed by the Kaplan-Meier analysis ( $P = 0.020$ ; Figure 2). Additionally, BMI at the time of transplantation was higher in patients who died within 15 years of follow-up in comparison to patients who survived ( $P = 0.014$ ; Figure 3). This was confirmed in the Eurotransplant control cohort ( $n = 2971$ ). Patients with a BMI below 24 kg/m<sup>2</sup> showed an improved survival rate in comparison with patients with a BMI above this threshold ( $P \leq 0.001$ ).

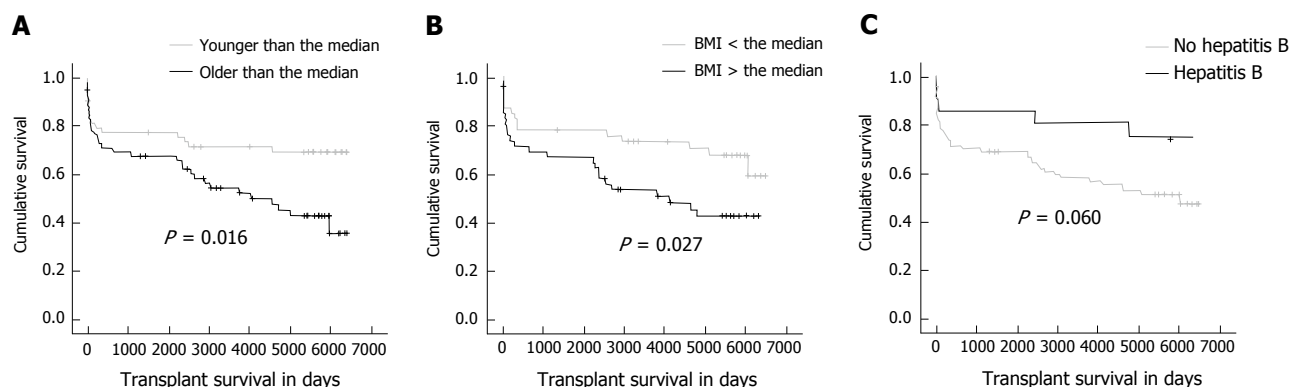
In detail, 61% with a BMI below 24 kg/m<sup>2</sup> survived, while 53% with a BMI above 24 kg/m<sup>2</sup> survived (Table 2).

A sub-analysis of patients with severe obesity and a BMI above 30 kg/m<sup>2</sup> was not possible as only two patients fulfilled this criterion.

There was no significant association between patients who survived more than 1 year and had BMI above the median ( $\chi^2$  test  $P = 0.449$ ). Notably, there was no significant association between age and BMI of the recipient ( $R = 0.114$ ,  $P = 0.278$ ), so that BMI seemed to be independent of age. Unfortunately, a multivariate analysis did not confirm BMI as an independent factor associated with decreased survival; perhaps, significance was missing due to the limited number of factors.

In contrast, the BMI of the donor was not associated





**Figure 4** Kaplan-Meier survival analysis reveals increased transplant survival for patients younger than the median (53 years) (A), with body mass index lower than the median (24 kg/m<sup>2</sup>) (B) and hepatitis B as underlying disease (C). BMI: Body mass index.

with survival of the recipient ( $P = \text{ns}$ ).

#### Association of graft survival and BMI

Patients having a BMI above the median (24 kg/m<sup>2</sup>) had a significantly worse graft survival, compared to patients with a BMI lower than the median ( $\chi^2$  test: 0.009, Mann-Whitney test: 0.047). On the other hand, in this study, donor's BMI did not have an influence on graft survival.

#### Association of patient and graft survival with the underlying liver diseases

The only underlying aetiology of cirrhosis which was statistically significantly associated with outcome was hepatitis B. Patients with hepatitis B as an underlying disease tended to have an improved patient survival in comparison to patients with other underlying diseases ( $P = 0.049$  in the categorical analysis and  $P = 0.055$  in the Kaplan-Meier analysis; Figure 2C). Three out of 21 liver transplant recipients with hepatitis B suffered from acute, fulminant hepatitis B, leading to acute liver failure and transplantation, while the majority ( $n = 18$ ) had been transplanted due to chronic hepatitis B with cirrhosis. Regarding the BMI, there was no difference between HBV-positive and HBV-negative patients ( $t$ -test, 2-sided, unequal variance,  $P = 0.38$ ), so that other reasons must be responsible for the survival benefit.

All HBV-positive liver transplant recipients received intravenous immunoglobulins, hepatitis B immune globulin (HBIG), to avoid reinfection of the graft.

In addition to patient survival, graft survival of patients with hepatitis B as underlying disease was also improved, compared to patients with other diagnoses ( $\chi^2$  test: 0.018, Mann-Whitney test: 0.018). The Euro-transplant control cohort confirmed that patients with hepatitis B had an improved survival in comparison to the remaining patients (Table 2).

#### Remaining factors

Neither recipient's laboratory parameters prior to transplantation (ALT, gamma-GT, bilirubin, creatinine) nor warm ischemia time or cold ischemia time influenced

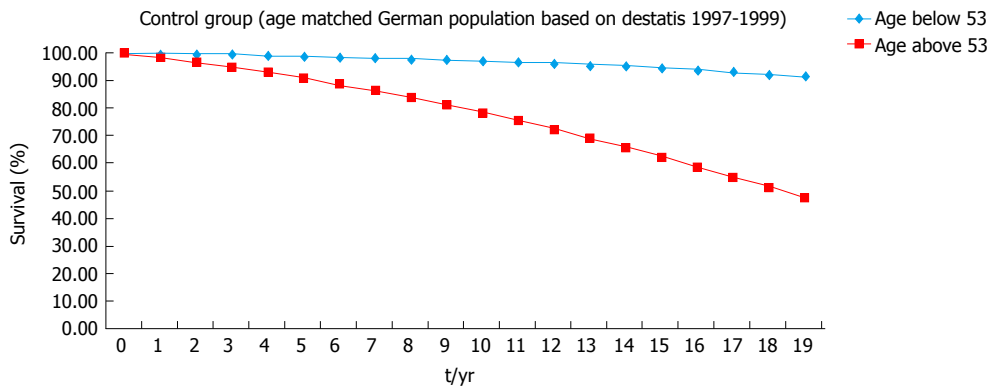
patient survival significantly.

## DISCUSSION

In the current situation of tremendous organ shortage, it is important to identify patients who benefit most from a liver transplantation and also to detect risk factors associated with poor outcome. The main findings of this study were that recipients' age and BMI are relevant for prediction of long-term patient survival as well as graft survival. Interestingly, neither other recipient factors such as bilirubin, creatinine, ALT nor donor factors, such as age and BMI, were associated with decreased survival. Another interesting finding was that OLT recipients with hepatitis B as underlying disease had improved survival rates.

The association of recipients' age and BMI and patient and graft survival was proven by univariate analysis for both factors. However, in multivariate analysis only age remained a significant predictor. On the other hand, it was unexpected that there was no significant association between survival of recipient's and donor's age and BMI. This finding is in contrast to numerous previous studies which demonstrated a significantly decreased survival in recipients of older donations within a large ET-DRI study<sup>[8]</sup>. Recently, a large analysis of more than 41000 liver transplant recipients receiving a donation after circulatory death showed that recipients of livers from donors with an age below 50 years had a higher survival rate, compared to recipients of livers from donors with an age above 60. However, several studies indicated that older grafts can be used safely with a careful selection of patient and donor in the majority of cases<sup>[9-13]</sup>. Based on the published literature, strict recommendations for the acceptance or refusal of potential liver donors cannot be made. The authors concluded that careful donor organ and recipient selection can lead to excellent results<sup>[14]</sup>.

In contrast to donor's age, our study highlighted the value of recipient's age as a predictor of survival. We identified a threshold of 53 years for recipient's age and a BMI of 24 kg/m<sup>2</sup> as relevant risk factors. These findings were confirmed in the analysis of the Eurotransplant



**Figure 5** Percentage of survival in an age-matched control of the German general population (age range: 18-67 years). This age-matched control cohort was constructed based on historical data about the German healthy population (<https://www.destatis.de>).

cohort of 2971 patients. Perhaps a larger cohort might also confirm a relevant aspect of donor age on survival. However, our study did not find such an association.

Within a previous German study with a follow-up period of 20 years and 313 liver transplant recipients, the survival of elderly transplant recipients (> 55 years) was reduced within the first year after transplantation, but long-term survival was similar to the general population<sup>[2]</sup>. Our observation that there is a relevant difference regarding survival between OLT recipients above and below the median age of 53 years (Figure 2A) is well in line with this study. However, we could not find a significant association between 1-year patient survival and age or BMI above the median. Therefore, these factors might be associated with long-term but not with short-term survival. Further studies are needed to elucidate this aspect.

Earlier studies showed inconsistent results concerning BMI and survival. A study by Fujikawa *et al.*<sup>[15]</sup> investigated the impact of obesity on clinical and financial outcome after liver transplantation and showed no influence on either patient survival or hospital costs. Also, it is conceivable that obese recipients were selected more carefully with respect to other risk factors. In contrast, the study by Rustgi *et al.*<sup>[16]</sup> observed a worse survival rate in patients having a BMI > 35. Our study confirms the finding that a higher BMI of the recipient is associated with a decreased survival. Only three of the patients in our study displayed malnutrition with a BMI < 18; thus, no interpretation of a possible effect of malnutrition and survival was possible for our cohort.

In order to strengthen our data, we compared the survival rate of our patients (younger or older than the median of 53 years) with two control groups (as described in the methods). There were no significant differences between all three groups (Figure 5 and Table 2). However, these are hypothetical control cohorts and more detailed statistical analyses were not possible.

Three independent statistical tests (Kaplan-Meier survival analysis/Log rank,  $\chi^2$  test, Mann-Whitney test) confirmed the association between recipient's age or BMI and decreased patient and graft survival rates. However,

there was no correlation between age and BMI indicating that these factors are independently associated with lower survival. Unfortunately, a multivariate analysis makes no sense due to the low number of significant factors in the univariate analysis. It is not surprising that older or overweight patients depict a shorter survival. This has been a well-known fact for many years.

Interestingly, hepatitis B was associated with an improved long-term patient survival in our cohort. This should be interpreted carefully as there are only 21 HBV patients in our study population. However, this observation might be due to the regularly applied immunoglobulin preparations, HBIG, that these patients still get at our institution<sup>[17-19]</sup>. However, currently this is only one hypothetical explanation of the observed survival benefit of hepatitis B patients.

In addition, our study cohort analysis of the Euro-transplant control cohort also shows an increased survival for transplant recipients with underlying hepatitis B in comparison to the remaining patients ( $P < 0.001$ ). This observation is in line with an analysis of the survival of liver transplant recipients with hepatitis B, based on the European liver transplant registry<sup>[20]</sup>. Within this study investigating the outcome of liver transplant recipients with hepatitis B as underlying disease within a period of approximately 20 years (1988-2010), it could be shown that the survival of HBV-positive transplant recipients strongly improved within these 2 decades<sup>[20]</sup>. This has been assumed to be caused by the prevention of hepatitis B re-infection by immunoglobulins<sup>[20]</sup>. However, this hypothesis still needs to be confirmed by further studies.

The results of this study might be helpful to identify patients with better chances of long-term survival. Our overall 15-year patient survival rate (Figure 1) of 58% is well in line with previous reports depicting a 20-year survival rate of approximately 50% after liver transplantation<sup>[2,3]</sup>. However, in the current era of model of end-stage liver disease (MELD)-allocation, which favours the sickest patients, such survival rates might not be met in future studies. Upcoming studies are needed to investigate not only short but also long-term survival of patients who received a liver transplantation in the

MELD-era. Perhaps the MELD score is a valuable tool for identifying the sickest patients, but it might not be the best predictor of long-term outcome. Furthermore, according to previous studies, it has been shown that prognosis of the patient is far more related to clinical parameters than laboratory data<sup>[17]</sup>. The study of Aloia *et al.*<sup>[18]</sup> also showed a decreased value of the MELD score in contrast to parameters such as ventilator status, diabetes mellitus, HCV, creatinine levels and recipient's and donor's age.

Our study has some limitations. It is based on patients who underwent liver transplantation in the pre-MELD era and at a time when less patients received organs with extended donor criteria. Furthermore, the number of patients with HCC was only 4% (5/114, 4%). In our study, at present these numbers are much higher.

Unfortunately, multivariate analysis of our data was prone to errors due to the small number of patients in comparison to the multiple variables. Thus, it can be said that the analysed cohort was too small for the investigation of the variables. This was a retrospective analysis and, therefore, there is some lack of information considering the long time period of observation (15-17 years). However, there are not many studies dealing with such long-term data as presented in this collective. In the future, more research, especially on the potential influence of immunoglobulins on the HBV patient's outcome, is necessary.

In conclusion, age and BMI of OLT recipients were predictors of long-term survival, while pre-transplant bilirubin, creatinine, ALT and gamma-GT were not associated with patient survival or graft survival (pre-MELD era). Age and BMI of the donor had no relevant influence on patient or graft survival in this cohort. OLT recipients with hepatitis B as underlying disease displayed an improved survival. The relevance of this observation still needs to be determined.

## ACKNOWLEDGMENTS

We thank Eurotransplant for providing data for the control cohort of 2971 patients.

## COMMENTS

### Background

Predictive factors associated with long-term patient and graft survival (> 15 years) in liver transplant recipients are not well defined. This study evaluates the possible association between various factors and survival.

### Research frontiers

The role of age and body mass index (BMI) for the outcome of liver transplant recipients still needed to be shown.

### Innovations and breakthroughs

This is the first study demonstrating a relevant association between age above 53 years or a BMI above 24 kg/m<sup>2</sup> with decreased graft survival. These thresholds were confirmed in an independent large Eurotransplant cohort to be associated with decreased graft survival. Furthermore, there was a weaker association between underlying hepatitis B and improved graft survival. The

pathological mechanism and relevance of this finding still needs to be shown.

## Applications

Future studies will focus in detail on patients with an age above 53 years or a BMI above 24 kg/m<sup>2</sup> to verify the authors' findings. If their data can be confirmed, this will help transplant physicians worldwide to predict the risk of liver transplant recipients.

## Terminology

Liver transplant recipients and their survival as well as graft survival, defined as period until death or re-transplantation were studied.

## Peer-review

Pischke *et al* analyzed the clinical data of the patients who underwent liver transplantation during 1997 to 1999. This article is interesting.

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Observational Study

## Concordance of non-invasive mechanical and serum tests for liver fibrosis evaluation in chronic hepatitis C

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### Abstract

#### AIM

To determine the sensitivity and specificity of liver stiffness measurement (LSM) and serum markers (SM) for liver fibrosis evaluation in chronic hepatitis C.

#### METHODS

Between 2012 and 2014, 81 consecutive hepatitis C virus (HCV) patients had METAVIR score from liver biopsy compared with concurrent results from LSM [transient elastography (TE) [FibroScan®/ARFI technology (Virtual Touch®)] and SM [FIB-4/aspartate aminotransferase-to-platelet ratio index (APRI)]. The diagnostic performance of these tests was assessed using receiver operating characteristic curves. The optimal cut-off levels of each test were chosen to define fibrosis stages  $F \geq 2$ ,  $F \geq 3$  and  $F = 4$ . The Kappa index set the concordance analysis.

#### RESULTS

Fifty six percent were female and the median age was 51 years (30-78). Fifty-six patients (70%) were

treatment-naïve. The optimal cut-off values for predicting  $F \geq 2$  stage fibrosis assessed by TE were 6.6 kPa, for acoustic radiation force impulse (ARFI) 1.22 m/s, for APRI 0.75 and for FIB-4 1.47. For  $F \geq 3$  TE was 8.9 kPa, ARFI was 1.48 m/s, APRI was 0.75, and FIB-4 was 2. For  $F = 4$ , TE was 12.2 kPa, ARFI was 1.77 m/s, APRI was 1.46, and FIB-4 was 3.91. The APRI could not distinguish between F2 and F3,  $P = 0.92$ . The negative predictive value for  $F = 4$  for TE and ARFI was 100%. Kappa index values for  $F \geq 3$  METAVIR score for TE, ARFI and FIB-4 were 0.687, 0.606 and 0.654, respectively. This demonstrates strong concordance between all three screening methods, and moderate to strong concordance between them and APRI (Kappa index = 0.507).

## CONCLUSION

Given the costs and accessibility of LSM methods, and the similarity with the outcomes of SM, we suggest that FIB-4 as well as TE and ARFI may be useful indicators of the degree of liver fibrosis. This is of particular importance to developing countries.

**Key words:** Elastography; Serum markers; Hepatitis C virus; Liver stiffness; Liver biopsy

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**Core tip:** Liver fibrosis evaluation in hepatitis C virus (HCV) patients has critical impact on prognosis and treatment strategies. Despite liver biopsy (LB) remains the gold standard for its evaluation, non invasive methods has improved in recent years. We evaluated 81 HCV patients with elastography methods [Fibroscan and acoustic radiation force impulse (ARFI)] and serum markers (APRI and FIB-4) compared to LB, and found that Fibroscan, ARFI, and FIB-4 independently identify advanced fibrosis. We suggest that FIB-4 alongside Fibroscan and ARFI may be good tools for the prediction of severity of liver fibrosis. This may be of particular importance to developing countries.

Paranaguá-Vezozzo DC, Andrade A, Mazo DFC, Nunes V, Guedes AL, Ragazzo TG, Moutinho R, Nacif LS, Ono SK, Alves VAF, Carrilho FJ. Concordance of non-invasive mechanical and serum tests for liver fibrosis evaluation in chronic hepatitis C. *World J Hepatol* 2017; 9(8): 436-442 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i8/436.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i8.436>

## INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most frequent etiologies of cirrhosis, and is therefore responsible for most of its complications, including hepatocellular carcinoma, which is the sixth most common cancer worldwide<sup>[1]</sup>. Despite the recent advances in HCV therapy, the prevalence of advanced liver disease will continue to

increase as well as the corresponding healthcare burden<sup>[2]</sup>. In Brazil, although the hepatitis C viremic prevalence is about 1%, only 15% of the estimated infected patients are diagnosed, usually with advanced fibrosis. This is partly explained by the scarcity of specialist centers compared with the societal needs. Of those who are diagnosed, only 60% receive specialized treatment<sup>[3,4]</sup>.

The stage of liver fibrosis in HCV patients is associated with prognosis, and has a resulting impact on treatment strategy and follow-up. Liver biopsy (LB) is still the gold standard procedure for fibrosis assessment, but non-invasive new approaches have been strongly recommended for evaluation of fibrosis, mainly in HCV. They require less operator expertise, have no complications and have good diagnostic accuracy<sup>[5-7]</sup>. The most extensively used non-invasive mechanical methods based on ultrasound are transient elastography (TE or FibroScan<sup>®</sup>) and acoustic radiation force impulse (ARFI) technology, Virtual Touch<sup>®</sup>. There are several laboratorial markers in development, and validated scores such as aspartate aminotransferase-to-platelet ratio index (APRI) and FIB-4 [based on age, platelet count, aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], that are easily calculated with routine laboratory tests.

Use of liver biopsy has decreased following the introduction of non-invasive tests, especially among chronic HCV patients<sup>[8]</sup>. Although, according to the recently published EASL Guideline for the evaluation of HCV patients, a perfect marker (AUROC > 0.90) for liver disease could not be achieved, the use of non-invasive tests reduce, but do not abolish the need for liver biopsy<sup>[8]</sup>.

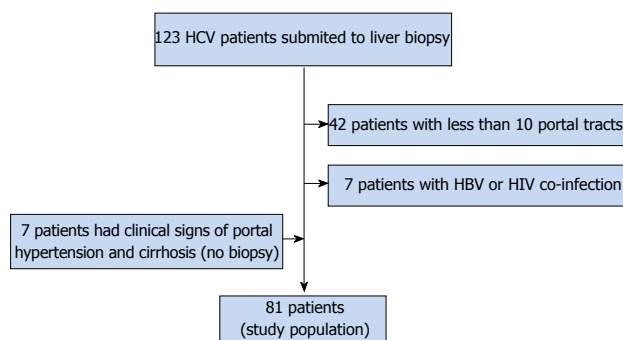
Many biological tests, including APRI, PGA index, Forns' index, Fibrotest, FIB-4 and Hepascore have been compared with TE and/or ARFI and LB for initial evaluation of liver fibrosis in HCV patients<sup>[9]</sup>. They have compared favorably, although some are difficult to calculate and others use specialized expensive commercially produced markers. However, APRI and FIB-4 are more accessible and easier to apply than others. As TE and ARFI show a representative result of different parts of the liver, accuracy studies have been developed to evaluate performance compared to LB, which evaluates only a small sample of the liver. As the best cut-off points of each fibrosis stage varies according to different cirrhosis etiologies and populations, these cut-off points need to be validated.

The aim of this study is to identify optimal cutoff values for TE, ARFI, APRI and FIB-4 compared with LB in a Brazilian HCV cohort, according to levels of significant fibrosis ( $F \geq 2$ ), advanced fibrosis ( $F \geq 3$ ) and cirrhosis ( $F = 4$ ).

## MATERIALS AND METHODS

### Ethical considerations

The Ethics Committee of the Hospital das Clínicas (CAPPesq number 1276/09) reviewed and approved this study,



**Figure 1** Flowchart of study population enrollment. HCV: Hepatitis C virus; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus.

that was conducted following the ethical guidelines of the 1975 Declaration of Helsinki. The requirement for informed written consent was waived.

### Study design

We performed an observational study of diagnostic accuracy for TE, ARFI, APRI and FIB-4 compared with LB. Between 2012 and 2014, 123 consecutive HCV patients followed by Hepatology Outpatient Center of Hospital das Clínicas, University of São Paulo School of Medicine, Brazil, that would be submitted to liver biopsy had liver stiffness measurement (LSM) [FibroScan®, EchoSens, Paris, France]/ARFI, Siemens AG, Erlangen, Germany) and serum markers (SM) [FIB-4/APRI] exams done, in order to compare the data with METAVIR score.

Inclusion criteria were: (1) HCV polymerase chain reaction (PCR) RNA positivity for at least 6 mo, and clinical or histopathological diagnosis of chronic HCV; and (2) representative liver biopsy (minimum of 10 portal spaces, non subcapsular fragment) carried out until 30 d prior to LSM and SM. Exclusion criteria were: (1) patient under 18 years of age; (2) hepatitis B virus (HBV) or human immunodeficiency virus (HIV) co-infection; (3) other chronic liver disease (cholestasis, non-alcoholic steatohepatitis, autoimmune hepatitis, hemochromatosis, Wilson's disease); (4) decompensated cirrhosis; (5) biopsies performed for more than 30 d of the evaluation; and (6) non-representative liver biopsy.

Results from TE and ARFI® were blinded for the results from LB. FibroScan® and ARFI were performed by an experienced ultrasonographer with more than 80000 liver ultrasounds, more than 2000 FibroScan® and more than 2000 ARFIs.

Forty-nine patients were excluded, as shown in Figure 1. Seven patients without LB, but with clinical signs of portal hypertension and cirrhosis (Metavir F = 4) were included. In the end, 81 were selected for the study. Three patients had hepatocellular carcinoma, with less than 2 cm. They were included in the study.

### Clinical and biological data

Anthropometric, clinical and laboratorial data were collected: Gender, age, weight, height, body mass index (BMI), smoking status, alcohol consumption, hypertension, diabetes, dyslipidemia, and serum enzymes such as AST,

ALT, bilirubin, albumin, glucose levels and platelet count, all taken from medical charts.

### Transient elastography

LSM were performed using the FibroScan® 402 device powered by VCTE (EchoSens, Paris, France), equipped with the standard M probe. The examination procedure have been previously described<sup>[9-11]</sup>. A valid LSM examination included 10 valid measurements, a success rate of 70%, and an interquartile range of measurements (IQR) below 30% of the median value. Controlled attenuation parameter (CAP) was also evaluated.

### ARFI

ARFI technology measures the shear wave speed in a precise anatomical region, with a predefined size, provided by the system. Measurement value and depth are also reported and elasticity results are represented in m/s<sup>[12]</sup>. ARFI elastography was performed using a Siemens Acuson S2000® ultrasound system, a Virtual Touch® quantification elastography technology (Siemens AG, Erlangen, Germany). The patients were examined in dorsal decubitus, with the right arm in maximum abduction. Scans were performed in a right inferior intercostal space over the right liver lobe (e.g., segment 8), 2 cm under the capsule, with minimal scanning pressure applied by the operator, while patients were asked to stop breathing temporarily. Ten measurements per patient were performed and a median and IQR values were calculated by the machine. Only when an IQR 30% was reached was the median value accepted.

### APRI and FIB-4

APRI and FIB-4 were calculated through the following scores: APRI score = {[AST/upper limit of normal (ULN)] 100}/platelet count 10<sup>9</sup>/L. FIB-4 score = {[age (yr) × AST (U/L)] / [platelet count (10<sup>9</sup>/L) × ALT (U/L)]}.

### LB

LB was performed in all but 7 patients, who had clinical or ultrasonographic signs of portal hypertension and cirrhosis. They were judged to have Metavir F4 histology. The LB was guided with 14 G - TruCut needle (Medical Technology, Gainesville, FL, United States). LB fragments including at least 10 portal tracts were considered adequate for pathological interpretation, and were included in our study. Liver specimens were fixed in formalin and embedded in paraffin. Two micron sections were stained with hematoxylin-eosin, Masson's trichrome and Sirius red for histological assessment. The liver biopsies were assessed according to the METAVIR score, by a senior pathologist and classified as: F0 - no fibrosis; F1 - portal fibrosis without septa; F2 - portal fibrosis and few septa extending into lobules; F3 - numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4 - cirrhosis.

### Statistical analysis

Statistical analyses were performed by using R statistics

**Table 1** Demographic, laboratory and liver fibrosis characteristics

Characteristics	Patients (n = 81)
Gender (male/female)	40 (49.4%)/41 (50.6%)
Age (yr) - median (Q1;Q3)	51 (30-78)
BMI (kg/m <sup>2</sup> ) - median (Q1;Q3)	26.5 (24.3-29.6)
ALT (IU/L) - median (Q1;Q3)	50 (32.5-85)
AST (IU/L) - median (Q1;Q3)	42 (28.5-61.5)
Platelet count ( $\times 10^3/\text{mm}^3$ ) - median (Q1; Q3)	202 (148-247)
Histological fibrosis stage, n (%)	
F0	5 (6.2)
F1	33 (40.7)
F2	20 (24.7)
F3	12 (14.8)
F4	11 (13.6)
TE (kPa) - median (Q1; Q3)	6.9 (5-12.2)
TE success rate (mean $\pm$ SD)	0.92 $\pm$ 0.21
CAP (dB/m) - mMedian (Q1; Q3)	237 (204-263)
ARFI (m/s) - median (Q1; Q3)	1.25 (0.65-2.89)
APRI - median (Q1; Q3)	0.66 (0.41-1.29)
FIB-4 - median (Q1; Q3)	1.41 (0.90-2.45)

BMI: Body mass index; ALT: Alanine amino transferase; AST: Aspartate amino transferase; TE: Transient elastography; CAP: Controlled attenuation parameter; ARFI: Acoustic radiation force impulse; APRI: Score index (AST/LSN AST/platelet); FIB-4: Score index (age  $\times$  AST/platelet  $\times$  ALT).

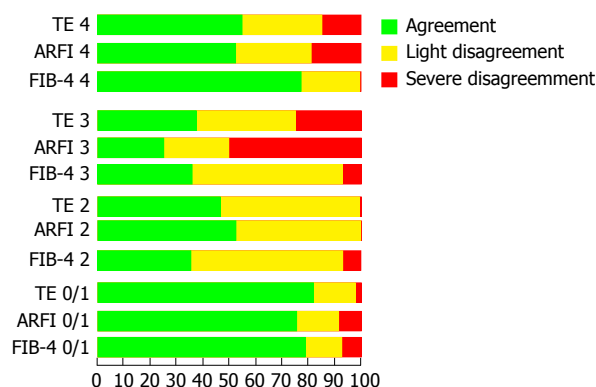
version 3.2.5. (R Core Team, Vienna, Austria). The STARD Statement guidelines were followed. Quantitative characteristics were expressed as mean (SD), median (first and third quartile) and range. Variables were compared using non-parametric Wilcoxon test.  $P$  value  $< 0.05$  was considered significant.

The ANOVA or Kruskal-Wallis tests were used for comparison of two or more groups, whether or not the data were normally distributed, respectively. We set that mild disagreement was when only one class was different, and severe disagreement when two or more class were wrongly misclassified.

The diagnostic performance of FibroScan<sup>®</sup>, ARFI, APRI and FIB-4 tests was assessed using receiver operator curves (ROC). The optimum cut-off levels, defined as area under ROC (AUROC), of each test was chosen to define fibrosis stages  $F \geq 2$ ,  $F \geq 3$  and  $F = 4$ . The Kappa index set the concordance analysis. The best sensitivity values ( $> 80\%$ ) have been chosen in order to identify all HCV patients with METAVIR  $F \geq 3$  (prioritized for treatment according to Brazilian Ministry of Health recommendations)<sup>[13]</sup>. Positive and negative predictive values (PPV and NPV) were calculated using the prevalence of liver fibrosis stages ( $F > 2$ ) in the Hepatology Outpatient Center from Hospital das Clinicas of the University of Sao Paulo School of Medicine, Brazil. A statistical review of the study was performed by a biomedical statistician (João Ítalo França).

## RESULTS

A total of 81 patients with HCV were included, 41 (50.6%) were female. Anthropometric and laboratorial chara-



**Figure 2** Rate of agreement of transient elastography, acoustic radiation force impulse, aspartate aminotransferase-to-platelet ratio index and FIB-4 according to Metavir fibrosis stage (%). TE: Transient elastography; ARFI: Acoustic radiation force impulse; FIB-4: Score index (age  $\times$  AST/platelet  $\times$  ALT).

cteristics are shown in Table 1. The median age was 51 years (30-78). Eleven (13.6%) patients had diabetes, 22 (27.2%) hypertension, 20 (24.7%) were smokers and 11 (13.6%) consumed alcohol ( $> 20$  g/d). The median BMI was 26.5 (24.3-29.6), and 70% of the patients were HCV treatment-naïve. Most of the patients had Metavir F1 on LB fibrosis stage (33 patients, 40.7%), followed by F2 (20 patients, 24.7%). The mean success rate of TE was 92%.

The best cut-off values of each test (LSM and SM) are found in Table 2. For predicting  $F \geq 2$  stage fibrosis with TE was 6.6 kPa, for ARFI 1.22 m/s, for APRI 0.75 and for FIB-4 1.47. For  $F \geq 3$ , TE was 8.9 kPa, ARFI was 1.48 m/s, APRI was 0.75, and FIB-4 was 2. For  $F = 4$ , TE was 12.2 kPa, ARFI was 1.77 m/s, APRI was 1.46, and FIB-4 was 3.91. The APRI could not distinguish between F2 and F3 ( $P = 0.92$ ). The NPV for  $F = 4$  for TE and ARFI was 100%. Kappa Index values for  $F \geq 3$  METAVIR score for TE, ARFI and FIB-4 were 0.687, 0.606 and 0.654, respectively. This demonstrates strong concordance between the TE, ARFI and FIB-4 methods, but moderate concordance between them and APRI (Kappa index = 0.507). Figure 2 shows the rate of agreement of TE, ARFI, APRI and FIB-4 according to Metavir stage on LB. Since alcohol consumption and severity of liver inflammation could affect TE measurements, patients were also analyzed individually. Of the 11 patients with alcohol consumption, 2 patients had discordant results between TE and liver biopsy. One had Metavir F1A1 and TE of 10 kPa and the other had Metavir F1A2 and TE of 16.3 kPa. With regard to patients with more intense inflammatory activity on hepatic biopsy (Metavir A3-4), we had 6 patients with Metavir A3, and none with Metavir A4. Of these Metavir A3 patients, 5 were Metavir F3 and 1 had cirrhosis. TE discordance with liver biopsy could be found in all Metavir F3A3 patients, with overestimation of TE results (mean TE results: 25.3 kPa). One Metavir F3A3 patient had ALT of 148 U/L and a TE of 28.4 kPa. All other patients with Metavir F3A3 had ALT results between 32 and 86 U/L.



**Table 2** Summary of cut-off values, area under de curve, sensitivity, specificity, positive predictive value, negative predictive value and accuracy

Method	AUC	AUC CI (95%)	Se	Sp	PPV	NPV	Accuracy
TE (kPa)							
> F2 (6.6)	0.8716	0.7953-0.948	82.90%	77.50%	89.30%	66.70%	80.20%
> F3 (8.9)	0.9187	0.8319-1.000	87%	86.20%	87.30%	85.90%	86.40%
= F4 (12.2)	0.9675	0.9321-1.000	100%	87.10%	79.30%	100%	88.90%
ARFI (m/s)							
> F2 (1.22)	0.7701	0.6653-0.8749	78%	70%	85.50%	58.40%	74.10%
> F3 (1.48)	0.8669	0.7756-0.9583	82.60%	82.80%	83.90%	81.40%	82.70%
= F4 (1.77)	0.9188	0.8592-0.9784	100%	85.70%	77.50%	100%	87.70%
APRI							
> F2 (0.75)	0.8107	0.7136-0.9077	75.60%	87.50%	93.20%	61.30%	81.50%
> F3 (0.75)	0.8272	0.7140-0.9405	87%	72.40%	77.40%	83.60%	76.50%
= F4 (1.46)	0.9143	0.8387-0.9899	81.80%	90%	80.10%	91%	88.90%
FIB-4							
> F2 (1.47)	0.8652	0.7844-0.9461	78%	82.50%	91%	62.40%	80.20%
> F3 (2.0)	0.8703	0.7634-0.9773	82.60%	86.20%	86.70%	82%	85.20%
= F4 (3.91)	0.9636	0.9211-1.000	90.90%	95.70%	91.30%	95.50%	95.10%

AUC: Area under de curve; CI: Confidence interval; Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value.

## DISCUSSION

Our results show that three methods, ARFI, TE and FIB-4, independently identify advanced fibrosis. Non-invasive methods have been studied and compared to other methods of liver fibrosis evaluation in order to diminish complications of liver biopsy and costs involved<sup>[8]</sup>. We evaluated the AUROC and the inter-agreement of LSM (TE and ARFI) as well as SM (APRI and FIB-4) compared with liver biopsy in a population of HCV-infected patients. The best cut-offs were established based on METAVIR fibrosis stages  $F \geq 2$ ,  $F \geq 3$  and  $F = 4$ , considering not only the recent international consensus (EASL-ALEH 2015) but the recommendation for HCV treatment in Brazil, which prioritize  $F \geq 3$  patients according to the 2015 Brazilian Protocol for HCV treatment<sup>[4,8,13]</sup>. The results of this study do not conflict with previous findings (Table 2). The TE sensitivity (Se) and specificity (Sp) in a recent FibroScan® meta-analysis<sup>[14]</sup> ranged from about 0.70 and 0.81 for  $F \geq 2$ , 0.80 and 0.85 for  $F \geq 3$ , and from 0.86 and 0.88 for  $F = 4$ . These are similar to our results. Although TE had better results for  $F \geq 2$ , the overall accuracy for ARFI and TE were comparable, as previously demonstrated by Crespo *et al.*<sup>[15]</sup>. For the F4 group we found an almost perfect correlation between ARFI, TE and FIB-4, suggesting that only one method is sufficient to identify cirrhosis. It is important to note that on 2 out of 11 patients who reported alcohol consumption ( $> 20$  g/d), TE values were overestimated. The influence of alcohol intake on liver stiffness measurement should be taken into account when interpreting TE results, as shown by Bardou-Jacquet *et al.*<sup>[16]</sup>. Hepatic inflammation can also be a confounding factor when evaluating liver fibrosis by TE<sup>[17]</sup>. We could demonstrate that all Metavir F3A3 patients had overestimation of liver fibrosis by TE, with median values of 25.3 kPa.

APRI is a good reproducible marker of cirrhosis, with a high applicability ( $> 95\%$ ), it is easy to perform and

is a non-patented score<sup>[18]</sup>. In our study APRI could not differentiate between  $F = 2$  and  $F = 3$ . This is possibly because it uses fewer variables than FIB-4. APRI uses AST and platelet count, while FIB-4 also incorporates ALT and age of the patient.

The F2 group is less clearly defined than other stages of fibrosis, as shown in the literature by Rizzo *et al.*<sup>[19]</sup>, and all methods identified it less accurately. In our study however, there was less disagreement than Afdhal *et al.*<sup>[20]</sup> which shows that for F2 group, application of both methods, TE and ARFI are necessary to identify these patients.

Although LB is the reference standard, its reproducibility is poor, owing to heterogeneity in liver fibrosis, operator bias and sample size. This can account for an margin of error of up to 20% in disease staging<sup>[20]</sup>.

A limitation of this study is that it identifies and selects cut-off points, but is not prospectively validated, warranting further studies to confirm these results. However, from this study, we can consider that a combined use of FibroScan® and FIB-4 or FibroScan® and ARFI in the follow-up of HCV patients can be a surrogate for fibrosis assessment through LB, which can be held in reserve for cases with significant diagnostic doubt. This is especially important in the intermediate stages of fibrosis (F2 and F3), where each individual non-invasive method is not sufficiently accurate to make a diagnosis, and so should be performed in combination. Further studies are necessary to identify whether they should be performed simultaneously or in parallel, and identify the best cutoffs for each combination of methods.

In conclusion, given the higher cost and reduced accessibility of LSM methods, and the similarity with the outcomes of SM in evaluation of liver fibrosis, we suggest that FIB-4 used alongside TE and ARFI may be good tools for the prediction of severity of liver fibrosis. This may be of particular importance to developing countries.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The evaluation of liver fibrosis is not a simple task and demands the use of different methods. Liver stiffness measurement (LSM) and serum markers (SM) provide a non-invasive source of diagnosis with good correlation with the gold standard method, liver biopsy.

### Research frontiers

The F2 group is less clearly defined than other stages of fibrosis. In the authors' study there was however less disagreement, which shows that for F2 group, application of both methods, transient elastography (TE) and acoustic radiation force impulse (ARFI) is necessary to identify these patients. Further studies are necessary to identify whether they should be performed simultaneously or in parallel, and identify the best cutoffs for each combination of methods.

### Innovations and breakthroughs

This results show that three methods, ARFI, TE and FIB-4, independently identify advanced fibrosis.

### Applications

Given the higher cost and reduced accessibility of LSM methods, and the similarity with the outcomes of SM in evaluation of liver fibrosis, the authors suggest that FIB-4 used alongside TE and ARFI may be good tools for the prediction of severity of liver fibrosis. This may be of particular importance to developing countries.

### Terminology

Non-invasive tests for liver fibrosis evaluation: (1) Mechanical markers: liver stiffness measurement according to transient elastography (FibroScan®) or ARFI; and (2) Serum markers: Aspartate aminotransferase-to-platelet ratio index (APRI) and FIB-4 (based on age, platelet count, aspartate aminotransferase and alanine aminotransferase). Liver biopsies were assessed according to the METAVIR score classified as: F0 - no fibrosis; F1 - portal fibrosis without septa; F2 - portal fibrosis and few septa extending into lobules; F3 - numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4 - cirrhosis.

### Peer-review

This study is addressed to evaluate the diagnostic performance and the concordance of different noninvasive methods for the evaluation of liver fibrosis (APRI, FIB-4, transient elastography, ARFI) in 81 patients with chronic hepatitis C, most of them with biopsy-proven diagnosis. The authors concluded that FIB-4, ARFI and transient elastography are useful tests for the noninvasive assessment of liver fibrosis, with a good concordance.

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# Meta-analysis reveals up-regulation of cholesterol processes in non-alcoholic and down-regulation in alcoholic fatty liver disease

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## Abstract

### AIM

To compare transcriptomes of non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) in a meta-analysis of liver biopsies.

### METHODS

Employing transcriptome data from patient liver biopsies retrieved from several public repositories we performed a meta-analysis comparing ALD and NAFLD.

### RESULTS

We observed predominating commonalities at the transcriptome level between ALD and NAFLD, most prominently numerous down-regulated metabolic pathways and cytochrome-related pathways and a few up-regulated pathways which include ECM-receptor interaction, phagosome and lysosome. However some pathways were regulated in opposite directions in ALD and NAFLD, for example, glycolysis was down-regulated in ALD and up-regulated in NAFLD. Interestingly, we found rate-limiting genes such as *HMGCR*, *SQLE* and *CYP7A1* which are associated with cholesterol processes adversely regulated between ALD (down-regulated) and NAFLD (up-regulated). We propose that similar phenotypes in both diseases may be due to a lower level of the enzyme CYP7A1 compared to the cholesterol synthesis enzymes HMGCR and SQLE. Additionally, we provide a compendium of comparative KEGG pathways regulation in ALD and NAFLD.

### CONCLUSION

Our finding of adversely regulated cholesterol processes in ALD and NAFLD draws the focus to regulation of cholesterol secretion into bile. Thus, it will be interesting to further investigate CYP7A1-mediated cholesterol secretion into bile - also as possible drug targets. The list of potential novel biomarkers may assist differential diagnosis of ALD and NAFLD.



**Key words:** Non-alcoholic fatty liver disease; Alcoholic liver disease cholesterol; Bile; Alcohol dehydrogenase; CYP7A1

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**Core tip:** With a meta-analysis of newly published liver biopsy-derived transcriptome datasets we identified multiple key genes and pathways in common and mutually exclusive in alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). We provide a compendium of comparative regulation for all KEGG pathways in both diseases and propose a list of biomarkers distinguishing both diseases. One surprising finding was that cholesterol metabolism was up-regulated in NAFLD and down-regulated in ALD although leading to the same steatosis phenotype which might be explained by an insufficient conversion rate to bile acids under both conditions.

Wruck W, Adjaye J. Meta-analysis reveals up-regulation of cholesterol processes in non-alcoholic and down-regulation in alcoholic fatty liver disease. *World J Hepatol* 2017; 9(8): 443-454 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i8/443.htm> DOI: <http://dx.doi.org/10.4254/wjhl.v9.i8.443>

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) have nearly identical symptoms and in the first report non-alcoholic steatohepatitis (NASH) was described as histologically mimicking alcoholic hepatitis<sup>[1]</sup>. While the cause of ALD is excessive alcohol, the cause of NAFLD is excessive fat resulting from an imbalance between diet and physical activity often associated with insulin resistance and obesity.

We are working on the hypothesis that alcohol is metabolized to fat and beyond this pathway both diseases share a common phenotype. Therefore we place special emphasis on alcohol metabolism which naturally plays a crucial role in ALD. Associations of variants in alcohol and aldehyde dehydrogenases with alcoholism have already been proposed<sup>[2]</sup>. Most variants protective against alcoholism result in a higher acetaldehyde level either by accelerating alcohol dehydrogenase (most common variants in *ADH1B*) metabolizing alcohol to acetaldehyde or by reducing aldehyde dehydrogenase (most common variants in *ALDH2*) metabolizing acetaldehyde to acetic acid. Acetaldehyde is a carcinogen and causes severe reactions such as flushing, accelerated heart rate and nausea. These severe reactions will impose on most carriers of these variants to abstain from alcohol and thus reduce their risk of becoming alcohol addicts. Furthermore, it has been reported that aldehyde dehydrogenases are down-regulated in alcoholics<sup>[3]</sup> or animals continually exposed to alcohol had lower ethanol elimination rates<sup>[4]</sup>.

However, this is a matter of debate as no significant down-regulation of aldehyde dehydrogenases was reported by Vidal *et al.*<sup>[5]</sup> but instead a down-regulation in cirrhotic livers independent of alcoholism. Acetic acid - the product of ethanol metabolism, can be further metabolized by acyl-CoA synthetases (ACSS1 and ACSS2) to acetyl-CoA, the substrate for fatty acid synthesis<sup>[6]</sup>. The expression and activity of Acyl-CoA synthetases in turn are controlled by the sterol regulatory element-binding protein which has been reported to be activated by ethanol<sup>[7]</sup>.

The progression of NAFLD from mild steatosis up to severe NASH or from ALD to alcoholic hepatitis varies widely between individual patients. Oxidative stress and dysregulation of cytokines as a basis for inflammation appear to foster progression to NASH<sup>[8]</sup> as well as alcoholic hepatitis (AH)<sup>[9]</sup>. A two-hit progression from simple steatosis to steatohepatitis and fibrosis has been proposed<sup>[10]</sup>, and suggests that after fat accumulation in the liver, lipids are peroxidized by oxidative stress induced by factors such as CYP2E1. The microsomal enzyme CYP2E1 metabolizes ethanol to acetaldehyde under conditions of alcohol dehydrogenase overload and generates oxidative stress as a by-product, however fatty acids also can be a substrate of CYP2E1<sup>[9]</sup>.

Recently the role of the gut has attracted attention. Under alcoholic or high-fat conditions lipopolysaccharides can pass the border of the intestine to the portal vein and circulate to the liver where they trigger inflammation in ALD<sup>[11]</sup> and in NAFLD<sup>[12]</sup>.

Some studies have already compared ALD and NAFLD<sup>[13]</sup>, e.g., Wilfred de Alwis and Day<sup>[14]</sup> compared the genetics of both diseases addressing the question why only a small percentage of heavy drinkers and obese people progress from steatosis to severe liver disease. Here, we provide an analytical comparison of transcriptomic and metabolic processes involved in the progression of ALD and NAFLD. Employing transcriptome data derived from patient liver biopsies retrieved from several public repositories we performed a meta-analysis and report a signature of biomarkers distinguishing AH from NASH samples. Furthermore, we found predominating commonalities between both diseases at the level of biological pathways thus implying a large mechanistic similarity between both diseases.

## MATERIALS AND METHODS

### Transcriptome data analysis

Datasets of microarray gene expression data from liver biopsies were downloaded from the public repositories at NCBI GEO and EBI Array-Express. The compendium consisted of the ALD datasets GSE28619<sup>[15]</sup> and E-MTAB-2664<sup>[16]</sup> and the NAFLD datasets GSE61260<sup>[17]</sup>, GSE59045<sup>[18]</sup>, GSE48452<sup>[19]</sup> and GSE46300<sup>[12]</sup>. Illumina data was processed via R/Bioconductor<sup>[20]</sup> and packages lumi<sup>[21]</sup>, limma<sup>[22]</sup> and qvalue<sup>[23]</sup>. Background-corrected log2-transformed data was normalized via quantile

normalization from the lumi package. Affymetrix data was processed via R/Bioconductor and packages affy<sup>[24]</sup>, limma, qvalue employing the *rma* normalization method.

Measurements from the multiple platforms were brought together in terms of mean ratios between ALD cases and controls and between NAFLD cases and controls. As controls, healthy liver biopsies or liver biopsies with a low grade of fat accumulation were used. For details we refer to the methods sections of the publications associated with the employed datasets<sup>[12,15-19]</sup>. Heterogeneity of the datasets was assessed via the meta-analysis R package metafor<sup>[25]</sup> generating forest and funnel plots (supplementary Figure 1A and B). The ratios were transformed to a log2 scale and normalized via quantile normalization. The results were again assessed with forest and funnel plots (supplementary Figure 1C and D).

### Pathway analysis

In order to disentangle commonalities and differences between ALD and NAFLD, KEGG pathways<sup>[26]</sup> were analysed with respect to common pathways, up- and down-regulation and discordant up- and down-regulation. The ratios between ALD and control and NAFLD and control were employed to count the numbers of up- and down-regulated genes for each pathway. A pathway was considered up-regulated when it contained more up- than down-regulated genes. Genes with a ratio  $> t$  were termed up-regulated and genes with a ratio  $< 1/t$  were termed down-regulated. The threshold  $t$  was determined at the 95-quantile of the mean ratios between ALD and NAFLD vs control and was set accordingly to  $t = 4/5$ . Up- and down-regulation of a pathway was determined via the ratio of numbers of up- and down-regulated genes and via a binomial test assuming an equal probability of  $P = 0.5$  for a gene to be up- or down-regulated.

$$n_{up,pw,case} = |\{g | (\frac{x_{g,case}}{x_{g,control}} > t) \wedge (g \in g_{pw})\}|, \text{ case} \in \{ALD, NAFLD\} \quad (1)$$

$$n_{down,pw,case} = |\{g | (\frac{x_{g,case}}{x_{g,control}} < 1/t) \wedge (g \in g_{pw})\}|, \text{ case} \in \{ALD, NAFLD\} \quad (2)$$

$$n_{pw,case} = n_{up,pw,case} + n_{down,pw,case} \quad (3)$$

$$r_{pw,case} = \frac{n_{up,pw,case}}{n_{down,pw,case}} \quad (4)$$

Here,  $n_{up,pw,case}$  and  $n_{down,pw,case}$  are the numbers of up- and down-regulated genes in a pathway  $pw$ ,  $g_{pw}$  are the gene symbols associated with a pathway,  $x_{g,case}$  is the gene expression value in a case which can be ALD or NAFLD,  $x_{g,control}$  is the gene expression value in the control case,  $r_{pw,case}$  is the ratio indicating up-regulation ( $r_{pw,case} > 1$ ) or down-regulation ( $r_{pw,case} < 1$ ) of pathway  $pw$ . Significance of up- or down-regulation of a pathway is assessed via the Binomial test with the Null hypothesis  $H_0: p \leq p_0$  and the test statistic  $B(p_0, n_{pw,case})$ . Because of assumed equal distribution of up- and down-regulation the probability for the binomial distribution is set to  $p_0$

= 0.5.

Pathway charts of KEGG pathways indicating up- and down-regulation of genes in ALD and NAFLD were generated via the R/Bioconductor package pathview<sup>[27]</sup>.

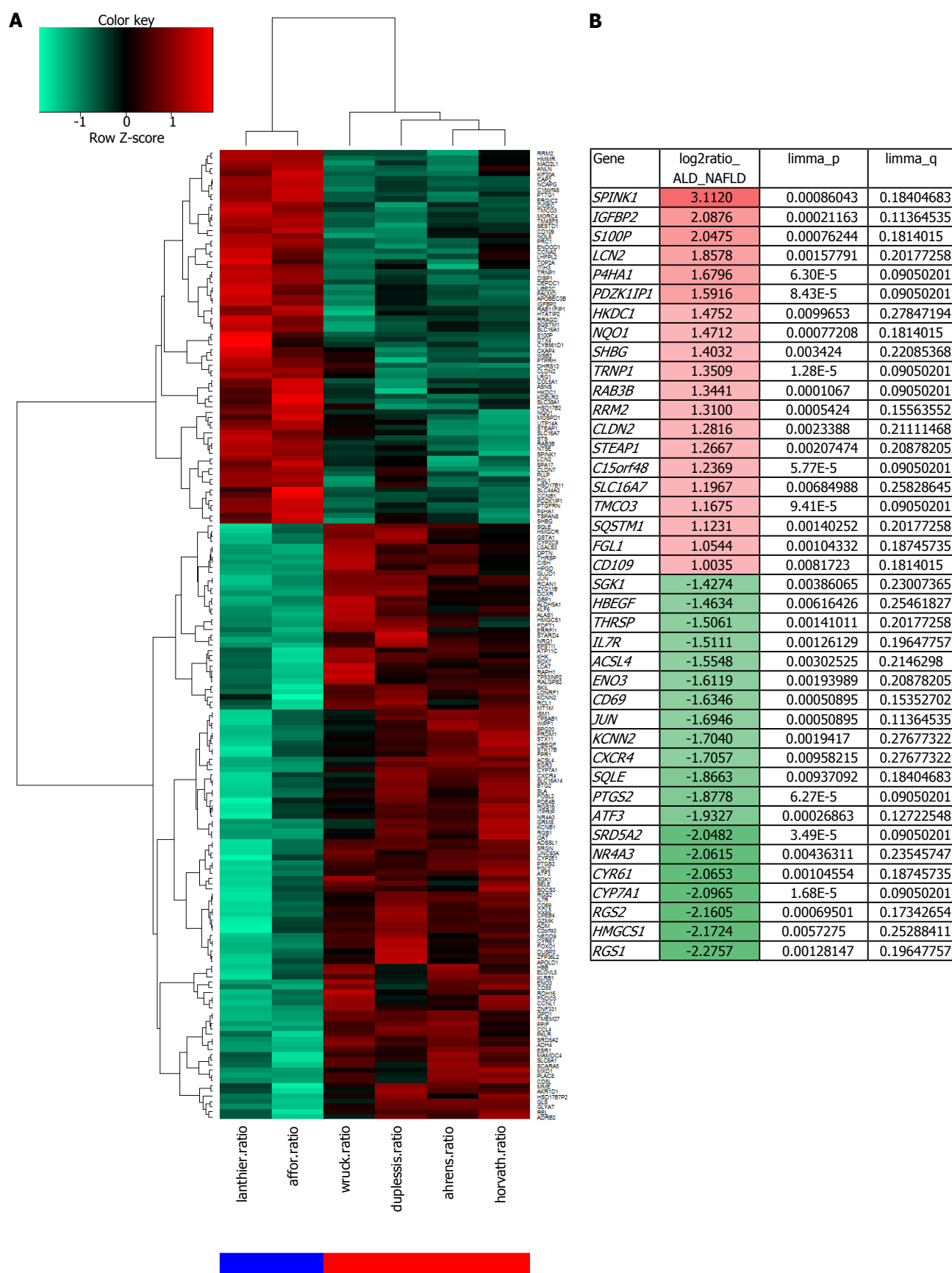
## RESULTS

### A gene signature distinguishes ALD from NAFLD

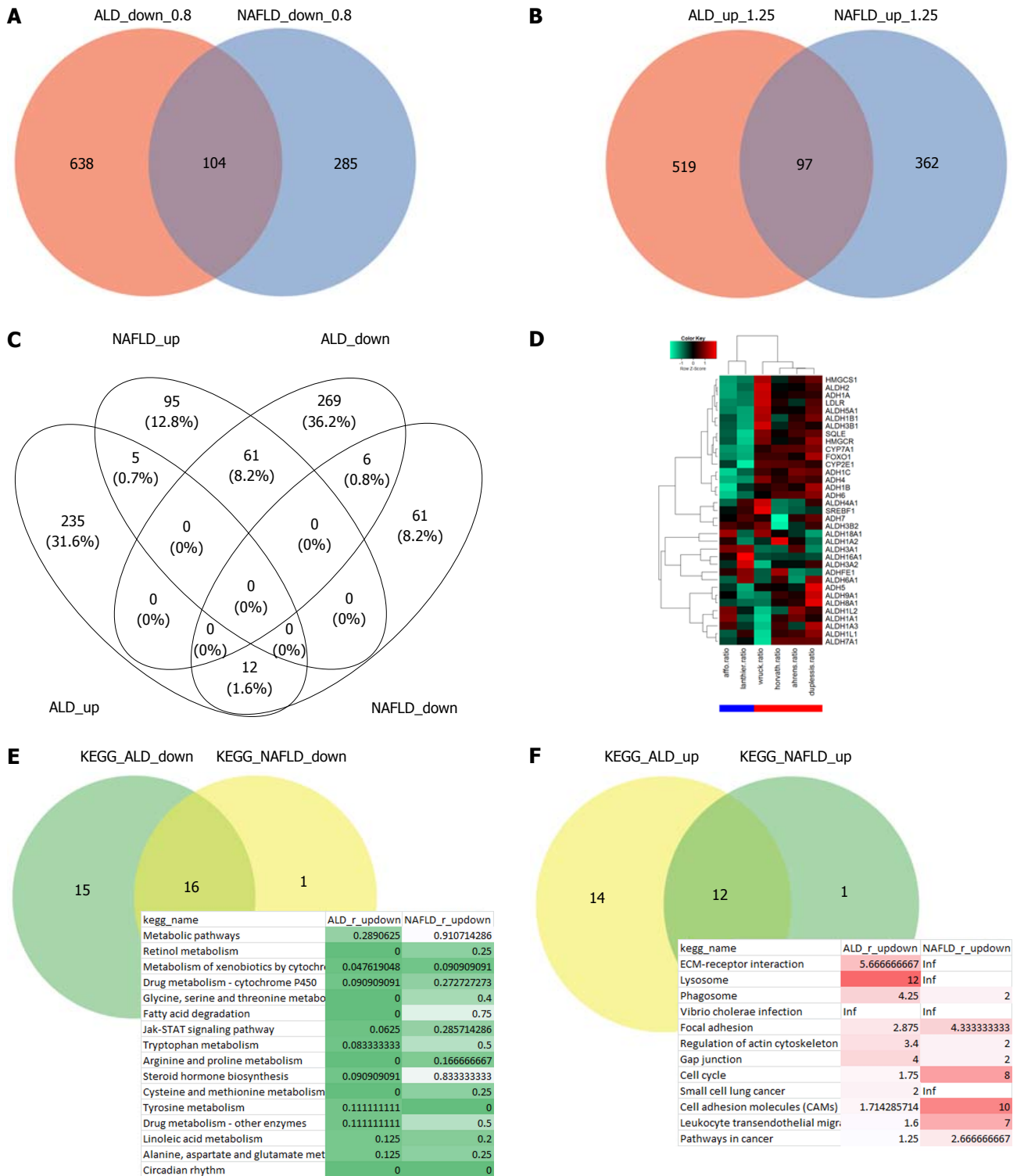
The differences between ALD and NAFLD at the transcriptome level could be condensed to a signature of 187 genes which are differentially expressed between both conditions with a  $P$ -value  $< 0.01$  from the limma test and a ratio  $> 3/2$  or a ratio  $< 2/3$ . The heatmap in Figure 1A shows a cluster analysis of this signature of gene expression data from ALD liver biopsies (blue bar) and NAFLD liver biopsies (red bar). The table in Figure 1B shows the 20 most up-regulated and 20 most-down-regulated genes from the signature indicating their log2-ratios and their  $P$ - and  $Q$ -values for the comparison ALD vs NAFLD. The most up-regulated gene between ALD and NAFLD was SPINK1. SPINK1 is secreted in the pancreatic juice to reversibly inhibit activated trypsin thus preventing pancreatic auto-digestion<sup>[28]</sup> and variants in this gene have been associated with pancreatitis<sup>[29]</sup>. Obesity and more prominent alcohol abuse are other causative factors for pancreatitis<sup>[28]</sup> which by its effects on insulin may contribute to liver disease. Lanthier *et al.*<sup>[16]</sup> revealed the association of SPINK1 with inflammation and proliferation via correlation with the inflammatory macrophage marker CD68 and the cell cycle markers Cdk1 and CyclinB1. At the lower part of the table in Figure 1B two RGS (regulator of G-protein signalling) encoding genes, RGS1 and RGS2 are down-regulated in ALD but up-regulated in NAFLD. Nunn *et al.*<sup>[30]</sup> reported reduced fat deposits, decreased serum lipids, and low Leptin levels in RGS2 deficient mice.

### Genes regulated in common between ALD and NAFLD

Analysis of the common genes between ALD and NAFLD was subdivided into analysis of down- and up-regulated genes. Figure 2A shows that 104 genes are down-regulated in ALD and NAFLD (ratio  $< 0.8$ ) while 638 genes are exclusively down-regulated in ALD and 285 in NAFLD. Figure 2B shows that 97 genes are up-regulated in ALD and NAFLD (ratio  $> 1.25$ ) while 519 genes are exclusively up-regulated in ALD and 362 in NAFLD. There are more distinctly expressed than overlapping genes - in contrary to the KEGG pathways where most pathways overlap (Figure 2E and F). Gene regulation was further restricted with a threshold for the limma test for differential expression of  $P < 0.05$ . Figure 2C shows a venn diagram of the four resulting sets of up/down-regulated genes in ALD and NAFLD. Here most genes are exclusively regulated but interestingly from the genes regulated in both diseases more genes are oppositely than commonly regulated: 61 genes are up-regulated in NAFLD but down-regulated in ALD and 12



**Figure 1 A gene signature distinguishes alcoholic liver disease from non-alcoholic fatty liver disease.** A: The heatmap shows a cluster analysis of logarithmic ratios of gene expression data from ALD liver biopsies vs control (blue bar) and NAFLD liver biopsies vs control (red bar); B: The table shows the 20 most up-regulated and 20 most-down-regulated genes from the signature indicating their log2-ratios and their *P*- and *Q*-values for the comparison ALD vs NAFLD. The full list of these genes can be found in Supplementary Table 2. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.



**Figure 2** Most biological pathways are regulated in the same direction in alcoholic liver disease and non-alcoholic fatty liver disease but a subset of metabolism-associated genes are oppositely regulated. A: Compares ALD and NAFLD in terms of down-regulated genes (ratio < 0.8); B: In terms of up-regulated genes (ratio > 1.25). There are more distinct than overlapping genes - in contrary to the KEGG pathways where most pathways overlap (E and F); C: Interestingly, when regulation is further restricted with a *P*-value < 0.05 more genes are oppositely than commonly regulated - but most are exclusively regulated. Many of the oppositely regulated genes are associated with cholesterol processes, e.g., HMGCR, SQLE and CYP7A1, and are co-expressed with alcohol (ADH) and aldehyde dehydrogenases (ALDH) as seen in the heatmap (ALD: Blue bar, NAFLD: Red bar) (D). A pathway is considered down-regulated (E) when it contains more down-regulated than up-regulated genes as tested by the binomial test and the ratio, analogously up-regulated pathways are determined (F). The table of common down-regulated pathways includes metabolic, retinol, cytochrome and fatty acid degradation pathways, the up-regulated include ECM-receptor, lysosome and phagosome. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

are up-regulated in ALD and down-regulated in NAFLD while only 5 were commonly up and 6 commonly

down-regulated. Supplementary Table 1 shows the corresponding gene sets. The genes up-regulated in



NAFLD but down in ALD refer to major players in cholesterol processes such as *HMGCS1*, *HMGCR*, *SQLE*, *CYP7A1* and *LDLR*. This would confirm the involvement of cholesterol biological processes in the etiology of NAFLD as we previously reported<sup>[31]</sup> and which distinguish it from the etiology of ALD. The opposite regulation of cholesterol processes as down in ALD and up in NAFLD can also be observed in the corresponding KEGG pathways Steroid biosynthesis, Primary bile acid biosynthesis and Terpenoid backbone biosynthesis (Supplementary file 1, p22, 34 and 84). These findings are in line with reports of a 29% decrease in *HMGCR* and a 56% decrease in cholesterol 7 $\alpha$ -hydroxylase alias *CYP7A1* by Lakshmanan *et al*<sup>[32]</sup>, they suggested that increased ethanol leads to a reduced rate of cholesterol degradation to bile acids and accumulation of cholesterol in the liver. We also found (Supplementary Table 2) a stronger down-regulation of *CYP7A1* (log2-ratio = -0.95) than of the upstream cholesterol genes *HMGCR* (log2-ratio = -0.429) and *SQLE* (log2-ratio = -0.33) in ALD while in NAFLD, *CYP7A1* (log2-ratio = 1.15) was weaker up-regulated than *HMGCR* (log2-ratio = 1.57) and *SQLE* (log2-ratio = 1.53). Thus although oppositely regulated in ALD and NAFLD in both diseases more cholesterol is produced than can be secreted by the bile *via* *CYP7A1*.

Amongst the genes up-regulated in ALD but down in NAFLD are *TNFSF14* in line with the major role of TNF-alpha in ALD<sup>[11]</sup> and *SPINK1* which was described above in "a gene signature distinguishes ALD from NAFLD".

To further investigate the mechanisms by which ethanol induces these changes in cholesterol processes we analysed expression clusters of genes involved in ethanol and cholesterol related processes. The analysis revealed a cluster of genes down-regulated in ALD and up-regulated in NAFLD including among others the genes encoding for *ALDH2*, *ADH1A*, *LDLR*, *SQLE*, *HMGCR*, *CYP7A1*, *CYP2E1* and *FOXO1* (Figure 2D). *FOXO* Transcription factors such as *FOXO1*, whose expression has been reported to be altered by ethanol<sup>[33]</sup> and may play a role in the regulation of several genes from this cluster. Interestingly, the heatmap (Figure 2D) shows a much higher degree of co-regulation of *FOXO1* with the rate-limiting cholesterol synthesis enzymes *HMGCR* and *SQLE* than of *SREBF1* which is known as the main regulator of cholesterol<sup>[34]</sup>.

The five genes up-regulated in common between ALD and NAFLD include two collagen encoding genes - *COL1A1* and *COL3A1*, thus demonstrating overlapping disease pathology in the development of fibrotic tissue. The six down-regulated genes in ALD and NAFLD include *HPRT1* which has been reported to be down-regulated in severe liver disease<sup>[35]</sup>.

### Pathway analysis

Most biological pathways are regulated in the same direction in ALD and NAFLD. A pathway is considered down-regulated (Figure 2E) when it contains more down-regulated than up-regulated genes as tested by the binomial test and the ratio is less than 1. Up-regulated pathways are determined accordingly (Figure 2F). The table of common

down-regulated pathways includes metabolic, retinol, cytochrome and fatty acid degradation pathways, the up-regulated pathways include ECM-receptor, lysosome and phagosome.

### Common pathways down-regulated in ALD and NAFLD

Sixteen common pathways are down-regulated in ALD and NAFLD. A pathway with high relevance to both diseases is Fatty acid degradation which is down-regulated in ALD and NAFLD but more so in ALD. The KEGG graph in Figure 3A shows down-regulation (green) in nearly all genes for ALD (left part of the gene boxes) while for NAFLD (right part of the gene boxes) there are up-regulated genes such as *ACSL1* and *ACAT1* but more are down-regulated. Interestingly, in the alcohol metabolism at the bottom of the chart, genes are down-regulated in ALD. At the bottom of Figure 3A, alcohol metabolism is shown in a schematic view. In a more detailed view we examined the behaviour of the alcohol dehydrogenase (*ADH*) encoding genes in the heatmap in Figure 3B and in the aldehyde dehydrogenase genes in Figure 3C. This resulted in a clear image for the *ADHs* which were down-regulated in ALD. The heatmap for the *ALDHs* (Figure 3C) looked more complex showing consistently ALD-down-regulated *ALDHs* only in a cluster at the top including *ALDH2* while most genes were heterogeneously regulated between ALD and NAFLD.

### Common pathways up-regulated in ALD and NAFLD

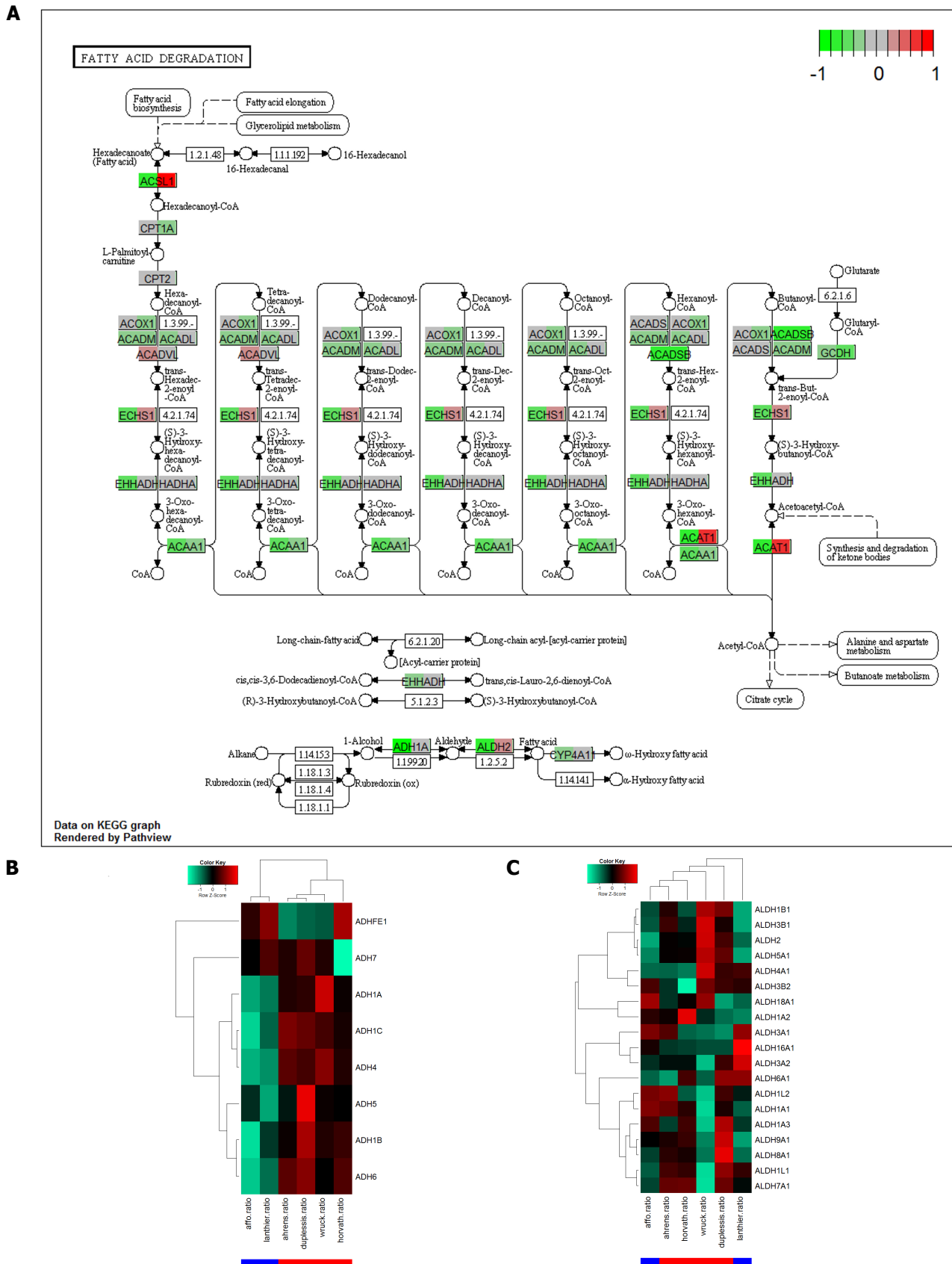
Few pathways (12) were up-regulated in ALD and NAFLD. One of these is ECM-receptor interaction (Supplementary file 1, p. 142). Up-regulation of this pathway might indicate the onset of fibrosis which is accompanied by excessive accumulation of extracellular matrix proteins including collagen<sup>[36]</sup>. Here, the involvement of the collagen *COL1A1* is shown.

### Pathways oppositely regulated in ALD and NAFLD

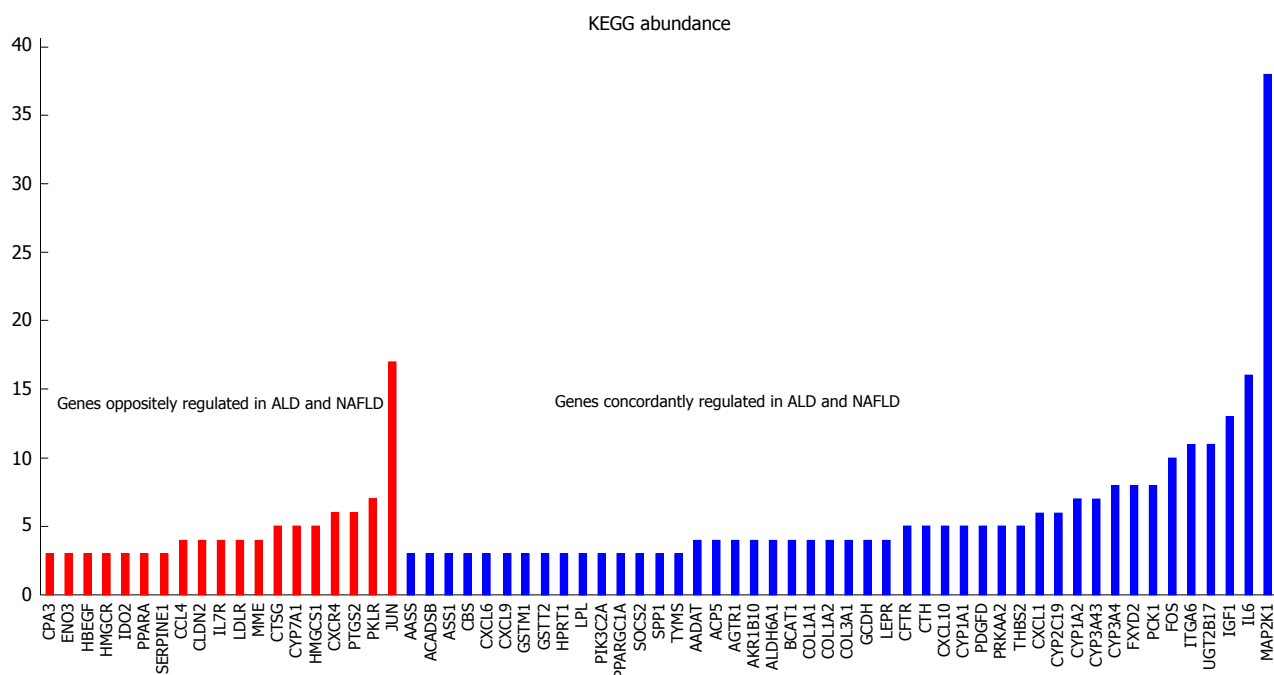
Of the oppositely regulated pathways, sixteen were down-regulated in ALD and up-regulated in NAFLD while only one was up-regulated in ALD and down in NAFLD (Supplementary Table 3). The Glycolysis pathway was down-regulated in ALD and up-regulated in NAFLD. The KEGG graph (Supplementary file 1, p. 11) shows more down- (green, *e.g.*, *PGM1*, *ENO1*) than up-regulated (red, *e.g.*, *PFKL*) genes for ALD (left part of gene boxes) while for NAFLD (right part of gene boxes) up-regulated genes predominate. Reduction of glycolysis by ethanol has been brought into context with consumption of oxygen for the alcohol metabolism and has been reported by several authors<sup>[37,38]</sup>. Berry *et al*<sup>[38]</sup> reported that ethanol oxidation inhibits glycolysis in rat hepatocytes *via* competition of the reducing equivalents generated during ethanol oxidation with those arising in glycolysis for transfer to the mitochondria.

### Pathway-based functional gene annotation

In "genes regulated in common between ALD and NAFLD" we described that after filtering genes with a



**Figure 3 Fatty acid degradation is down-regulated in alcoholic liver disease and non-alcoholic fatty liver disease but more pronounced in alcoholic liver disease.** A: The KEGG graph shows down-regulation (green) in nearly all genes for ALD (left part of the gene boxes) while for NAFLD (right part of the gene boxes) there are up-regulated genes such as ACSL1 and ACAT1 but more are down-regulated. Interestingly, in alcohol metabolism at the bottom of the chart, genes are down-regulated in ALD. Alcohol metabolism at the bottom of (A) is shown in detail in the alcohol dehydrogenase (ADH) genes in the heatmap in (B) and in the aldehyde dehydrogenase genes in (C). ADHs are down-regulated in ALD while only dedicated ALDHs, e.g., ALDH2 are down-regulated in ALD. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.



**Figure 4** More genes are concordantly than oppositely regulated in alcoholic liver disease and non-alcoholic fatty liver disease. The chart shows the abundance of concordantly and oppositely regulated genes in KEGG pathways (for abundances > 3). The most abundant MAP2K1 (MEK1) refers to the MAPK/RAS-signalling module acting in many KEGG-pathways. JUN which is appearing in 17 KEGG pathways and is down-regulated in ALD and up-regulated in NAFLD shows that there are mechanistic differences in disease pathologies. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

$P$ -value < 0.05 for differential expression more genes were oppositely than concordantly regulated in ALD and NAFLD. This filtering revealed the interesting genes described above but was very restrictive due to the low number of replicates in the condensed ratios - the  $P$ -values were relatively high. However, the condensed ratios were themselves based on numerous replicates so we consider them as reliable. In a second approach, we filtered genes only by fold change 1.25 and checked on the pathway-level if there were significantly more up- or down-regulated genes based on the binomial test. With this method more genes were concordantly than oppositely regulated in ALD and NAFLD. Figure 4 shows the abundance of concordantly and oppositely regulated genes in KEGG pathways (for abundances > 3). The most abundant MAP2K1 (MEK1) refers to the MAPK/RAS-signalling module acting in many KEGG-pathways. JUN which appears in 17 KEGG pathways and is down-regulated in ALD and up-regulated in NAFLD shows that there are mechanistic differences in molecular basis of these diseases. JUN which is directly connected to c-Jun N-terminal kinase (JNK) was down-regulated in ALD and up-regulated in NAFLD. The up-regulation of JUN in NAFLD is in line with reports from Samuel *et al.*<sup>[39]</sup> showing that activated PKC- $\epsilon$  and JNK can induce insulin resistance *via* impaired IRS1 and IRS2 tyrosine phosphorylation in rats fed with high fat diet.

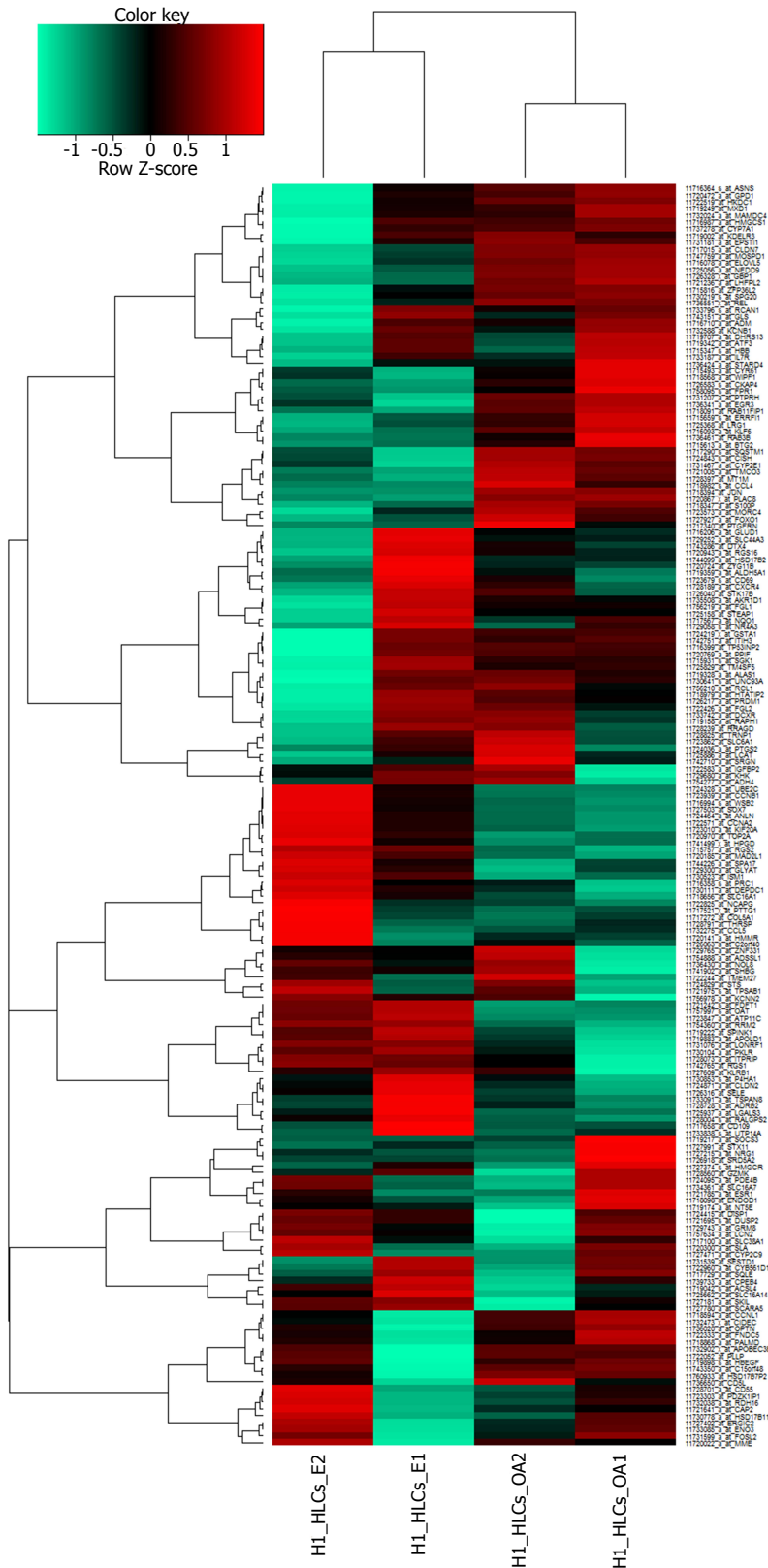
#### Pluripotent stem cell-based models of ALD and NAFLD

We recently described a disease-in-a-dish model of steatosis<sup>[40]</sup>. Pluripotent stem cells, both human embryonic stem cells and induced pluripotent stem cells were differentiated

into hepatocyte-like cells and afterwards challenged with ethanol (E) and oleic acid. In order to test how close these models are to the modeled disease we applied our gene signature distinguishing ALD from NAFLD to gene expression data described in Graffmann *et al.*<sup>[40]</sup>. Figure 5 demonstrates that our gene signature can clearly separate two clusters of the ALD and the NAFLD model in a heatmap generated from this gene expression dataset. Furthermore, relevant regulating or rate-limiting genes described above such as *CYP7A1*, *CYP2E1*, *HMGCS1*, *FOXO1* are down-regulated in the ALD-model and up-regulated in the NAFLD-model similar to the liver biopsy-derived dataset.

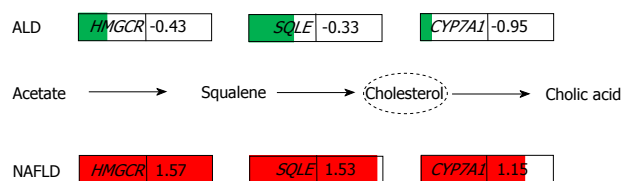
## DISCUSSION

In this comparative analysis of gene expression in ALD and NAFLD liver biopsies we unveiled many commonalities in pathways regulated in the same direction in both diseases. However, there were also pathways regulated in the opposite direction and maybe even more important, essential rate-limiting or regulating genes were adversely regulated. This adverse effect was unexpected as in our working hypothesis, we stated that alcohol is metabolized to fat and beyond this pathway both diseases share a common phenotype. It could hardly be brought together with the common phenotype that of the genes significantly dysregulated between ALD and NAFLD there were more genes regulated in the opposite than in the same direction. One major complex within the adversely regulated genes were cholesterol-related processes including the rate-limiting genes *HMGCR*, *SQLE*, *CYP7A1* and *LDLR*. These



**Figure 5** The pluripotent stem cell models of alcoholic liver disease and non-alcoholic fatty liver disease reflect the characteristics of the biopsy-derived gene signature. The gene signature condensed from the meta-analysis of multiple ALD and NAFLD gene expression datasets was applied to the steatosis-model by (Graffmann *et al*<sup>[40]</sup>) where pluripotent-stem-cell-derived hepatocyte-like cells (HLCs) were challenged with ethanol (E) and oleic acid (OA). The cluster analysis shows a clear separation into the ethanol model (red bar) and the oleic acid model (blue bar). ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.





**Figure 6 Rate-limiting genes of cholesterol metabolism are down-regulated in alcoholic liver disease and up-regulated in non-alcoholic fatty liver disease.** This schematic figure shows the log<sub>2</sub>-ratios of *HMGCR*, *SQLE* and *CYP7A1* indicating down-regulation in ALD (green) and up-regulation in NAFLD (red). There was stronger down-regulation of *CYP7A1* (log<sub>2</sub>-ratio = -0.95) than of the upstream cholesterol genes *HMGCR* (log<sub>2</sub>-ratio = -0.429) and *SQLE* (log<sub>2</sub>-ratio = -0.33) in ALD while in NAFLD, *CYP7A1* (log<sub>2</sub>-ratio = 1.15) was weaker up-regulated than *HMGCR* (log<sub>2</sub>-ratio = 1.57) and *SQLE* (log<sub>2</sub>-ratio = 1.53). The size of the arrows points to a disequilibrium between cholesterol production and secretion into the bile via *CYP7A1* in both diseases despite the opposite regulation in ALD and NAFLD. ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease.

were down-regulated in ALD and up-regulated in NAFLD (each compared vs healthy control). However, we found in both cases that the gene encoding *CYP7A1* - the enzyme responsible for cholesterol removal by catalysing the conversion of cholesterol to bile acids was regulated at a lower level than the genes encoding for the cholesterol synthesis determining enzymes *HMGCR* and *SQLE*. This would explain cholesterol accumulation in the liver because more cholesterol is produced than secreted into bile - regardless if the cholesterol processes are down-regulated in total (in ALD) or up-regulated (in NAFLD). Moreover, the strong down-regulation of *CYP7A1* in ALD might be a clue for the higher risk of cholestasis in ALD than in NAFLD<sup>[41]</sup>. Briefly, these findings emphasize the importance of cholesterol efflux from the liver *via* *CYP7A1* and may suggest that the cause of the disease might be that the rate of cholesterol efflux is too low. Negative feedback loops down-regulating *CYP7A1* by bile acids have already been described<sup>[42]</sup>: Bile acids can down-regulate *CYP7A1* *via* (1) FXR and SHP; or (2) by interaction with liver macrophages (Kupffer cells) whose role in fibrosis has been established as they produce cytokines such as transforming growth factor beta leading to the transformation of stellate cells into myofibroblasts<sup>[43]</sup>. Furthermore, Kupffer cells secrete cytokines, *e.g.*, tumor necrosis factor (TNF $\alpha$ ) and interleukin (IL-1 $\beta$ ) which in turn induce protein kinase, c-Jun N-terminal kinase and thus inhibit hepatocyte nuclear factor and consequently *CYP7A1*<sup>[44,45]</sup>. This gives rise to the question if the lower *CYP7A1* levels are a cause of steatosis or are a consequence of the profibrotic stage. Here, systems biology modelling of cholesterol fluxes in the liver including bile acids and regulatory mechanisms of *CYP7A1* could be useful in determining under which condition efflux rates are too low.

Beside the differences in cholesterol processes we could also confirm effects which had been much disputed before such as the ethanol-mediated down-regulation of glycolysis and of alcohol and aldehyde dehydrogenases.

The common up-regulated pathways might provide synergies for research into ALD and NAFLD. We found similar mechanisms underlying the progression of both diseases and could identify the common up-regulated ECM-receptor interactions and also associated collagen encoding genes *COL1A1* and *COL3A1* which indicate development of fibrotic tissue.

Finally, we provide a comprehensive compendium displaying comparative regulation of all KEGG pathways in ALD vs NAFLD which may serve as an encyclopaedic tool to lookup regulation of dedicated pathways associated with ALD and NAFLD.

In the current study we performed a meta-analysis of gene expression data of liver-derived biopsies from ALD and NAFLD patients, and report a gene signature which clearly separates the transcriptomes of ALD and NAFLD derived liver biopsies. Furthermore, we uncovered predominating commonalities between both diseases at the level of biological pathways, *e.g.*, common down-regulation of the Fatty acid degradation pathway and common up-regulation of the ECM-receptor interaction pathway which may explain common progression of both diseases by cytokines being exchanged between hepatocytes, Kupffer cells and stellate cells at the fibrosis stage. This is confirmed by the common expression of *COL1A1* and *COL3A1* which are associated with fibrotic tissue.

Interestingly, we found rate-limiting genes of cholesterol processes such as *HMGCR*, *SQLE* and *CYP7A1* adversely regulated (Figure 6) between ALD (down-regulated) and NAFLD (up-regulated). The fact that both diseases have the same phenotype may be due to a lower level of the enzyme *CYP7A1* compared to the cholesterol synthesis enzymes *HMGCR* and *SQLE*. Thus, it will be interesting to further investigate *CYP7A1*-mediated cholesterol secretion into bile - possibly by systems biology modeling of cholesterol fluxes in the liver. For future therapy, drugs able to adjust *CYP7A1* to levels amenable with cholesterol synthesized in or transported to the liver will be useful.

## COMMENTS

### Background

Non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) are highly prevalent liver diseases and in an increasing number of developed countries NAFLD is becoming the most common cause of liver disease. Although NAFLD and ALD have distinct etiologies the manifestation and the potential progression of both diseases to hepatitis, cirrhosis and cancer is similar.

### Research frontiers

A two-hit hypothesis is the established explanation for disease progression to alcoholic hepatitis (AH) and non-alcoholic steatohepatitis (NASH). After steatotic fat accumulation due to metabolic disorders such as insulin resistance (NAFLD) or due to alcohol (ALD) oxidative stress and dysregulation of cytokines initiate inflammation and hence the progression to NASH as well as AH.

### Innovations and breakthroughs

The authors found that rate-limiting enzymes of cholesterol metabolism such as *HMGCR*, *SQLE* and *CYP7A1* are down-regulated in ALD and up-regulated in

NAFLD compared to a healthy control. However, in ALD and NAFLD CYP7A1 - associated with conversion of cholesterol into bile acids - is regulated at a lower level than HMGCR and SQLE. That might explain the accumulation of cholesterol by the reduced efflux into bile acids.

## Applications

CYP7A1 is a potential drug target and the proposed gene signature distinguishing ALD from NAFLD consists of biomarkers which may be exploited for diagnostic tests. The compendium of KEGG pathway regulation in ALD and NAFLD and the finding of the adverse regulation of cholesterol metabolism in ALD and NAFLD are promising start points for future research.

## Terminology

NAFLD is the disease related to fat accumulation (steatosis) in the liver in the absence of alcohol abuse (usually the threshold is set at 30 g/d of alcohol for men and 20 g/d for women). It ranges from the relatively benign steatosis to NASH, cirrhosis and hepatocellular carcinoma.

## Peer-review

This manuscript was informative. The authors found commonalities between both ALD and NAFLD at the level of biological pathways implying some mechanistic similarity between both diseases.

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## Tumor reactive stroma in cholangiocarcinoma: The fuel behind cancer aggressiveness

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### Abstract

Cholangiocarcinoma (CCA) is a highly aggressive epithelial malignancy still carrying a dismal prognosis, owing to early lymph node metastatic dissemination and striking resistance to conventional chemotherapy. Although mechanisms underpinning CCA progression are still a conundrum, it is now increasingly recognized that the desmoplastic microenvironment developing in conjunction with biliary carcinogenesis, recently renamed tumor reactive stroma (TRS), behaves as a paramount tumor-promoting driver. Indeed, once being recruited, activated and dangerously co-opted by neoplastic cells, the cellular components of the TRS (myofibroblasts, macrophages, endothelial cells and mesenchymal stem cells) continuously rekindle malignancy by secreting a huge variety of soluble factors (cyto/chemokines, growth factors, morphogens and proteinases). Furthermore, these factors are long-term stored within an abnormally remodeled extracellular matrix (ECM), which in turn can deleteriously mold cancer cell behavior. In this review, we will highlight evidence for the active role played by reactive stromal cells (as well as by the TRS-associated ECM) in CCA progression, including an overview of the most relevant TRS-derived signals possibly fueling CCA cell aggressiveness. Hopefully, a deeper knowledge of the paracrine communications reciprocally exchanged between cancer and stromal cells will steer the development of innovative, combinatorial therapies, which can finally hinder the progression of CCA, as well as of other cancer types with abundant TRS, such as pancreatic and breast carcinomas.

**Key words:** Tumor microenvironment; Desmoplasia; Cancer-associated fibroblast; Inflammation; Tumor-



associated macrophage; Lymphatic endothelial cell; Mesenchymal stem cell; Extracellular matrix

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**Core tip:** Cholangiocarcinoma (CCA) is a typically worrisome malignancy, whose incidence has been steadily increasing. In CCA, as cancerous lesions are emerging, the surrounding stroma gradually undergoes a pathological remodeling, eventually becoming a paramount determinant of tumor growth and dissemination. Indeed, the different cell types populating the tumor microenvironment, also referred to as tumor reactive stroma, enable CCA cells to develop an aggressive phenotype, due to the secretion of a multitude of soluble factors. Therefore, functional insights into the harmful relationship between cancer and reactive stromal cells are of utmost importance, in order to unveil novel molecular targets amenable of therapeutic intervention.

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## INTRODUCTION

Cholangiocarcinoma (CCA) is a deadly malignancy originating from the epithelial cells lining the biliary tree, including the extrahepatic and intrahepatic portions either. Among primary liver cancers, it represents the second most common type after hepatocellular carcinoma, and its incidence and mortality rate have been steadily increasing for two decades. CCA still carries a very dismal prognosis (less than 5% of patients survives up to 5 years from diagnosis), due to a striking resistance to chemotherapy and a propensity for early intrahepatic or lymph node metastatization, making radical surgery suitable to less than one-third of patients. Furthermore, results from both surgical resection and liver transplant are limited by the high recurrence rates. To date, the pathophysiological mechanisms underlying CCA progression remain largely unknown, and consequently, the development of new effective treatments is a very awkward task<sup>[1-3]</sup>.

Whilst the majority of CCAs are thought to be sporadic, several geographically heterogeneous risk factors have been identified, mostly related to an inflammatory bile duct injury. They include hepatobiliary fluke infestations (*e.g.*, *Opisthorchis viverrini*, *Clonorchis sinensis*), hepatolithiasis, congenital abnormalities of the bile ducts (*e.g.*, Caroli's disease, choledochal cysts), primary sclerosing cholangitis (PSC), viral hepatitis B and C, and exposure to toxic compounds (*e.g.*, thorium dioxide, naphthenic acids). Furthermore, CCA development has been associated with genetic and epigenetic alterations in well-

known proto-oncogenes (*e.g.*, KRAS GTPase, isocitrate dehydrogenases 1 and 2) and tumor suppressor genes (*e.g.*, tumor protein p53, cyclin dependent kinase inhibitor 2A). More recent evidence indicates that cholangiocarcinogenesis is driven by chronic deregulation of various signaling pathways deeply involved in cholangiocyte biology, leading to uncontrolled proliferation, evasion of apoptosis, and loss of genome integrity. For instance, increased activity of cytokines and growth factors, such as interleukin (IL)-6, transforming growth factor (TGF)- $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , and platelet-derived growth factor (PDGF), is a common event in CCA, due to either enhanced production or increased cell responsiveness, and likely contributes to the malignant transformation of cholangiocytes<sup>[4,5]</sup>.

Evidence is mounting that the aggressive behavior of CCA is greatly influenced by paracrine cues released from the inflammatory and mesenchymal cell types populating the tumor microenvironment<sup>[6]</sup>. Indeed, as cancerous lesions are emerging, the surrounding stroma gradually develops profound and complex changes, undergoing a switch from key player in tissue homeostasis to pathological niche supporting tumor growth and dissemination<sup>[7,8]</sup>. Therefore, an in-depth insight into the actual contribution of the stromal microenvironment to CCA progression is imperative, with the ultimate goal to pave the way for innovative combinatorial treatments targeting both stromal and cancer cells. Hopefully, this may lead to a more effective management of this devastating malignancy.

## THE TUMOR REACTIVE STROMA: A SPECIALIZED COMPARTMENT ORCHESTRATING TUMOR PROGRESSION

Neoplastic bile ducts are tightly enveloped by a striking and diffuse desmoplastic, hypovascularized stroma that is usually referred to as tumor reactive stroma (TRS). This histopathological lesion is made up of a variety of stromal cells embedded in a dense collagenous extracellular matrix (ECM), encompassing myofibroblasts, inflammatory cells, endothelial cells and mesenchymal stem cells (MSCs)<sup>[6,9]</sup>. Once recruited, activated and co-opted by malignant cholangiocytes, the cellular components of the TRS can diffusely infiltrate the growing tumor and eventually support its progression by secreting a wide range of soluble factors. Indeed, these factors can directly trigger the emergence of malignant phenotypes and/or enhance the migration and aberrant activation of other stromal cells<sup>[10,11]</sup>. In addition to the plethora of cytokines, chemokines, growth factors and proteinases perpetually released by stromal cells, cell-extrinsic factors, such as hypoxia and abnormally remodeled ECM components, also provide the TRS with invasiveness-promoting properties<sup>[12]</sup>. Interestingly, it has been proposed that the pro-tumorigenic functions of the TRS could partially rely on the induction of epigenetic, and therefore heritable, changes in cancer cells<sup>[13,14]</sup>. In fact, gastric, ovarian

and breast cancer cell lines co-cultured with fibroblasts isolated from the tumor milieu, were found to undergo gene-specific DNA hypermethylation events, generally coupled with increased migratory abilities<sup>[15-17]</sup>.

Development of CCA is often associated with inflammation-related alterations, as observed in those cases arising in specific disease settings, such as PSC and congenital hepatic fibrosis. Moreover, recent observations indicate that in the last few years CCA is more often detected on a background of chronic inflammation associated to cirrhosis, regardless of its etiology<sup>[18]</sup>. As a general concept, the TRS may be regarded as an aberrant, over-healing reparative complex ("wound that does not heal" according to the old Dvorak's paradigm), wherein various types of inflammatory and stromal cells are somehow hijacked by the malignant compartment, whose immunomodulatory functions and metabolic needs eventually prevail over the physiological homeostasis of the normal tissue<sup>[6,19]</sup>. This behavior also reflects the inherent plasticity of the naïve stroma, which enables it to comply quickly the evolution of the adjacent transformed epithelium, in contrast with the self-limited response occurring in the wound repair<sup>[8]</sup>.

## FUNCTIONAL INSIGHTS INTO THE INFLUENCE OF THE TRS ON CCA PROGRESSION

In CCA, increasing evidence has highlighted the prognostic relevance of the molecular alterations related to the generation of the TRS. Indeed, gene expression profiling of microdissected stroma from both tumoral and peritumoral areas of resected human CCA revealed a TRS-specific gene signature, encompassing 1073 genes involved in cell metabolism, cell cycle, cell signaling pathways and ECM biology. In particular, the overexpression of representative genes (namely KIAA0101, TGF- $\beta$ 2, laminin subunit  $\gamma$ 2 and osteopontin) was found to significantly correlate, at different levels, with clinic-pathological features of CCA patients<sup>[20]</sup>. Andersen *et al.*<sup>[21]</sup> undertook a similar approach in order to compare the epithelial and stromal transcriptomic profiles in CCA tissues. They identified a stromal gene signature associated with poor clinical outcome. Interestingly, the 1442 differentially expressed genes revealed a stromal dysregulation of both chemokine (CXCR4, CCR7, CCL2, CCL5, CCL19, CCL21) and IL (IL3RA, IL7R, IL10RA, IL18RAP, IL6, IL16, IL33) receptors and ligands. In the next paragraphs, we will discuss in detail how the main components of the TRS are supposed to promote CCA progression.

## CANCER-ASSOCIATED FIBROBLASTS

The TRS is predominantly composed by a subpopulation of activated fibroblasts, called cancer-associated fibroblasts (CAFs). In stark contrast with the small number of lowly proliferating fibroblasts populating the naïve

stroma, CAFs are present in an exaggerated high number, and exhibit a permanent state of activation, resulting in a broad release of both biochemical signals and ECM components, in particular fibronectin and collagen type I<sup>[6,10,12,22]</sup>. The main phenotypic markers of CAFs are alpha-smooth muscle actin ( $\alpha$ -SMA), vimentin, S100A4 (also called fibroblast specific protein-1) and fibroblast activation protein alpha (FAP)<sup>[23]</sup>. CAFs are recognized as a heterogeneous population, likely reflecting the variety of cell precursors that they are supposed to originate from, including hepatic stellate cells (HSCs), portal fibroblasts and, to a lesser extent, bone marrow-derived MSCs<sup>[10]</sup>. The hypothesis that cancer cells themselves may represent an alternative source of CAFs by undergoing epithelial-to-mesenchymal transition (EMT) has gradually waned<sup>[14,24]</sup>. Nevertheless, neoplastic cells act in concert with inflammatory cells to secrete a vast array of growth factors, cytokines and chemokines ultimately responsible for the recruitment of fibroblasts to the TRS, as well as for their chronic activation state. In this regard, we recently showed that PDGF-DD is overexpressed by CCA cells under the effect of hypoxia, and acts as a key mediator of fibroblast recruitment nearby the tumoral mass. Indeed, PDGF-DD strongly induces fibroblast migration by binding its cognate receptor PDGFR $\beta$  (which is extensively expressed by CAFs), thereby activating the Rho GTPases, Rac1 (lamellipodia inducer) and Cdc42 (filopodia inducer), as well as the JNK pathway<sup>[24]</sup>. Furthermore, conditioned medium from CCA cells sustained the activation of both HSCs and liver myofibroblasts, which actually acquired a more elongated shape and up-regulated the expression of  $\alpha$ -SMA, *in vitro*<sup>[25,26]</sup>. Among the multitude of soluble factors triggering the persistent activation of CAFs, TGF- $\beta$ , fibroblast growth factor and, again, several PDGF family members, undoubtedly play a pivotal role<sup>[6,12]</sup>.

### **Evidence for the pro-neoplastic effects exerted by CAFs**

In CCA samples, the expression of  $\alpha$ -SMA is barely detectable in fibroblasts populating the peritumoral areas, whereas most, if not all, fibroblasts embedded in the tumor stroma are  $\alpha$ -SMA<sup>+</sup><sup>[27]</sup>. Consistently, in a hamster model of CCA, the density of  $\alpha$ -SMA<sup>+</sup> fibroblasts within liver tissue was clearly shown to increase during cholangiocarcinogenesis<sup>[28]</sup>. There is a strong evidence that an increased density of CAFs within the TRS correlates with increased tumor growth and poor outcome in CCA patients. Indeed, high stromal expression of  $\alpha$ -SMA was reported as an independent prognostic factor for overall and disease-free survival<sup>[27,29]</sup>. In line with these findings, both incubation of CCA cells with CAF conditioned medium and co-culture of CCA cells with CAFs resulted in increased cancer cell proliferation and migration, *in vitro*<sup>[27,30]</sup>. On the contrary, slighter pro-tumorigenic effects were elicited by liver fibroblasts isolated from the peritumoral areas, arguing for a deep biological gap between CAFs and their naïve counterpart (see below)<sup>[27]</sup>. Of further interest, Campbell *et al.*<sup>[31]</sup> developed a three-dimensional organotypic culture model of CCA by embedding to-

gether within a collagen gel matrix clonal strains of CCA cells and CAFs, both derived from a syngeneic rat model of CCA generated by orthotopic inoculation of spontaneously transformed cholangiocytes. Clearly, these culture conditions more accurately reproduce the complex biological interactions occurring *in vivo* within the desmoplastic tumor. Interestingly, the authors observed that CCA cells co-cultured with CAFs exhibited markedly distinct growth features as compared to CCA cells cultured alone. In particular, the number of duct-like structures formed in the gel matrix by CCA cells dramatically increased in direct proportion to initial CAFs plating density. The *in vitro* ability of primary and established HSCs (that is, major CAF precursors) to boost CCA proliferation, survival and migration/invasion has been widely reported as well<sup>[25,29,32-36]</sup>. Moreover, it was shown that co-transplantation of CCA cells with either HSCs or liver myofibroblasts in immunodeficient mice resulted in accelerated tumor growth, compared with mice inoculated with cancer cells alone<sup>[25,26]</sup>. On the other hand, in a syngeneic rat model of CCA, selective CAF depletion in the tumor microenvironment, obtained by unleashing the specific CAF pro-apoptotic protein Bax by navitodax, suppressed tumor growth and improved host survival<sup>[37]</sup>. Overall, these data indicate that myofibroblastic-like cells populating the tumor stroma are leading actors in fueling CCA progression.

### **Molecular players underlying the tumor-promoting effects of CAFs**

Gene expression profiling of CAFs from human CCA samples revealed profound genetic changes as compared to normal liver fibroblasts. Most of the differentially expressed genes are involved in cell metabolism, likely reflecting the biologically active role of CAFs in supporting tumor growth. In addition, some of the up-regulated genes encode secreted proteins exerting pro-tumorigenic functions in multiple carcinomas (*i.e.*, amphiregulin, epiregulin, Jagged 1, PDGF-AA, periostin, secretogranin 2 and ADAM metallopeptidase domain 12), thus emerging as potential candidates underlying the harmful cross-talk between CAFs and CCA cells<sup>[38]</sup>. Below, we will summarize the most prominent CAF-derived molecules fostering CCA aggressiveness. It is also worth noting that, beyond paracrine soluble factors, extracellular vesicles, especially exosomes, nano-sized molecular shuttles of about 40-100 nm of diameter, are also claimed to mediate the paracrine communications between cancer cells and neighboring stromal components. Indeed, exosomes can transfer functional proteins, lipids and nucleic acids from one cell to another, thereby modulating gene expression programs<sup>[11,19]</sup>. In this regard, it was recently showed that microRNA-loaded vesicles derived from myofibroblastic-like cells can selectively target CCA cells, thus influencing their neoplastic properties, both *in vitro* and *in vivo*<sup>[39]</sup>. However, a detailed characterization of their cargo is still missing, thus further studies are needed to better elucidate their role in tumor progression.

**IL-1 $\beta$ :** Chemokines can be secreted by many cell types, such as epithelial cells, fibroblasts and endothelial cells, either constitutively or upon inflammatory conditions. Besides their role in the immune system, chemokines are also implicated in tumor biology, owing to their ability to recruit specific subsets of leukocytes, stimulate angiogenesis, and directly promote cancer cell proliferation and invasiveness in an autocrine or paracrine fashion<sup>[40]</sup>. In particular, a mass spectrometry analysis of conditioned media from co-cultures of CCA cells and HSCs revealed a striking increase in C-X-C chemokine ligand (CXCL)5 production by cancer cells, as compared to mono-culture media. Consistently, CXCL5 expression by neoplastic bile ducts positively correlated with stromal expression of  $\alpha$ -SMA, overall suggesting its active role in the interplay between tumor and stroma. In particular, IL-1 $\beta$ , a paramount inflammatory cytokine, has been pointed out as the most likely HSC-derived inducer of CXCL5 production. Interestingly, IL-1 $\beta$  secretion by HSCs can be further enhanced by CCA cells themselves through paracrine signals. Autocrine production of CXCL5 promotes CCA cell proliferation, migration and invasion, by activating phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinases (ERK)1/2 pathways in a CXCR2-dependent manner, *in vitro*<sup>[41]</sup>. Moreover, CXCL5 provides cancer cells with the ability to massively recruit tumor-infiltrating neutrophils, which in turn enhance CCA growth and invasiveness, *in vivo*<sup>[42]</sup>. In line with these findings, high CXCL5 expression negatively affected the overall survival of CCA patients<sup>[41,42]</sup>.

**Stromal cell-derived factor 1:** Stromal cell-derived factor (SDF)-1, also known as CXCL12, acts as ligand for the G protein-coupled receptors CXCR4 and CXCR7. SDF-1 binding to its receptors triggers a variety of downstream signaling pathways, governing cell proliferation, survival and chemotaxis. Besides its well-established role in embryogenesis and tissue homeostasis, the SDF-1/CXCR4 axis is also diffusely implicated in the pathogenesis of autoimmune and inflammatory diseases, as well as in cancer progression<sup>[43]</sup>. In CCA, SDF-1 is solely expressed by CAFs, and not by cancer cells, which overexpress its cognate receptor CXCR4. In contrast, fibroblasts in the peritumoral stroma weakly express SDF-1, suggesting that SDF-1 expression may markedly increase following their recruitment to the TRS, likely upon angiotensin II stimulation<sup>[33]</sup>. SDF-1 secretion by cultured HSCs was demonstrated to enhance CCA cell survival and invasiveness (along with EMT-like changes), *via* up-regulation of the anti-apoptotic protein Bcl-2, and activation of ERK1/2 and PI3K/Akt pathways, respectively<sup>[32,33]</sup>. In addition, SDF-1 could also promote the activation and proliferation of HSCs in an autocrine fashion, thus supporting further CAF enrichment. Consistent with these data, high stromal expression of SDF-1 predicted poor prognosis in CCA patients<sup>[33]</sup>. Noteworthy, CCA cells become hyper-

responsive to SDF-1 due to the overexpression of CXCR4, likely induced by either TNF- $\alpha$  released from TAMs<sup>[44]</sup> or hepatocyte growth factor produced by CAFs<sup>[31]</sup>. This clearly outlines the wide web of communications sustaining the pro-tumorigenic function of the TRS, allowing multidirectional paracrine loops among its different cellular components, which support each other in speeding up tumor progression.

**PDGF-BB:** PDGF family includes five dimeric ligands (PDGF-AA, -BB, -AB, -CC, -DD), acting *via* two receptor tyrosine kinases (PDGFR $\alpha$  and PDGFR $\beta$ ). The PDGF/PDGFR system is involved in various biological processes requiring mesenchymal cell activation, mostly related to tissue repair and wound healing. Moreover, overexpression of PDGF ligands and receptors has been documented in a huge variety of epithelial cancers, and usually predicts poor outcome<sup>[45]</sup>. Among growth factors commonly produced by cultured HSCs, PDGF-BB is one of the most abundantly expressed. HSCs secrete PDGF-BB at much higher levels compared with CCA cells, which, from their side, express its cognate receptor PDGFR $\beta$ . Co-culture experiments demonstrated that HSC-derived PDGF-BB promoted CCA cell resistance to TNF-related apoptosis-inducing ligand-mediated apoptosis, by activating the Hedgehog (Hh) signaling cascade<sup>[35,36]</sup>, a morphogen pathway directing several cholangiocyte functions critical for liver repair<sup>[46,47]</sup>. Specifically, PDGF-BB binding to PDGFR $\beta$  increases intracellular levels of cyclic adenosine monophosphate, resulting in a protein kinase A-dependent translocation of the Hh signaling activator Smoothened (SMO) to the plasma membrane, which eventually leads to the activation of GLI transcription factors<sup>[35]</sup>. Importantly, both cyclopamine (SMO inhibitor) and imatinib mesylate (PDGFR $\beta$  inhibitor) were able to reduce tumor growth by promoting cancer cell apoptosis in an orthotopic syngeneic rat model of CCA<sup>[35,36]</sup>. Kim *et al.*<sup>[34]</sup> further confirmed that paracrine signals from HSCs (which, actually, may include Sonic Hh as well) are of paramount importance for the activation of Hh signaling within CCA cells, whereas autocrine activation only plays a minor role. Furthermore, they also outlined the involvement of Hh signaling in CCA cell proliferation, migration and invasiveness.

**Heparin-binding epidermal growth factor:** In CCA, overexpression of epidermal growth factor receptor (EGFR) is one of the most common genetic aberrations, and, most relevantly, it was associated with poor survival and tumor recurrence after resection<sup>[48]</sup>. In CCA xenografts derived from subcutaneous co-injection of cancer cells and liver myofibroblasts, EGFR activation was shown to promote tumor growth and metastasis, and, above all, to be strictly dependent on the presence of activated fibroblasts. Indeed, cultured myofibroblasts secrete high amounts of heparin-binding epidermal growth factor (HB-EGF), a well-known EGFR ligand, thereby triggering the activation of EGFR signaling in CCA cells, *in vitro*. The HB-EGF/EGFR axis promotes CCA

cell proliferation, migration and invasion, along with EMT-like changes, through activation of signal transducer and activator of transcription (STAT)-3 and ERK1/2 pathways. Of note, HB-EGF expression in fibroblasts can be further enhanced by TGF- $\beta$ 1 released from CCA cells, whose production is in turn triggered by EGFR activation, thus outlining the presence of a self-perpetuating paracrine loop<sup>[26]</sup>.

### ***The deleterious interplay between CAFs and endothelial cells: emerging evidence***

Importantly, the paracrine signals released by CAFs not only directly exacerbate the malignancy of cancer cells, but also participate in the recruitment of other stromal components, including inflammatory cells and endothelial cells, thereby further supporting cancer growth and progression<sup>[6]</sup>. In particular, we recently unveiled that CAFs may cooperate with CCA cells in driving the development of a rich lymphatic vasculature within the tumor stroma. CCA is characterized by a striking expansion of the intratumoral and peritumoral lymphatic vessels, which represents a key determinant of the early metastasization to the regional lymph nodes, often precluding curative surgery<sup>[6]</sup>. Consistently, a high lymphatic microvessel density in CCA tissues correlated with significantly reduced overall and disease-free survival of patients<sup>[49]</sup>. Our recent findings demonstrated that, within the TRS, lymphatic endothelial cells (LECs) localize in close spatial relationship with either CCA cells or CAFs. Indeed, besides recruiting fibroblasts around the neoplastic ducts, PDGF-DD produced by CCA cells can also provide CAFs with the ability to secrete lymphangiogenic growth factors, namely vascular endothelial growth factor (VEGF)-A and VEGF-C, which eventually promote the recruitment of LECs, along with their tubular assembly in highly anastomosed structures<sup>[50]</sup>. Overall, these observations are consistent with the concept that CAFs are able to generate a pro-invasive microenvironment conducive to the lymphatic metastatic behavior of CCA.

It is important to note that in CCA, the large expansion of the lymphatic vasculature is not paralleled by an equal increase in blood vessels<sup>[6]</sup>. Nevertheless, angiogenesis has been also associated with a high risk of recurrence after surgery<sup>[51]</sup>. In this regard, it is likely that CAFs, especially those originated from HSCs, contribute to generate a pro-angiogenic microenvironment, as reported in other cancer types<sup>[52]</sup>. Indeed, HSCs likely behave as liver-specific pericytes, participating to vascular remodeling during both liver regeneration and tumor-associated angiogenesis. In this context, PDGF has been pinpointed as a relevant player. Specifically, PDGF ligands (especially PDGF-BB) released from the vascular endothelium are able to drive the recruitment of PDGFR- $\beta$ -expressing HSCs, along with their subsequent adhesion to the vessel wall, similar to what occurring in embryogenesis. From their side, activated HSCs promote vascular tube formation by secreting VEGF ligands and angiopoietins under the effect of hypoxia, and mecha-



nically stabilize the sprouting vessels by providing a tight envelope around the sinusoidal endothelial cell layer<sup>[53-56]</sup>.

## TUMOR-ASSOCIATED MACROPHAGES

Among the several immune cell types populating the TRS, macrophages are the most represented. Tumor-associated macrophages (TAMs) are mainly derived from circulating monocytes (CD14<sup>+</sup>/CD16<sup>+</sup>), rather than from resident macrophages (CD68<sup>+</sup>) or Kupffer cells in the liver. They are efficiently recruited to the tumor mass by a range of chemoattractants variably secreted by neoplastic and stromal cells, including C-C motif ligand (CCL) chemokines [e.g., monocyte chemoattractant protein (MCP)-1, also known as CCL2], colony stimulating factor (CSF)-1 and VEGF<sup>[6,57,58]</sup>. For instance, CAFs, especially FAP<sup>+</sup> CAFs, are a major source of MCP-1<sup>[59]</sup>. In contrast to T cells, which can exert both tumor-promoting and tumor-suppressive functions, TAMs are almost exclusively implicated in boosting cancer aggressiveness, a function exemplified by their predominant localization at the tumor front. TAMs mostly display a M2 (or alternatively activated) phenotype, manipulated by paracrine signals originating from both malignant cells and specific subsets of T cells (including IL-10, CSF-1 and TGF- $\beta$ ), as well as by tumor hypoxia. Pro-tumorigenic effects of the M2 phenotype rely on a range of properties, including limited antigen-presenting functions, strong tissue remodeling and immune tolerance abilities, and production of pro-angiogenic and pro-lymphangiogenic growth factors; furthermore, TAMs directly provide cancer cells with pro-migratory inputs. TAMs are characterized by low expression of major histocompatibility complex class II molecules and IL-12, and high expression of IL-10, arginase-1 and multiple scavenging, mannose, and galactose receptors. Conversely, the so-called classically activated M1 macrophages, which are usually less represented within the TRS, possess strong antigen-presenting abilities, prime tissue destruction and anti-tumor immune responses, and possess tumoricidal activities<sup>[10,12,58,60-63]</sup>.

Conditioned medium from CCA cells fostered the emergence of the M2 phenotype in cultured macrophages, which actually up-regulated the expression of the M2 specific marker CD163, as well as of the M2-related molecules IL-10 (immunosuppressive cytokine), TGF- $\beta$  (pro-fibrotic cytokine), VEGF-A (pro-angiogenic growth factor) and matrix metalloproteinase (MMP)-2<sup>[64]</sup>. Growing interest has also been drawn on the interplay between TAMs and CAFs, as it was recently found that conditioned medium from HSCs affected the differentiation of macrophages, stimulating the production of pro-inflammatory (IL-6) and pro-fibrotic cytokines (TGF- $\beta$ )<sup>[65]</sup>. Furthermore, within the CCA stroma, the density of M2 TAMs positively correlated with the number of regulatory T cells, suggesting that they contribute to macrophage polarization toward the pro-neoplastic phenotype<sup>[64]</sup>. Interestingly, cholangiocyte ability to finely orchestrate a macrophage-centric inflammatory response was also

reported by our group in a mouse model of congenital hepatic fibrosis, a disease of the biliary epithelium at increased risk for CCA development. In this model, dysfunctional biliary epithelial cells (due to a genetic defect in the ciliary protein fibrocystin) secrete a range of chemokines (CXCL1, CXCL10, CXCL12) able to recruit and activate bone marrow-derived macrophages, which then progressively switch from an M1 to an M2 phenotype as the disease progresses<sup>[66]</sup>. However, it is worth considering that the macrophage phenotype is extremely plastic, showing a continuum of activation states, in which M1 and M2 types only represent the extreme points<sup>[62]</sup>. In line with this concept, many tumor-promoting cytokines that are actually M1 cytokines, such as IL-6, are even produced by TAMs<sup>[61]</sup>. Recently, Raggi *et al.*<sup>[67]</sup> revealed that the CCA stem-like compartment is actively involved in both the recruitment of circulating monocytes and their differentiation into TAMs, owing to the release of IL-13, IL-34 and osteoactivin. Of note, cancer stem cell (CSC)-associated TAMs display unique phenotypic and functional features, namely mixed expression of M1 and M2 markers (e.g., M1-related chemokines CXCL9 and CXCL10, and M2-related chemokines CCL17 and CCL18), increased adhesive and invasive abilities, *in vitro*, and enhanced tumor-promoting functions, *in vivo*. This clearly highlights the existence of different TAM subsets within the tumor, depending on the multitude of microenvironmental cues originating from various cell niches.

### Evidence for the pro-neoplastic effects exerted by TAMs

In CCA tissues, M2 macrophages are definitely much more abundant than in the peritumoral areas, and TAM are mostly located at the leading edge of the tumor<sup>[67]</sup>. Consistently, immunohistochemical analyses in *Opisthorchis viverrini*-associated CCA in a hamster model revealed a progressive, dramatic increase in M2 macrophages through carcinogenesis<sup>[28]</sup>. Studies from different groups showed that a high density of TAMs at the invasive front correlated with poor survival of CCA patients after resection<sup>[64,68,69]</sup>. However, it is important to underline that not all of these studies provided evidence that the observed TAM actually exhibited the pro-neoplastic, M2 phenotype. Whereas Subimerb *et al.*<sup>[68]</sup> evaluated the expression of MAC387, a marker of recently infiltrated, bone marrow-derived, macrophages, Atanasov *et al.*<sup>[69]</sup> evaluated the expression of resident, CD68<sup>+</sup> macrophages. On the contrary, Hasita *et al.*<sup>[64]</sup> sought to distinguish M2 TAMs from total resident macrophages based on their expression of CD163, in order to highlight their specific contribution to tumor progression. They found that, in CCA tissues, the number of CD163<sup>+</sup> M2 cells was, as expected, lower than the number of CD68<sup>+</sup> cells, and that high infiltration of M2 macrophages, but not of total macrophages, was significantly associated with poor disease-free survival of patients. Of further interest, the density of M2 macrophages within CCA stroma also correlated with the presence of extrahepatic metastases<sup>[28]</sup>, the tumor pathological grade<sup>[67]</sup>, and the microvascular density<sup>[64]</sup>. Although these findings are

based on the evaluation of different phenotypic markers, overall, they suggest that TAMs strongly influence CCA progression, with a major role played by M2 TAMs, thus confirming what observed in other cancer types. In accordance with these immunohistochemical findings, conditioned medium from M2 macrophages boosted the migratory abilities of CCA cells by inducing EMT-like changes, *in vitro*<sup>[28]</sup>.

As previously mentioned, recruitment of circulating monocytes, rather than proliferation of resident macrophages, is the mechanism responsible for TAM accumulation in the TRS<sup>[57]</sup>. In fact, in CCA patients, levels of circulating CD14<sup>+</sup>/CD16<sup>+</sup> monocytes were increased, and correlated with high density of MAC387<sup>+</sup> TAMs, and with poor survival rates. CD14<sup>+</sup>/CD16<sup>+</sup> monocytes represent a minor subset of total monocytes, whose expansion is usually associated with acute or chronic inflammation. They are classically regarded as more mature cells than CD14<sup>+</sup>/CD16<sup>-</sup> monocytes, and thought to be the major precursors of tissue macrophages. Besides expressing a larger number of adhesion molecules, enabling them to strongly adhere to vascular endothelium, CD14<sup>+</sup>/CD16<sup>+</sup> monocytes also up-regulate the EGFR ligand epiregulin, and the angiogenic chemokine CXCL3. Overall, these features are consistent with the adoption of a pro-tumorigenic phenotype, likely induced by tumor-derived molecules, which may also drive their recruitment into the TRS<sup>[70]</sup>.

#### **Molecular players underlying the tumor-promoting effects of TAMs**

**MMP-9:** MMPs, in particular MMP-9, are the most important proteolytic enzymes in the context of tumor spread, and their overexpression tends to be predictive of worst outcome in human cancers. Besides underpinning cancer cell invasion through the selective deletion of ECM integrity, MMPs can also elicit the post-translational activation of growth factors and cytokines, thereby influencing key cellular processes<sup>[71]</sup>. Subimerb *et al.*<sup>[68]</sup> found that TAMs (especially those located at the tumor-host interface) rather than cancer cells represent the main source of MMP-9 in CCA. Moreover, CCA patients with high numbers of MMP-9<sup>+</sup> TAMs displayed significantly shorter survival times than those with low numbers, thus pointing out MMP-9 production as a key driver of CCA progression promoted by TAMs. Furthermore, a broad expression of other pivotal ECM remodeling-related genes, namely *MMP-2*, *ADAM10*, and *ADAM17* was reported in CSC-associated TAMs<sup>[67]</sup>.

**TNF- $\alpha$ :** TNF- $\alpha$  is a pleiotropic cytokine, acting as a central pro-inflammatory mediator in human carcinogenesis, wherein it was reported to play both anti-tumoral and pro-tumoral effects<sup>[72]</sup>. In CCA, as well as in the majority of carcinomas, TNF- $\alpha$  is widely expressed by macrophages located at the tumor edge, whereas it is only focally expressed by cancer cells<sup>[44]</sup>. Lipopolysaccharide (LPS)-activated macrophages were able to elicit EMT-like phenotypic changes in CCA cells

(namely, down-regulation of the epithelial markers E-cadherin and cytokeratin 19, along with up-regulation of the mesenchymal markers S100A4 and MMP-9), probably mediated by a TNF- $\alpha$ -induced activation of Snail and ZEB2 transcription factors<sup>[73-75]</sup>. Consistently, upon TNF- $\alpha$  stimulation, CCA cells gained increased migratory functions in conjunction with the activation of ERK, Akt and nuclear factor (NF)- $\kappa$ B<sup>[74,76]</sup>.

**IL-6:** Aberrant activation of the IL-6 classical downstream effector STAT3 is described in many epithelial cancers, and is currently regarded as a major oncogenic event<sup>[77]</sup>. For instance, in CCA patients, high expression of STAT3 by cancer cells was associated with poorly differentiated tumor phenotypes, as well as with low survival rates<sup>[78]</sup>. In particular, in CCA cells, increased cell survival by up-regulation of the anti-apoptotic protein myeloid cell leukemia-1 is the fundamental mechanism triggered by the IL-6/STAT3 axis<sup>[79]</sup>. In the hamster experimental model of *Opisthorchis viverrini*-induced CCA, STAT3 activation peaked at the pre-cancerous stage, in association with a high degree of inflammation. Consistently, conditioned medium from LPS-activated macrophages led to a robust STAT3 activation in CCA cells<sup>[78]</sup>, likely mediated by IL-6, whose secretion is potently stimulated by LPS<sup>[73]</sup>. Although IL-6 can be secreted even by CCA cells themselves, paracrine signaling is probably essential to reach broad STAT3 activation<sup>[79]</sup>, and TAMs may actually be central in this process.

**YKL-40:** YKL-40, also called chitinase 3-like 1, is a secreted glycoprotein, which is supposed to play key roles in different aspects of tumorigenesis, from cell proliferation and survival, to angiogenesis and ECM remodeling. Interestingly, YKL-40 serum levels are dramatically increased in patients with multiple chronic inflammatory diseases, such as liver cirrhosis, as well as in patients with several malignancies, including breast, lung and colorectal carcinomas<sup>[80]</sup>. In CCA patients, YKL-40 serum levels were actually much higher than those of healthy subjects, and also negatively correlated with overall survival. Importantly, within the tumoral area, CCA cells represent only minor contributors to YKL-40 production, which is primarily caused by infiltrating inflammatory cells, especially TAMs. Of further interest, exogenous YKL-40 stimulated CCA cell growth and migration, by triggering Akt and ERK1/2 activation<sup>[81]</sup>.

**Wnt3:** Involvement of Wnt/ $\beta$ -catenin pathway in CCA pathogenesis and progression is a well-established concept for many years<sup>[82,83]</sup>. Wnt family ligands are secreted glycoproteins modulating fundamental transcriptional programs by stimulating the nuclear translocation of  $\beta$ -catenin. In the basal conditions,  $\beta$ -catenin is mainly located at the cell-cell junctions, whereas a minor pool is sequestered in the cytoplasm by a destruction complex, where phosphorylation at specific residues (Ser 33/37 and Thr 41) is a pre-requisite to allow its inactivation

and proteasomal degradation. Binding of Wnt ligands to Frizzled receptors lets  $\beta$ -catenin to detach from the membrane, accumulate within the cytoplasm, and then translocate into the nucleus, where it interacts with several co-activators, among which T-cell factor and lymphoid enhancer-binding factor 1 are the main partner in gene regulation.  $\beta$ -catenin target genes encompass well-known proto-oncogenes relevant for CCA growth, such as c-Myc, cyclin D1 and ZEB1<sup>[84,85]</sup>. In CCA tissues,  $\beta$ -catenin is constitutively expressed at high levels either in the cytoplasm or in the nucleus of cancer cells, whereas its membranous expression is decreased, consistent with the activation of the Wnt signaling. Among Wnt ligands, whereas Wnt5a and Wnt7b are overexpressed by neoplastic bile ducts, TAMs represent a major source of Wnt3. Notably, conditioned medium from LPS-activated macrophages elicited  $\beta$ -catenin nuclear translocation in CCA cells, resulting in enhanced cell growth<sup>[86]</sup>.

## MSCs

MSCs are non-hematopoietic stem cells primarily resident in the bone marrow, where they are recruited from by chemotactic signals mainly originating from injured tissues and inflammatory sites. Indeed, MSCs are multipotent cells able to differentiate in a variety of cell types, thus being classically regarded as a valuable source of tissue replacement. However, under the influence of cancer-derived chemokines, MSCs can also home to primary tumor sites, wherein they eventually become an additional component of the tumor microenvironment. Tumor-resident MSCs have been also reported to perform several activities supporting cancer progression. For instance, they can interfere with anti-tumor immunity, promote angiogenesis, and directly enhance the aggressiveness of malignant cells through secreted factors<sup>[8,87,88]</sup>.

In nude mice bearing subcutaneous human CCA xenografts, it was shown that, upon infusion into the venous circulation, MSCs were able to selectively reach both the primary tumor and the metastatic liver, thus confirming their pronounced tumor tropism. Furthermore, exposure of CCA cell to conditioned medium from MSCs resulted in increased proliferation, apoptosis resistance, and invasiveness, likely due to the activation of the Wnt/ $\beta$ -catenin signaling. Consistently, subcutaneous co-injection of CCA cells with MSCs in immunodeficient mice led to accelerated tumor growth, and higher incidence of liver metastases, compared with mice inoculated with cancer cells alone<sup>[87]</sup>. Interestingly, the ability of MSCs to promote CCA cell proliferation was further strengthened by preliminary exposure of MSCs to cancer cell-derived extracellular vesicles (with features consistent with exosomes). Indeed, these vesicles induced profound changes in the MSC secretome, including increased secretion of IL-6, CCL2/MCP-1, CXCL1/GRO- $\alpha$ , CX3CL1/Fractalkine and PDGF-AA. Besides directly favoring tumor cell growth, MSCs may also represent an additional (although minor) source of CAFs, as conditioned medium from CCA cells prompted a phenotypic switch from MSCs

into myofibroblastic-like cells<sup>[88]</sup>.

## ECM

Besides providing a physical support to cells, the ECM (mainly consisting of collagens, glycoproteins and proteoglycans) also communicates straight with them, thereby modulating a variety of cellular functions, and acts as a paramount reservoir of cell-derived soluble factors<sup>[6]</sup>. Throughout carcinogenesis, the ECM gradually undergoes stiffening and profound compositional changes, resulting from the accumulation of secreted structural and non-structural proteins, in particular collagen type I and fibronectin, as well as of matrix modifying enzymes<sup>[19,22,89]</sup>. An abnormal ECM leads to a dysregulated behavior of both cancer and stromal cells, thereby affecting several processes related to tumor biology, including cancerous fibrogenesis, inflammation and angiogenesis<sup>[11,90]</sup>. Interestingly, ECM stiffness is emerging as a driving force behind cancer progression. As previously mentioned, tumor-associated ECM is typically stiffer than the normal matrix, due to a pathological remodeling mainly driven by neoplastic cells and CAFs. This stiff, collagen enriched ECM can signal to cells through specific mechanosensors, thus activating intracellular pathways regulating the acquisition of malignant phenotypic traits<sup>[90,91]</sup>. Among the intracellular sensors of ECM-driven mechanical stress, the transcriptional co-activator yes-associated protein (YAP) and its paralog, transcriptional co-activator with PDZ-binding motif (TAZ), are emerging as master directors of cancer cell reprogramming and enhanced invasiveness<sup>[92]</sup>. Indeed, high levels of cytoskeletal contractility, resulting from increased ECM rigidity, are generally coupled with the activation of YAP/TAZ, which can profoundly affect epithelial cell behavior, including the balance between proliferation and apoptosis<sup>[93,94]</sup>. Interestingly, in CCA, YAP overexpression was reported to enhance cancer cell proliferation, invasion (*via* EMT-like changes) and resistance to chemotherapeutic drugs, both *in vitro* and in tumor xenografts<sup>[95]</sup>. Therefore, it is tempting to speculate that, after being recruited by CCA cells through PDGF-DD<sup>[24]</sup>, CAFs may gradually manipulate ECM stiffening within the TRS, thereby inducing YAP/TAZ activation in cancer cells, leading to the emergence of a particularly aggressive tumor phenotype.

In CCA, interactions between tumor cells and specific molecular components of the ECM may trigger additional pathways of tumor invasiveness. In fact, CCA cells cultured on a reconstituted basement membrane preparation (mainly composed of collagen type IV and laminin), showed enhanced invasive properties compared with cells grown on uncoated culture plates. This was dependent on the dysregulated expression of a wide range of proteins, especially L-plastin, which is an actin-bundling protein supporting cell motility and adhesion. L-plastin is dramatically up-regulated in many types of malignant cells and, in CCA tissue, it is primarily expressed at the tumor front, thereby indicating its involvement in tumor invasion<sup>[96]</sup>. The ability of the TRS-associated ECM to

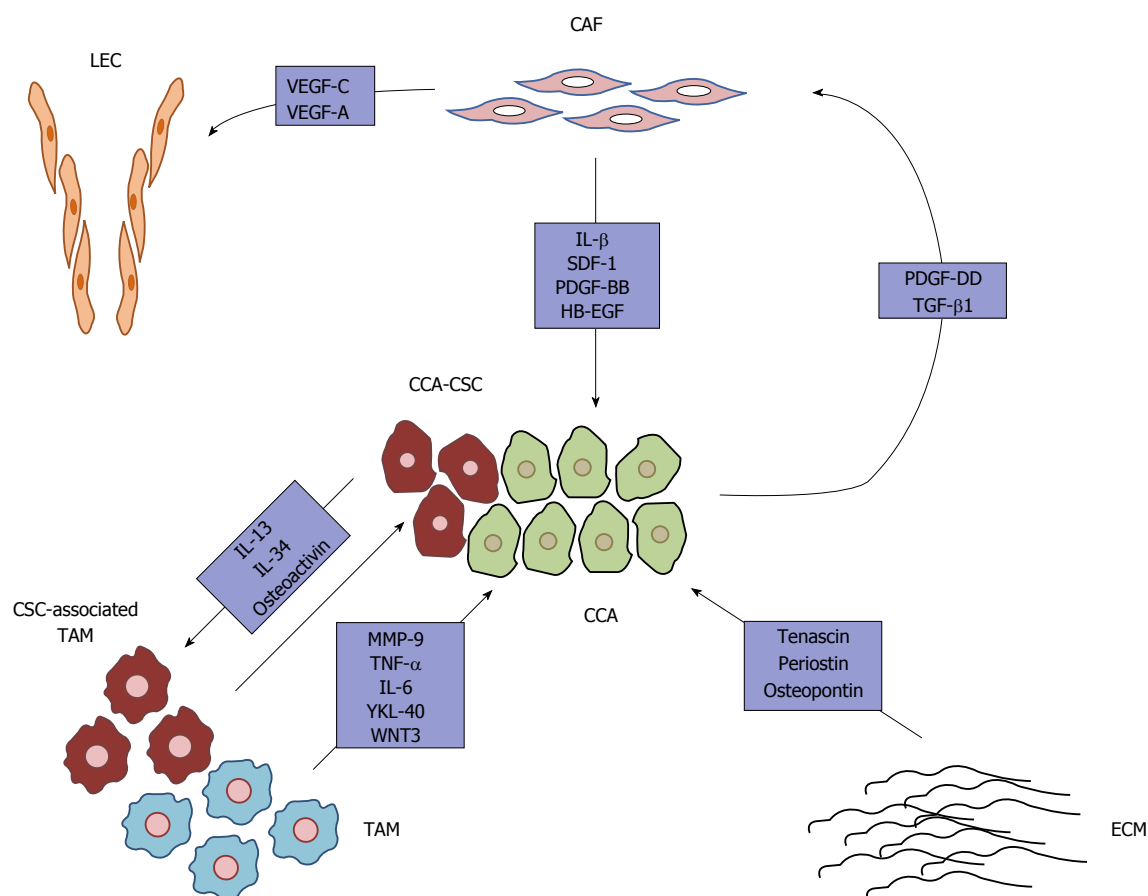
support cancer aggressiveness is also well exemplified by three fundamental non-structural ECM proteins, namely tenascin, periostin and osteopontin, reported as poor prognostic biomarkers for CCA patients. In CCA samples, tenascin is abnormally expressed in the intratumoral stroma, as well as at the tumor leading edge. Although CAFs undoubtedly represent the main source of tenascin, carcinoma cells can contribute to its biosynthesis. In CCA patients, aberrant deposition of tenascin at the invasive front positively correlated with tumor size and lymph node metastasis, and also predicted poor survival. It is worth noting that the expression pattern of tenascin roughly parallels that of EGFR, which tenascin can bind to, likely underpinning its tumor-promoting functions<sup>[97]</sup>. Similarly, high expression of periostin within the TRS, which is solely due to CAFs, was an independent prognostic factor for overall survival of CCA patients. Moreover, serum periostin levels were significantly higher in CCA patients compared with both healthy subjects and patients with other hepatic malignancies. Consistent with these findings, exogenous periostin induced CCA cell proliferation and invasion through its interaction with integrin receptors  $\alpha 5\beta 1$  and  $\alpha 6\beta 4$ , leading to the activation of the PI3K/Akt pathway, *in vitro*<sup>[38,98,99]</sup>. High stromal expression of osteopontin is also an independent risk factor for reduced overall and disease-free survival in CCA patients, positively correlating with both tumor size and the presence of lymph node or macrovascular invasion<sup>[20]</sup>.

## THE TRS AS POTENTIAL THERAPEUTIC TARGET

Classically, anticancer therapies aim at targeting intrinsic traits of neoplastic cells, which, until recently, were actually seen as the only players deserving attention in the context of clinical management. However, in CCA, a lethal malignancy paradigmatic of the strong resistance to conventional chemotherapy, mounting evidence supports the role of tumor microenvironment in dictating tumor growth, progression and metastatic dissemination. Indeed, CCA cells establish intense, mutual, paracrine communications with neighboring stromal components, in particular CAFs and TAMs, which are a rich source of signals promoting malignancy (Figure 1). Therefore, combinatorial therapies that both directly tackle tumor growth and turn off the tumor-promoting functions of the TRS might represent an important step forward in anticancer treatment, especially in CCA. In addition to provide a number of druggable targets, TRS may help to identify (by gene expression profiling) molecular signatures serving as novel prognostic biomarkers, useful for predicting therapeutic response or monitoring tumor recurrence, as it could be the case with periostin<sup>[8,63,89]</sup>. It is worth noting that, unlike cancer cells, which undergo multiple genetic/epigenetic changes giving rise to a tremendously heterogeneous population, stromal cells represent a genetically stable, more uniform compartment,

and thus stand out as viable and compelling therapeutic targets<sup>[12,37]</sup>. Basically, TRS-oriented therapeutic approaches should aim at: (1) hampering the recruitment of reactive stromal cells by counteracting tumor-derived chemokines; (2) promoting TRS depletion by eliciting apoptosis of its cellular components; (3) interfering with the intracellular pro-oncogenic pathways triggered by the TRS within the cancer cell; and (4) interfering with the paracrine communications between stromal and cancer cells, by neutralizing specific soluble factors or antagonizing their cognate receptors<sup>[6]</sup>. The study performed by Mertens *et al.*<sup>[37]</sup> is an archetype of these potential new strategies. Using the BH3 mimetic navitoclax (a small molecule mimicking the pro-apoptotic protein Bad), the authors were able to selectively induce Bax-dependent apoptosis in CCA-derived CAFs, but not in normal fibroblasts or CCA cells, *in vitro*. By translating these findings in an *in vivo*, orthotopic syngeneic rat model of CCA, navitoclax markedly reduced tumor growth and metastasis, and significantly improved survival, an effect related to a quantitative depletion of CAFs from the stroma. Taking a different approach, the mammalian target of rapamycin inhibitor everolimus, in addition to directly reduce CCA cell proliferation and invasion<sup>[100]</sup>, was reported to hamper the cross-talk between CAFs and CCA cells, by both impairing the activation of CAF-induced mitogenic pathways in cancer cells, and inhibiting the secretion of tumor-promoting cyto/chemokines by CAFs<sup>[30]</sup>. Interestingly, everolimus is already an FDA-approved drug for the treatment of breast, neuroendocrine and renal cell carcinomas<sup>[101]</sup>. By turning to TAMs, it was shown that liposome-encapsulated clodronate, a selective macrophage-depleting agent, as well as GW2580 or AZD7507, small molecules preventing monocyte-to-macrophage differentiation, significantly reduced the growth of subcutaneous human CCA xenografts. Moreover, the tumor-suppressive effect of liposomal clodronate was also confirmed in a non-transgenic, thioacetamide-induced rat model of CCA, which faithfully reproduces the inflammatory and desmoplastic microenvironment associated with human CCA<sup>[102]</sup>. Noteworthy, besides priming TAMs for apoptosis or blocking monocyte recruitment, it might be possible to harness the inherent plasticity of macrophages in order to revert their polarization from the pro-neoplastic M2 phenotype to the anti-tumoral M1 phenotype<sup>[12,57]</sup>. However, the development of combinatorial therapies targeting both tumor and stromal cells must be rooted in a deep knowledge of the epithelial-mesenchymal interactions occurring within the CCA microenvironment, which is not possible without proper experimental models. In this regard, two-dimensional co-culture systems and, even more, three-dimensional organotypic culture models represent powerful tools for investigation, but, of course, they cannot fully reproduce the complexity of the TRS, which integrate a multitude of cell elements. On the other hand, rodent models of CCA more closely mimic the structural and functional heterogeneity of the TRS, even though the murine environment may not accurately





**Figure 1 Soluble factors derived from reactive stromal cells, along with tumor-associated extracellular matrix, sustain cholangiocarcinoma cell malignancy.** CCA cells shape the surrounding microenvironment to meet their highly demanding needs, thus providing CAFs and TAMs with the ability to secrete a broad range of cyto/chemokines, growth factors, morphogens and proteinases, which boost cancer cell proliferation, survival and invasiveness. In this model, CAFs are recruited by PDGF-DD released by CCA cells. In addition, TGF-β1, also derived by CCA cells, stimulates CAFs to produce the EGFR ligand, HB-EGF, which triggers the acquisition of malignant behaviors (*i.e.*, EMT-like changes) by cancer cells. CAF-derived tumor-promoting molecules also include IL-1β, SDF-1 and PDGF-BB. Moreover, PDGF-DD induces CAFs to acquire pro-lymphangiogenic functions (exerted by VEGF-A and VEGF-C). On the other hand, TAMs, displaying a predominant M2 phenotype, also support tumor survival and invasiveness by secreting several soluble factors, including MMP-9, TNF-α, IL-6, YKL-40 and Wnt3. Of note, the CCA stem-like compartment molds a specific subset of TAMs (through secretion of IL-13, IL-34 and osteoactivin), displaying a mixed M1/M2 phenotype, to promote self-renewal and drug-resistance properties. In addition, non-structural proteins expressed by the abnormally remodeled ECM (tenascin, periostin, osteopontin) further enhance CCA aggressiveness. CAF: Cancer-associated fibroblast; CCA: Cholangiocarcinoma; CSC: Cancer stem cell; ECM: Extracellular matrix; LEC: Lymphatic endothelial cell; TAM: Tumor-associated macrophage; IL: Interleukin; TGF: Transforming growth factor; TNF: Tumor necrosis factor; PDGF: Platelet-derived growth factor; EGFR: Epidermal growth factor receptor; MMP-9: Matrix metalloproteinase 9.

reproduce the wide range of paracrine communications occurring in the human disease setting, all the more so in xenograft models, where the host is immunodeficient<sup>[8,11]</sup>.

## CONCLUSION

Unravelling the complex mechanisms underlying the mutual interactions between the tumoral and stromal compartments is indeed a topic of great translational significance, worth being pursued further in the next future. Based on the data discussed above, specific targeting of the signals operating in the tumor micro-environment, coupled with conventional anticancer treatments, could actually open new promising and feasible therapeutic avenues in CCA, hopefully expandable to other aggressive desmoplastic epithelial malignancies, such as pancreas and breast carcinomas. It is tempting to speculate that these innovative, multitargeted the-

rapies might more effectively eradicate tumor cells, owing to concurrent switching-off actions on intrinsic (cancer cell-dependent) as well as extrinsic (TRS-derived) tumor-promoting mechanisms, eventually leading to improved patient outcomes.

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Case Control Study

## Annexin A2 as a biomarker for hepatocellular carcinoma in Egyptian patients

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### Abstract

#### AIM

To investigate the clinical utility of serum annexin A2 (ANXA2) as a diagnostic marker for early hepatocellular carcinoma (HCC).

#### METHODS

This study was performed in HCC Clinic of Ain Shams University Hospitals, Cairo, Egypt and included: Group 1: Fifty patients with early stage HCC (Barcelona Clinic Liver Cancer stage A); Group 2: Twenty five patients with chronic liver disease; and Control Group: Fifteen healthy, age- and sex-matched subjects who were seronegative for viral hepatitis markers. The following

laboratory investigations were done: Viral hepatitis markers [hepatitis B surface antigen and hepatitis C virus (HCV) antibodies], HCV RNA in HCV antibody-positive patients, serum alpha fetoprotein (AFP), and serum ANXA2 levels.

## RESULTS

In this study, 88% of HCC patients ( $n = 44$ ) were HCV-positive, while HBV infection represented only 8% of all HCC patients ( $n = 4$ ); and two patients were negative for both viral markers. A highly significant difference was found between patients with HCC and chronic liver disease as well as controls with regard to serum ANXA2 levels (130, IQR 15-240; 15, IQR 15-17; and 17, IQR 15-30 ng/mL, respectively). The area under the curve of ANXA2 was 0.865; the cut-off value was established to be 18 ng/mL with a diagnostic sensitivity of 74% and a specificity of 88%, while the sensitivity and specificity of AFP at the cut-off value of 200 ng/dL were 20% and 100%, respectively.

## CONCLUSION

Serum ANXA2 may serve as a biomarker for the early detection of HCC.

**Key words:** Hepatocellular carcinoma; Hepatitis C virus; Annexin A2; Alpha-fetoprotein; Tumor markers

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**Core tip:** Thirty percent of hepatocellular carcinoma (HCC) patients present with normal serum alpha fetoprotein, which highlights the need for new biomarkers for HCC. In the present study, a highly significant difference was observed among patients with HCC and chronic liver disease as well as controls with regard to serum annexin A2 (ANXA2) levels (130, IQR 15-240; 15, IQR 15-17; and 17, IQR 15-30 ng/mL, respectively). The area under the curve of ANXA2 was 0.865; the cut-off value was 18 ng/mL with a diagnostic sensitivity of 74% and specificity of 88%. Thus, ANXA2 may serve as a useful biomarker for the early detection of HCC.

Shaker MK, Abdel Fattah HI, Sabbour GS, Montasser IF, Abdelhakam SM, El Hadidy E, Yousry R, El Dorry AK. Annexin A2 as a biomarker for hepatocellular carcinoma in Egyptian patients. *World J Hepatol* 2017; 9(9): 469-476 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i9/469.htm> DOI: <http://dx.doi.org/10.4254/wj.h.v9.i9.469>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. According to the National Institute of Cancer in Egypt, HCC is considered one of the commonest malignancies in Egypt as a result of the high prevalence of hepatitis B and C infections, since these

represent approximately 45.3% of all new cases of this type of cancer<sup>[1]</sup>.

Because of the asymptomatic nature of early HCC as well as the lack of its effective screening strategies; most patients (> 80%) present with an overt advanced disease<sup>[2]</sup>. Approximately 30% of HCC cases with normal serum alpha fetoprotein (AFP) levels are diagnosed before the appearance of clinical manifestations, and this highlights the need for new and early reliable biomarkers for the detection of HCC<sup>[3]</sup>.

Annexins are a family of proteins that bind anionic phospholipids in a calcium-dependent manner. Annexins were first discovered in animal cells and were named for their ability to "annex" or aggregate membranes. The annexins are expressed in vertebrates (ANXA), invertebrates (ANXB), fungi and protozoa (ANXC), plants (ANXD) and protists [e.g., algae (ANXE)]<sup>[4,5]</sup>.

Annexin A2 (ANXA2) is primarily expressed in human endothelial cells, mononuclear cells, macrophages, marrow cells and some tumor cells<sup>[6]</sup>. Moreover, ANXA2 is an inducible, calcium-dependent phospholipid-binding protein that is overexpressed in a variety of human malignancies and has emerged as an attractive candidate receptor for increased plasmin generation on the tumor cell surface<sup>[7]</sup>. It plays multiple roles in the regulation of cellular functions including angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion<sup>[8,9]</sup>.

ANXA2 is almost undetectable in the normal liver and in chronic hepatitis tissues, while it is highly expressed in HCC<sup>[10]</sup>; moreover, serum levels of ANXA2 are elevated in patients with early stage HCC who are AFP-negative<sup>[11]</sup>. ANXA2 was reported to promote HCC metastasis and invasion through its interaction with HAb18G/CD147 (a member of the immunoglobulin family of proteins)<sup>[6]</sup>. Nevertheless, the importance of the change in serum levels of ANXA2 in the early stages of HCC has yet to be elucidated.

This study aimed to determine the clinical utility of the serum level of ANXA2 as a diagnostic biomarker of HCC and to correlate its level with that of AFP.

## MATERIALS AND METHODS

This prospective case control study was conducted at the HCC clinic, Departments of Tropical Medicine and Clinical Pathology; Ain Shams University Hospitals (Cairo, Egypt), after approval from the Research and Ethics Committee of Ain Shams University was obtained in accordance with local research governance requirements. This study was performed in accordance with the 1964 Declaration of Helsinki and all subsequent revisions.

This study included two patient groups. Group 1: Fifty patients with early stage HCC on top of chronic liver disease (CLD) Child-Pugh class A and B. They were diagnosed according to the Barcelona Clinic Liver Cancer (BCLC) staging system (BCLC A)<sup>[12]</sup>; and Group 2: Twenty-five patients with CLD (without HCC), their diagnosis was based on clinical, laboratory, and ultrasonographic findings.

**CLD in this study represented patients with:** (1) persistent viral infection [hepatitis C virus (HCV) and/or hepatitis B virus (HBV)] or affected liver functions for more than 6 mo; and (2) ultrasound features suggestive of CLD: Coarse liver echo-texture, dilated portal vein, attenuated hepatic veins, splenomegaly and/or ascites.

**Inclusion criteria for HCC group:** (1) confirmed diagnosis of HCC according to the European Association for the Study of Liver Diseases<sup>[12]</sup>; (2) early stage HCC (Stage A) according to the BCLC staging system (single or 3 nodules < 3 cm; Performance Status 0)<sup>[12]</sup>; and (3) informed consent from all participants before enrollment in the study.

**Exclusion criteria for HCC group:** (1) intermediate or advanced stage HCC as defined by the BCLC staging system; (2) major vascular tumor invasion or metastasis as confirmed by radiological imaging studies; (3) patients with other suspected solid malignancies or liver metastasis; and (4) other types of CLD as autoimmune hepatitis.

In addition, fifteen age- and sex-matched healthy persons were enrolled, constituting the control group. The healthy controls were collected from the outpatient clinics among those coming for pre-employment screenings. Liver and systemic diseases were excluded by history, physical examination, laboratory and radiologic assessment.

**All included patients and control subjects were subjected to the following:** (1) full medical history and thorough clinical examination; and (2) laboratory investigations included: Liver function tests to determine the levels of aspartate aminotransferase, alanine aminotransferase, serum bilirubin (total and conjugated), serum albumin, and prothrombin time; viral markers hepatitis B surface antigen, and HCV by enzyme linked immunosorbent assay (ELISA) and detection of HCV RNA by real-time polymerase chain reaction; detection of the serum AFP level by electro-chemiluminescence; and determination of the serum concentration of ANXA2 by ELISA.

### Samples

A total of 5 mL of venous blood was withdrawn from each subject under complete aseptic conditions. Then, 1.8 mL was placed into sodium citrate (3.2%) tubes in a ratio of 9:1 (blood: Citrate) for the PT assay. The remainder of the blood was placed in sterile vacutainers with a clot activator and was left to clot for 30 min. The serum was then separated by centrifugation at  $1000 \times g$  for 15 min and was divided into two aliquots: One aliquot was used for the immediate routine liver function tests and serum AFP detection, while the remaining portion of sera was stored as an aliquot at  $-20^{\circ}\text{C}$  until future use (*i.e.*, the detection of the serum level of ANXA2). Frozen samples

were allowed to thaw to room temperature just prior to the analysis. Hemolyzed samples were discarded, and repeated freezing and thawing was avoided.

### Analytical methods

**AFP:** Quantitative determination of AFP was conducted in an Immulite immunoassay auto analyzer using an AFP kit supplied by DPC (DIAGNOSTICA Product Corporation, Los Angeles, CA, United States). This assay is based on an electro-chemiluminescence immunoassay technique. The antigen (sample), a biotinylated monoclonal AFP-specific antibody and a monoclonal AFP-specific antibody labeled with a ruthenium complex react to form a sandwich complex. Streptavidin-coated microparticles were added, and the complex was then bound to a solid phase *via* the interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell. The application of a voltage to the electrode then induced chemiluminescent emission, which was measured by a photomultiplier. The results were determined *via* a calibration curve that was specifically generated by 2-point calibration and a master curve provided by the reagent barcode.

**ANXA2 assay:** This assay was performed with a commercially available ELISA kit supplied by Glory Science (Glory Science Co., Del Rio, TX, United States). This assay employs a quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ANXA2 is pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ANXA2 that is present becomes bound by the immobilized antibody. After any unbound substances are washed away, an enzyme-linked monoclonal antibody specific for ANXA2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of ANXA2 bound in the initial step. The color development is then stopped and the intensity of the color is measured at  $450\text{ nm}$ <sup>[13]</sup>.

Radiological investigations included abdominal ultrasound and triphasic spiral abdominal computed tomography (CT) to confirm the diagnosis and staging of HCC.

### Statistical analysis

IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, United States) was used for the data analysis. The quantitative variables were presented as the mean and the standard deviation, while the qualitative variables were presented as frequencies and percentages. The values of skewed parameters were expressed as the median and IQR ( $25^{\text{th}}$ - $75^{\text{th}}$ ). An unpaired *t* test (*t* value) was used to compare a quantitative variable between two independent groups for parametric data, while a Mann-Whitney test (*Z* value)



**Table 1** Descriptive statistical data of the various parameters in the three studied groups<sup>1</sup>

Parameter	HCC (n = 50)	CLD (n = 25)	Control (n = 15)
ALT (U/L)	43 (31-72.5) <sup>2</sup>	31 (22.5-41) <sup>2</sup>	25 (17-29) <sup>2</sup>
AST (U/L)	60 (42.25-97.25) <sup>2</sup>	45 (41-61.5) <sup>2</sup>	26 (21-35) <sup>2</sup>
PT (s)	13.8 ± 1.53	16 ± 3.37	12 ± 0.1
Alb (g/dL)	3.25 ± 0.53	2.75 ± 0.65	3.8 ± 0.28
T.Bil (mg/dL)	1.33 (1-2.2) <sup>2</sup>	2 (1.15-2.9) <sup>2</sup>	0.8 (0.6-0.9) <sup>2</sup>
D.Bil (mg/dL)	0.55 (0.29-0.9) <sup>2</sup>	0.6 (0.4-1.3) <sup>2</sup>	0.1 (0.1-0.2) <sup>2</sup>
AFP (ng/mL)	41.5 (8.4-191.25) <sup>2</sup>	8.5 (4.1-12.5) <sup>2</sup>	3.1 (2.3-4.6) <sup>2</sup>
ANXA2 (ng/mL)	130 (15-240) <sup>2</sup>	15 (15-17) <sup>2</sup>	17 (15-30) <sup>2</sup>

<sup>1</sup>Values are given as the mean ± SD; <sup>2</sup>Values are given as the median (IQR). HCC: Hepatocellular carcinoma; CLD: Chronic liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; INR: International normalized ratio; Alb: Albumin; T.Bil: Total bilirubin; D.Bil: Direct bilirubin; AFP: Alpha-fetoprotein; ANXA2: Annexin A2; IQR: Interquartile range.

**Table 2** Comparison of the different studied groups with regard to alpha-fetoprotein and annexin A2<sup>1</sup>

Parameter	HCC vs control		CLD vs control		HCC vs CLD	
	Z value	P value	Z value	P value	Z value	P value
AFP (ng/mL)	-5.006	< 0.01 <sup>2</sup>	-3.4	< 0.01 <sup>2</sup>	-4.17	< 0.01 <sup>2</sup>
ANXA2 (ng/mL)	-3.5	< 0.01 <sup>2</sup>	-1.6	> 0.05	-4.8	< 0.01 <sup>2</sup>

<sup>1</sup>Wilcoxon Rank Sum test (non-parametric data); <sup>2</sup>Statistically significant difference. AFP: Alpha-fetoprotein; ANXA2: Annexin A2; HCC: Hepatocellular carcinoma; CLD: Chronic liver disease.

was used instead of the *t* test to compare a quantitative variable between two independent groups when the data were non parametric (SD > 25% of mean). A  $\chi^2$  test ( $\chi^2$  value) was used to compare a qualitative variable between two independent groups. The Spearman correlation test (rho value) was used to rank different non parametric variables against each other, either positively or inversely.

A *P* value < 0.05 was considered significant. The diagnostic accuracy of AFP and ANXA2 was determined by a receiver operating characteristic (ROC) curve analysis, the area under the curve (AUC) and its 95% confidence interval. The diagnostic cut-off, the related sensitivity and specificity, and the positive and negative predictive values (PPV, NPV) were also determined.

The statistical methods of this study were reviewed by Ahmed Mohamed Kamal, consultant in Biostatistics, Ain Shams University; Cairo, Egypt.

## RESULTS

The demographic features of the included population were as follows: 33 males (66%) and 17 females (34%) with an age range from 28 to 62 years in Group 1 and 13 males (52%) and 12 (48%) females with an age range from 22 to 68 years in Group 2. The control group included 15 healthy subjects (11 males and 4 females) with an age range between 22 and 60 years.

Group 1 included 50 patients with early stage HCC on top of CLD. Among them, 44 patients (88%) were HCV-positive, 4 patients (8%) had HBV infection and two patients (4%) were negative for both viral markers and were diagnosed as cryptogenic cirrhosis.

Group 2 (CLD group) included 25 patients with

CLD only (without HCC). All patients (100%) in this group were having HCV infection.

Descriptive statistics of the different laboratory parameters in all of the patient groups and the controls are shown in Table 1, Figures 1 and 2.

Comparative statistics between various groups in terms of AFP and ANXA2 using the Wilcoxon Rank Sum test (non-parametric data) are shown in Table 2. In regard to AFP, highly significant increases were observed in patients with HCC compared with patients with CLD (*P* < 0.01) and compared with controls (*P* < 0.01). In addition, the level of AFP was significantly higher in patients with CLD compared with the control group (*P* < 0.01). In regard to ANXA2, highly significant increases were observed in patients with HCC compared with patients with CLD (*P* < 0.01) and compared with controls (*P* < 0.01). However, a non-significant difference was observed between patients with CLD and controls with respect to ANXA2 (*P* > 0.05).

The correlation analysis between ANXA2 and AFP in both HCC (*r* = 0.22; *P* = 0.124) and CLD (*r* = 0.28; *P* = 0.173) patients using Spearman's rank correlation test revealed no statistically significant differences.

ROC curve analysis was performed to assess the diagnostic performance of AFP and ANXA2 in the discrimination of patients with HCC from those with CLD. This analysis revealed that the best cut-off value for AFP was 19.8 ng/mL, with a diagnostic sensitivity of 70%, a specificity of 96%, a PPV of 97.2%, a NPV of 61.5% and an efficacy of 78.7%. While the sensitivity and specificity of AFP at the cut-off value 200 ng/mL (the standard cut-off value used to diagnose HCC) was 20% and 100%, respectively, and the PPV was 100% and the NPV was 50%. The best cut-off value for ANXA2 was 18 ng/mL,

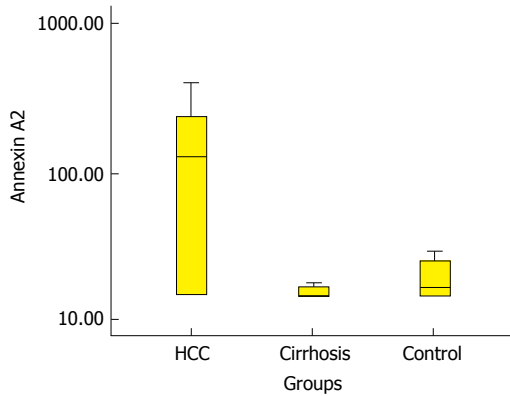


Figure 1 Box-plot diagram that shows the annexin A2 level in the three groups. HCC: Hepatocellular carcinoma.

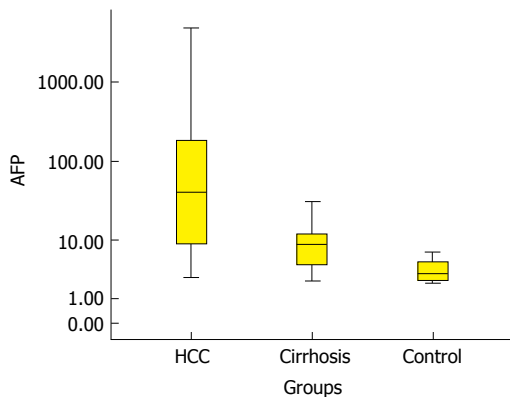


Figure 2 Box-plot diagram shows the alpha-fetoprotein level in the three groups. AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

the diagnostic sensitivity was 74%, the specificity was 88%, the PPV was 92.5%, the NPV was 62.9% and the efficacy was 78.7% (Figure 3 and Table 3).

## DISCUSSION

HCC is the fifth most common cancer and is the third leading cause of cancer death worldwide<sup>[14]</sup>. Unlike other solid malignancies, the coexistence of inflammation and cirrhosis makes an early diagnosis and prognostic assessment of HCC much more difficult<sup>[15]</sup>. In addition, the conventional tests of hepatic function do not distinguish HCC from cirrhosis, and thus they contribute little to the diagnosis of such tumor<sup>[16]</sup>.

Detection of circulating markers is the most effective method because it is simple, accurate and cost-effective, but no ideal biomarker has been found thus far<sup>[7]</sup>. For this reason, early diagnosis of HCC is critical to ensure a good prognosis. Worldwide, ongoing and continuous studies will determine and evaluate sensitive and specific new diagnostic markers for HCC.

The imaging-based diagnosis of small tumors is relatively inaccurate, as cirrhotic and dysplastic nodules can resemble HCC, and hence, a given imaging modality cannot always differentiate between benign hepatic

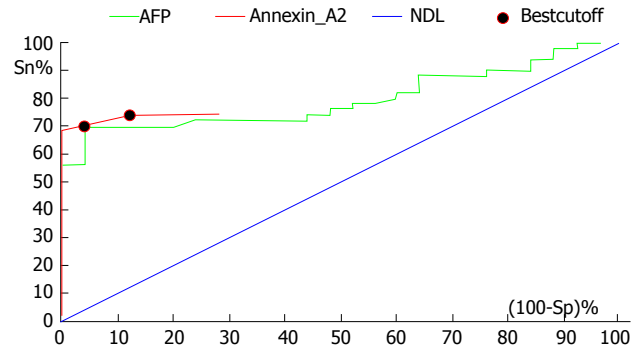


Figure 3 Receiver operating characteristic curve analysis shows the diagnostic performance of alpha-fetoprotein and annexin A2 in the discrimination of patients with hepatocellular carcinoma from those with chronic liver disease. AFP: Alpha-fetoprotein; NDL: Non-discriminating line (Diagonal line).

lesions and HCC; as a result, early and small lesions might be overlooked<sup>[17]</sup>.

Since AFP was discovered in the serum of individuals with HCC in 1964, it has been regarded as the most useful serum protein marker for patients at risk for HCC. However, its sensitivity for the detection of HCC ranges between 25%-60%, and its specificity is also low since serum AFP can also be detected in patients with cirrhosis (11%-47%) and chronic hepatitis (15%-58%)<sup>[15]</sup>. In addition, highly and poorly differentiated HCC cells usually produce little AFP in contrast to the high levels that are synthesized by moderately differentiated HCC cells<sup>[18]</sup>. Therefore, the positive rate of AFP in the diagnosis of HCC is generally only 60%-70%. The availability of a more sensitive serological marker that distinguishes between HCC and benign hepatic lesions would therefore be very useful and essential for early and specific diagnosis<sup>[19]</sup>. Unfortunately, surveillance programs are hindered by the poor performance of the commonly used serum markers, namely AFP<sup>[20]</sup>, even in combination with abdominal ultrasound. A great effort has been put forth and continues to be applied in the search for better HCC biomarkers.

ANXA2 is a 36-kDa calcium and phospholipid binding cytoskeletal protein of the Annexin superfamily that is localized to the extracellular surface of endothelial cells and various types of tumor cells<sup>[21]</sup>. Many reports have shown that ANXA2 is differentially expressed between normal and malignant tissues and is potentially involved in tumor progression<sup>[22]</sup>. The increased expression of ANXA2 was reported in cancers of the breast, liver, prostate and pancreas. ANXA2 has also been demonstrated to play a role in processes that are essential for cancer metastasis, such as tumor cell migration, invasion, and adhesion<sup>[5]</sup>.

The purpose of this study was to determine the clinical utility of the serum level of ANXA2 as a diagnostic marker of HCC and to correlate its level with that of alpha fetoprotein, which is currently the most widely used marker for HCC.

Our study revealed that 88% of patients with HCC were HCV-positive, while only 8% of patients with

**Table 3** Diagnostic performance of serum alpha-fetoprotein and annexin A2 in the discrimination of patients with hepatocellular carcinoma from those with chronic liver disease

Variable	Cut-off	Sn (%)	Sp (%)	NPV (%)	PPV (%)	Efficacy
AFP (ng/mL)	19.8	70	96	61.5	97.2	78.7
ANXA2 (ng/mL)	18	74	88	62.9	92.5	78.7

AFP: Alpha-fetoprotein; ANXA2: Annexin A2; Sn: Sensitivity; Sp: Specificity; NPV: Negative predictive value; PPV: Positive predictive value.

HCC were HBV-positive. This was in agreement with the results of El-Serag<sup>[2]</sup>, Zidan *et al.*<sup>[23]</sup> and Zekri *et al.*<sup>[24]</sup> and reflects the close relationship between HCV and HCC. The prevalence of HCV infection in Egypt is high and its percentage in patients who develop HCC is higher than that in patients in other countries<sup>[25,26]</sup>.

Our results revealed highly significant increases in the levels of AFP in patients with HCC compared with patients with CLD and subjects in the control group; this result was in agreement with that of El-Tayeh *et al.*<sup>[27]</sup> and Awadallah *et al.*<sup>[28]</sup>. They explained their results by an increase in the selective transcriptional activation of the AFP gene in malignant hepatocytes, which resulted in the increased secretion of AFP during the development of HCC.

Additionally, a highly statistically significant difference was observed between patients with CLD and control subjects with respect to AFP; this was in agreement with the result of Page *et al.*<sup>[14]</sup>, who declared that one of the limitations in the use of AFP for the diagnosis of HCC is its increase in patients who have hepatitis and CLD but who do not have HCC. El-Serag<sup>[2]</sup>, stated that hepatic injury and regeneration alone (such as during active hepatitis C virus infection) can increase the serum levels of AFP in patients who do not have HCC. In 2011, AASLD guidelines<sup>[29]</sup> omitted AFP from the algorithm for surveillance and diagnosis of HCC.

By contrast, we found that ANXA2 levels were highly and significantly increased in patients with HCC compared with the levels in patients with CLD and in controls; however, no statistical significance was found between patients with CLD and the controls with respect to ANXA2 expression. This was in agreement with that of Zhang *et al.*<sup>[6]</sup>, Liu *et al.*<sup>[22]</sup>, Ibrahim *et al.*<sup>[30]</sup> and Wang and Lin<sup>[31]</sup>.

This was explained by Zhang *et al.*<sup>[6]</sup> who stated that the ANXA2 gene is up-regulated in HBV- and/or HCV-associated HCC. In addition, Mohammad *et al.*<sup>[32]</sup>, stated that ANXA2 is rarely detected in either normal or chronic hepatic tissues but is over expressed at both the mRNA and protein levels in tumor and non-tumor regions of HCC (primarily localized within cancer cells).

It has been shown that the increased ANXA2 in HCC featured phosphorylation of its tyrosine residues. Interestingly, the tyrosine phosphorylation of ANXA2 was detected in HCC but not in cirrhotic tissue. These data suggest that tyrosine phosphorylation is an important event in hepatocarcinogenesis. It has been reported that ANXA2 is an excellent substrate for Src kinase. Mohammad *et al.*<sup>[32]</sup> reported that the level of Src kinase

activity in HCC is higher than that in cirrhotic tissues. These data suggest that ANXA2 in HCC may be tyrosine-phosphorylated *via* the elevated tyrosine kinase activity of Src or other kinases, and that increased levels of ANXA2 and the phosphorylation of its tyrosine residues may be related to human hepatocarcinogenesis.

The present study revealed no significant correlation between ANXA2 and AFP in either the CLD or the HCC group; this agrees with the study by Sun *et al.*<sup>[9]</sup>.

The clinical utility of AFP and ANXA2 in the discrimination of patients with HCC from those with CLD was assessed by ROC curve analysis. This revealed that the best diagnostic cut-off value of AFP for the discrimination of patients with HCC from those with CLD was 19.8 ng/mL. This had a diagnostic sensitivity of 70%, a specificity of 96%, a PPV of 97.2%, a NPV of 61.5% and an efficacy of 78.7% (AUC = 0.822). In regards to ANXA2, the best cut-off value was 18 ng/mL. This had a diagnostic sensitivity of 74%, a specificity of 88%, a PPV of 92.5%, a NPV of 62.9% and an efficacy of 78.7% (AUC = 0.873). In accordance with our results, Ibrahim *et al.*<sup>[30]</sup> found that the AUC for AFP was 0.84 and that for ANXA2 was 0.89. In another Egyptian study, AUC for ANXA2 was 0.910 (95%CI: 0.84-0.97). Combining both ANXA2 and AFP increased the diagnostic efficiency (98% specificity and 97.6% PPV)<sup>[33]</sup>.

In conclusion, our data show that the serum level of ANXA2 might be a good biomarker for the early detection of HCC since it had a higher sensitivity, specificity, and positive and negative predictive values than AFP. ANXA2 could differentiate between HCC and CLD since we found that the levels of ANXA2 were significantly higher in patients with HCC than in CLD patients and in controls. An in-depth analysis of the dynamic changes in serum ANXA2 in both normal and disease conditions as well as a future trial that includes a larger number of patients are emphasized.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. Approximately 30% of individuals with HCC present with normal levels of

serum alpha fetoprotein (AFP), and therefore, this highlights the need for new biomarkers for HCC.

## Research frontiers

This research study was conducted at the HCC clinic, Ain Shams University Hospitals, Faculty of Medicine, Cairo, Egypt to investigate the potential role of annexin A2 (ANXA2) as a new biomarker for HCC. Compared with AFP, the results were encouraging. A future trial that involves a larger number of patients and the combination of both markers to increase the diagnostic accuracy is strongly recommended.

## Innovations and breakthroughs

The literature suggests a benefit for ANXA2 as a potential tumor marker for HCC. The current trial adds that the level of ANXA2 in patients with chronic liver disease and healthy controls was significantly lower than that in patients with HCC.

## Applications

The authors' data show that the serum level of ANXA2 might be a good marker for HCC because it has a higher sensitivity, specificity, and positive and negative predictive values than AFP. ANXA2 may serve as a marker for the early detection of HCC and for the differential diagnosis between HCC and CLD because they found that the levels of ANXA2 were significantly higher in patients with HCC than in patients with CLD and in controls. An in-depth analysis of the dynamic changes in serum ANXA2 in both normal and disease conditions is therefore warranted.

## Terminology

ANXA2 is an inducible, calcium-dependent phospholipid-binding protein that is overexpressed in a variety of human malignancies and has emerged as an attractive candidate receptor for increased plasmin generation on the tumor cell surface. It plays multiple roles in the cellular angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion.

## Peer-review

The authors prospective case control study investigated 50 early stage HCC, 25 CLD and 15 healthy age-, sex- matched subjects with seronegative viral markers of hepatitis and normal liver function. AFP and ANXA2 were measured from each subject. Authors concluded that ANXA2 at cut-off value of 18 ng/mL was a good diagnostic marker for early HCC.

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Observational Study

# Characteristics of escape mutations from occult hepatitis B virus infected patients with hematological malignancies in South Egypt

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## Abstract

### AIM

To investigate the prevalence and virological characteristics of occult hepatitis B virus (HBV) infections in patients with hematological malignancies in South Egypt.

## METHODS

Serum samples were collected from 165 patients with hematological malignancies to monitor titers of HBV DNA, hepatitis B surface antigen (HBsAg), and antibodies to HBV core (anti-HBc) and surface antigens. Serum samples negative for HBsAg and positive for anti-HBc were subjected to nucleic acid extraction and HBV DNA detection by real-time polymerase chain reaction. DNA sequences spanning the S region were analyzed in cases with occult HBV infection. *In vitro* comparative study of constructed 1.24-fold wild type and S protein mutant HBV genotype D clones was further performed.

## RESULTS

HBV DNA was detected in 23 (42.6%) of 54 patients with hematological malignancies who were HBsAg negative, but anti-HBc positive, suggesting the presence of occult HBV infection. The complete HBV genome was retrieved from 6 occult HBV patients, and P120T and S143L were detected in 3 and 2 cases, respectively. Site directed mutagenesis was done to produce 1.24-fold genotype D clones with amino acid mutations T120 and L143. The *in vitro* analyses revealed that a lower level of extracellular HBsAg was detected by chemiluminescence enzyme immunoassay (CLEIA) with the clone containing T120 mutation, compared with the wild type or the clone with S143L mutation despite the similar levels of extracellular and intracellular HBsAg detected by Western blot. Southern blot experiments showed that the levels of intracellular HBV DNA were not different between these clones.

## CONCLUSION

Occult HBV infection is common in patients with hematological malignancies and associated with P120T and S143L mutations. 120T mutation impairs the detection of HBsAg by CLEIA.

**Key words:** Occult hepatitis B infection; Hematological malignancies; Escape mutation

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**Core tip:** The present study was conducted to investigate the prevalence and virological characteristics of occult hepatitis B virus (HBV) infections in patients with hematological malignancies in Egypt. Serum samples were collected from 165 patients with different hematological malignancies and screened for occult HBV infection. In the present study, occult HBV infection was detected in 23 (42.6%) of 54 patients with hematological malignancies who were hepatitis B surface antigen (HBsAg) negative, but antibodies to HBV core (anti-HBc) positive. Based on *in vitro* study of clones inserted with 120T and 143L, it was found that the 120T mutation could impair HBsAg detection by changing its conformation. Patients with hematological malignancies should be screened and closely monitored for anti-HBc and HBV DNA.

Elkady A, Iijima S, Aboulfotuh S, Mostafa Ali E, Sayed D, Abdel-Aziz NM, Ali AM, Murakami S, Isogawa M, Tanaka Y. Characteristics of escape mutations from occult hepatitis B virus infected patients with hematological malignancies in South Egypt. *World J Hepatol* 2017; 9(9): 477-486 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i9/477.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i9.477>

## INTRODUCTION

Occult hepatitis B virus (HBV) infection is defined by the presence of HBV DNA in the liver (with or without HBV DNA in the serum) in hepatitis B surface antigen (HBsAg) negative individuals<sup>[1]</sup>. Apart from posing diagnostic challenges, several studies indicated that occult HBV infection also associates with flares of liver disease in hepatitis C virus (HCV) infected patients who do not exhibit changes in HCV RNA levels and reduces the response rate to interferon therapy<sup>[2]</sup>. Furthermore, occult HBV infection is frequently detected in cryptogenic liver diseases and autoimmune hepatitis<sup>[3-5]</sup>. HBV reactivation is a well-known complication in patients with occult infection under immune suppression, such as anticancer therapy, and human immunodeficiency virus (HIV) infection<sup>[6,7]</sup>. In addition, occult HBV infection increases the risk of HBV transmission through blood transfusion<sup>[8]</sup>.

Significant advances have been achieved in understanding the molecular basis for occult HBV infection, and several factors have been implicated in the pathogenesis of occult HBV infection<sup>[9,10]</sup>. A variety of mutations in HBsAg have been reported to affect *in vitro* antigen detection, *in vivo* immune recognition, HBV infectivity, cell tropism and virion morphology<sup>[11-14]</sup>. The aim of this study was to determine the prevalence of occult HBV infection in patients with hematological malignancies in South Egypt. An *in vitro* study was performed to assess the virological characteristics of prevalent HBsAg mutations detected in patients with occult HBV infection.

## MATERIALS AND METHODS

### Patients

Serum samples were collected consecutively from 165 patients with hematological malignancies hospitalized in the Oncology Department of the Sohag Faculty of Medicine and South Egypt Cancer Institution from November 2010 to October 2011. All patients started their treatment regimen at the time of conduction of the study.

The serum samples were stored at -80 °C for future examination of HBsAg, antibodies to HBsAg (anti-HBs), antibodies to HBV core (anti-HBc), and HBV DNA.

### Serological markers of HBV infection

HBsAg was measured by enzyme immunoassay (AxSYM; Abbott Japan, Tokyo, Japan) and chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan).

The IgG class of anti-HBc was determined by radioimmunoassay (Abbott Japan). Anti-HBs was tested by enzyme immunoassay (AxSYM; Abbott Japan, Tokyo, Japan). Anti-HCV was tested by CLEIA (Fujirebio, Tokyo, Japan). All serologic assays were performed according to the manufacturer's instructions.

#### **DNA extraction**

DNA was extracted from serum samples (200  $\mu$ L) using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and re-suspended in 100  $\mu$ L of the storage buffer provided by the kit manufacturer.

#### **Quantitation of serum HBV DNA**

HBV DNA sequences spanning the entire S gene were amplified by real-time polymerase chain reaction (PCR) according to a previously described protocol with a slight modification<sup>[15]</sup>. The detection limit of the assay was 100 copies/mL (equivalent to 20 IU/mL).

#### **HBV genomic amplification, sequencing and molecular evolutionary analysis of HBV**

Extracted DNA was subjected to PCR for amplifying the complete genome of HBV as previously described<sup>[16]</sup>. Amplicons were sequenced directly using the ABI Prism Big Dye ver. 3.1 kit on the ABI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, United States). All sequences were analyzed in both forward and reverse directions. HBV genotypes were determined by molecular evolution analysis. Target sequences were aligned by CLUSTALX with reference sequences retrieved from the DDBJ/EMBL/GenBank databases, and genetic distances were estimated by the 6-parameter method in the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/>)<sup>[17]</sup>. Based on the obtained distances, phylogenetic trees were constructed by neighbor-joining method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times for analysis by the ODEN program of the National Institute of Genetics.

#### **Plasmid constructs of HBV DNA and sequencing**

Various plasmids were constructed based on a consensus clone named D-IND 60 in which 1.24-fold HBV DNA genome of wild type genotype D (nt 1413-3215/1-2185) was inserted into a pUC19 vector (Invitrogen Corp., Carlsbad, CA, United States)<sup>[18]</sup>.

For site-directed mutagenesis, plasmid D-IND 60 was digested by HindIII and EcoO65I, and ligated with the fragments carrying P120T and S143L amino acid mutations to produce the 1.24-fold HBV genome. Cloned HBV DNA sequences were confirmed using ABI Prism Big-Dye (Applied Biosystems, Foster City, CA, United States) on an ABI 3100 automated sequencer.

#### **Cell culture and DNA transfection**

The hepatoma derived cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium containing 10%

fetal bovine serum. For the standard replication assay,  $1 \times 10^6$  Huh-7 cells were seeded onto a 10-cm-diameter dish, and 16 h later, transfected with 5  $\mu$ g DNA constructs using a Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, United States). Transfection efficiency was monitored by cotransfecting 0.5  $\mu$ g of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and measuring SEAP activity in the culture supernatant. The supernatants and cell lysates of the transfected cells were collected 3 d after the transfection to analyze HBV markers. Three independent experiments were conducted for each clone<sup>[19]</sup>.

#### **Determination of HBV markers**

HBsAg and HBcAg were determined by CLEIA using commercial assay kits (Fujirebio Inc., Tokyo, Japan). To detect the intracellular replicative intermediates of HBV, the core particle-associated HBV DNA in the cells was isolated and measured by Southern blot<sup>[20,21]</sup>. Briefly, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA and 1% IGEPAL CA-630 (Sigma-Aldrich, Japan G.K.). Total cell lysate was treated with 120  $\mu$ g/mL of RNase A and 30  $\mu$ g/mL of DNase I for 3 h at 37 °C, in the presence of 6 mmol/L magnesium acetate. HBV DNA was then released by proteinase K digestion, extracted with phenol, and ethanol precipitated. DNA was separated on a 1.2% w/v agarose gel and then transferred to a positive-charged nylon membrane (Roche Diagnostics). The membrane was hybridized with digoxigenin (DIG)-dUTP-labeled full-length HBV genotype C fragment, which was generated using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH), and then detected by alkaline phosphatase-labelled anti-DIG antibody according to the manufacturer's instructions. Signals were analyzed using an ImageQuant LAS 4000 mini (GE Healthcare United Kingdom Ltd).

#### **Western blot**

Huh7 cells ( $1 \times 10^6$ ) were transfected with genotype D wild type clone or its mutants using Fugene6 (Promega, Madison, WI, United States). Transfected Huh7 cells and culture supernatants were harvested 72 h post transfection. Cells were washed with phosphate-buffered saline twice and lysed with lysis buffer (CellLytic M Cell lysis reagent; Sigma). The culture supernatants and cell lysates were quantified for HBsAg and HBcAg by CLEIA (Fujirebio, Japan). The protocol of Western blot was previously described<sup>[22]</sup>. To detect HBsAg and HBcAg in cell lysates and viral particles, we used monoclonal antibodies specific for PreS1 and Core (Institute of Immunology, Japan), respectively. Immunoreactive proteins were visualized using chemiluminescence reagents (Immobilon; Millipore).

#### **Statistical analysis**

Statistical analyses were performed by Fisher's exact probability test and independent *t*-test for continuous



**Table 1** Characteristics of patients with hematological malignant disease included in the study *n* (%)

Characteristic	Total ( <i>n</i> = 165)
Age (mean ± SD)	36.1 ± 23.1
Gender (M)	89 (55.6)
ALT (mean ± SD)	29.7 ± 32.8
AST (mean ± SD)	38.2 ± 49.2
Anti-HCV(+)	39 (23.6)
HBsAg(+)	13 (7.9)
Anti-HBc(+)/HBsAg(-)	54 (32.7)
Clinical characteristics	
Malignant lymphoma	88 (53.3)
Hodgkin's disease	8 (4.8)
Acute leukemia	43 (26.1)
Chronic leukemia	23 (13.9)
Multiple myeloma	3 (1.8)
Steroid containing treatment	110 (66.7)

ALT: Alanine transaminase; AST: Aspartate aminotransferase; HBsAg: Hepatitis B surface antibody; HBc: Hepatitis B virus core; M: Male.

variables using SPSS software (SPSS, Chicago, IL, United States). *P*-values (two-tailed) less than 0.05 were considered statistically significant.

### Ethical consideration

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from all patients.

## RESULTS

### Patient characteristics

The baseline characteristics of 165 patients with hematological malignant diseases are shown in Table 1. The mean age of the studied cohort was 36.1 ± 23.1 years old. Among the 165 patients with hematological malignancies, 13 (7.9%) were found positive for HBsAg, 39 (23.6%) positive for anti-HCV, and 152 (92.1%) negative for HBsAg, of whom 54 (35.5%) were serologically positive for anti-HBc. Male predominance was observed in the studied cohort (55.6%). Overt HBV and HCV co-infections were not detected in the studied cohort. Eighty-eight (53.3%) patients were diagnosed with malignant lymphoma, 43 (26.1%) diagnosed with acute leukemia, 23 (13.9%) with chronic leukemia, 8 (4.8%) with Hodgkin's disease, and 3 (1.8%) with multiple myeloma.

### Occult HBV infection in the studied cohort

HBV DNA was detected in 42.6% (23/54) of the patients with hematological malignancies who were negative for HBsAg, but positive for anti-HBc, suggesting the presence of occult HBV infection. Anti-HCV was detected in 26.1% (6/23) of occult hepatitis B cases. The complete genome of HBV was successfully obtained from 6 cases with occult HBV infection. The clinical and HBV virological aspects of these 6 patients are summarized

in Table 2. Four of these 6 patients were diagnosed with malignant lymphoma, and two patients were diagnosed with Hodgkin's disease and ALL each (Table 2). Their age ranged between 5 and 80 years. Two patients were serologically anti-HBs positive (Samples ID; Egl6 and EGL4). Three patients were serologically negative for anti-HBs (samples ID; U79, D1 and D14). There was insufficient serum sample to measure anti-HBs levels in one case (sample ID; A79).

Phylogenetic analysis of the retrieved complete HBV genomic sequences indicated that all isolates were of genotype D subtype D1 (Figure 1). Further analysis was applied to the major hydrophilic region (MHR) of the HBV genome, revealing the presence of two prevalent amino acid substitutions; P120T in patients Egl4, A79, D1, and D79 and S143L in U31 and Egl4 (Table 2).

HBV genomic short sequences encompassing the "a" determinant region were available in 12 of 17 samples. Of the 12 samples, escape mutation Q129R was present in 7, while P120T was detected in 4 and S143L was present in 1, suggesting that the clones with 129R mutation were from a minor population with relatively low HBV-DNA levels.

### In vitro analysis of P120T and S143L mutations

To elucidate the virological characteristics of P120T and S143L mutations obtained from the complete sequences in this study, these mutations were individually inserted by site mutagenesis into a 1.24-fold replication competent HBV clone based on genotype D. Wild type genotype D clone, and two variants containing P120T and S143L mutations were transfected to Huh7 cells, and supernatants (extracellular) and cell lysates (intracellular) were collected to compare HBsAg and HBcAg levels. As shown in Figure 2A, a lower concentration of HBsAg was detected in the supernatant of Huh7 cells transfected with the P120T variant compared with wild type or S143L variant transfected cells. No significant difference was observed in extracellular or intracellular HBcAg levels between these three clones (Figure 2B and C). Furthermore, Southern blot experiments showed that the levels of intracellular HBV single stranded DNA were not different between these clones (Figure 2D). Taken together, these results indicate that P120T strongly reduces HBsAg levels as detected by CLEIA without affecting the HBV DNA replicative capacity.

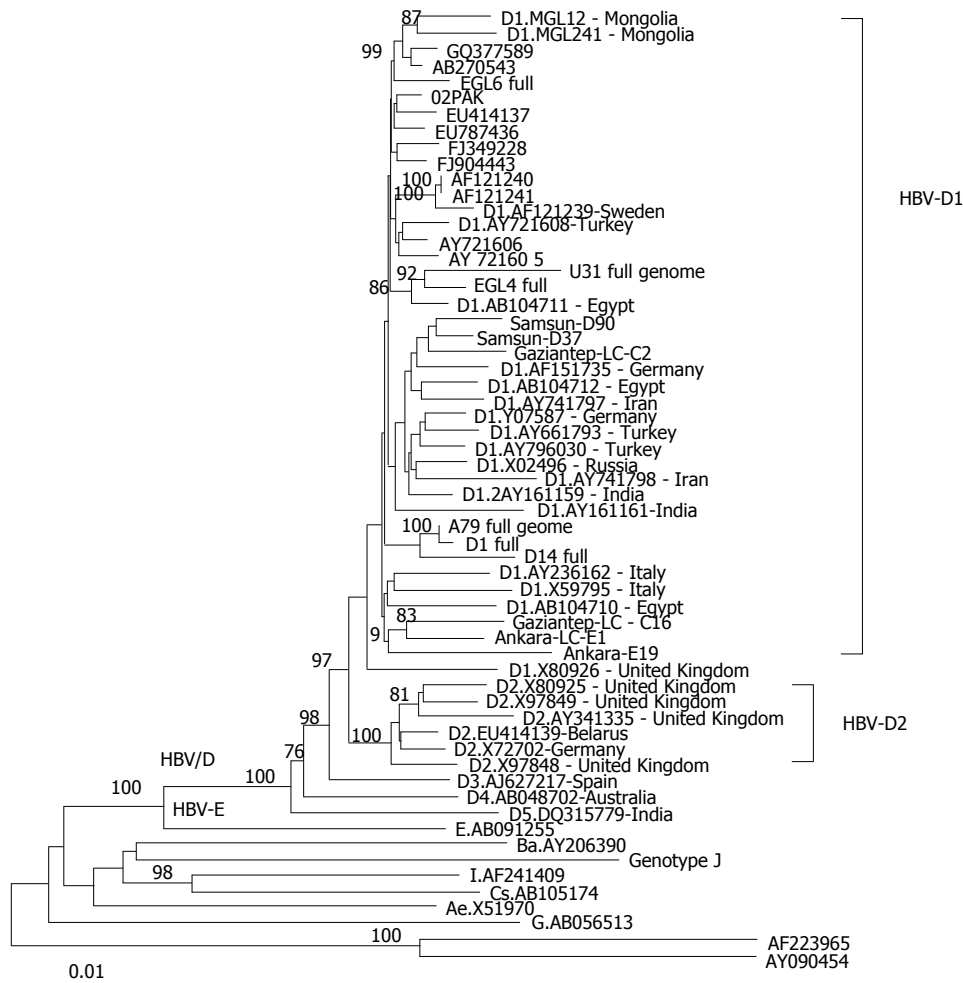
### P120T mutation reduces in vitro antigenicity of HBsAg

The above data suggest that the P120T mutation either prevents the secretion of HBsAg or reduces the antigenicity of HBsAg. To distinguish between these alternatives, we examined the extracellular and intracellular expression levels of denatured HBsAg by Western blot after transfecting Huh7 cell with the wild type genotype D clone, and both the P120T, and S143L mutants. As controls, we also monitored the extracellular and intracellular expression levels of the denatured HBcAg by Western blot. As shown in Figure 3, the extracellular

**Table 2 Clinical characteristics of patients with occult hepatitis B and detectable full hepatitis B virus genome**

Sample ID	Age	Gender	Diagnosis	Anti-HBs (mIU/L)	HBV DNA Log (copies/mL)	Mutation <sup>1</sup>
EGL6	38	M	Malignant lymphoma	(+)/15	3.5	-
U31	55	F	Malignant lymphoma	(-)	3.8	S143L
EGL4	80	F	Malignant lymphoma	(+)/18.9	2.4	P120S, S143L
A79	5	M	ALL	NT	4.4	P120T
D1	70	M	Malignant lymphoma	(-)	3.9	P120T
D14	5	M	HD	(-)	3.6	P120T

<sup>1</sup>Mutation in the "a" determinant region of the S gene product. ALL: Acute lymphoblastic leukemia; HD: Hodgkin disease; HBV: Hepatitis B virus; NT: Not tested; M: Male; F: Female.



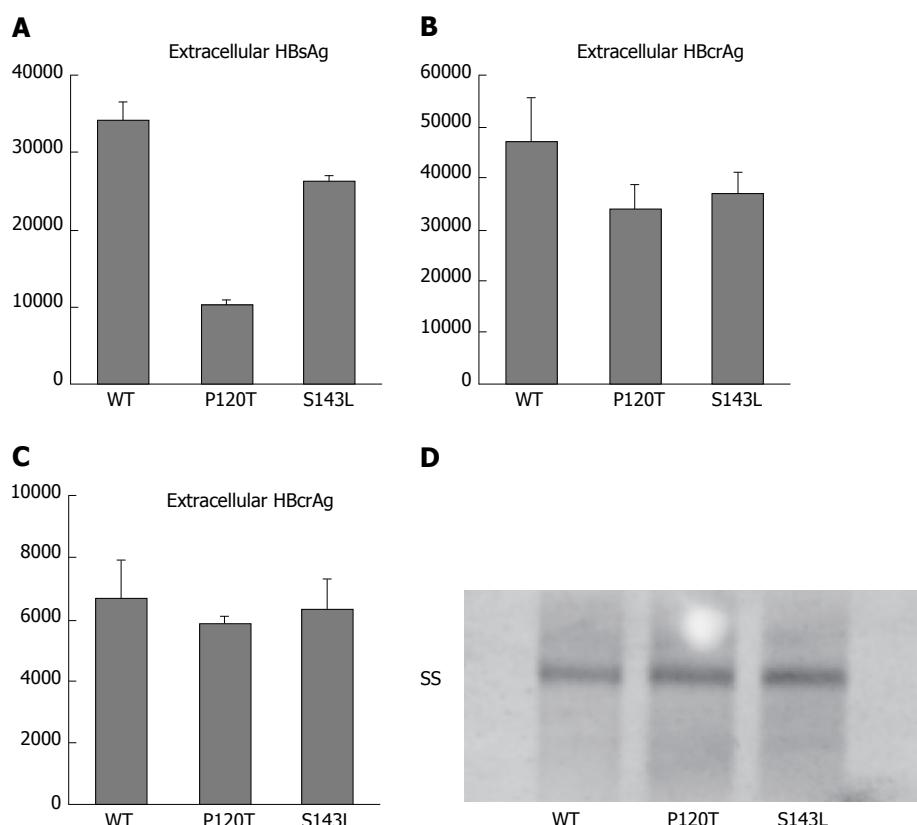
**Figure 1** Phylogenetic tree constructed from the nucleotide sequences of the full hepatitis B virus genome. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (> 75%) are indicated in the tree roots. HBV sequences isolated from the studied cohort are indicated in bold. Reference sequences retrieved from the GenBank/EMBL/DBJ are indicated by their accession numbers. The country origin of the reference cohort sequences is indicated in brackets. HBV genotypes A-H are indicated in the cluster roots. HBV: Hepatitis B virus.

expression levels of denatured HBsAg were not different between the wild type and mutants. The result suggests that the reduced HBsAg level detected by CLEIA in the supernatant of P120T mutant transfected cells was caused by reduced antigenicity *in vitro* rather than intracellular retention of the mutant HBsAg. In line with this notion, a similar amount of intracellular HBsAg was detected in cells transfected with either of the three clones (Figure 4). Collectively, these results suggest that the P120T mutation reduces the antigenicity of HBsAg *in*

*vitro*.

## DISCUSSION

Few studies have reported the characteristics of occult HBV infection with genotype D compared to reports of genotypes B and C. In the present study, we investigated the characteristics of occult HBV infections among patients with hematological malignancies in a population where genotype D infection is prevalent<sup>[23,24]</sup>.



**Figure 2** Expression of hepatitis B virus DNA and antigens after transfection of Huh 7 cells with wild type hepatitis B virus genotype D clone (WT) and mutants with S gene mutations P120T (120T) and S143L (143L). A and B represent the extracellular expression of HBsAg and HBcrAg, respectively, by WT and S gene mutants (120T and 143L); C represents the intracellular expression of the core gene product (HBcrAg) by WT and S gene mutant clones; D: The density of the single-stranded DNA in Southern blot analysis of cell lysates of Huh7 cells transfected with WT and mutants with S gene mutations.

We detected two previously reported escape mutations P120T and S143L that were associated with occult HBV infection<sup>[25-28]</sup>, and investigated the mechanism by which these mutations cause occult infection using an *in vitro* system.

No accurate estimate regarding the prevalence of occult HBV infections in different Egyptian cohorts has been reported, perhaps due to the high expense of PCR and low budget for scientific research in Egypt<sup>[29]</sup>. However, a high prevalence rate (17.2%; 52/303) of occult HBV was reported in Egyptian blood donors positive for anti-HBc<sup>[30]</sup>. Our results indicated that the frequency of occult HBV infection among patients with hematological malignancies was even higher. Therefore, patients with hematological malignancies might be more likely to develop occult HBV infections than those without. The highest prevalence of occult HBV was reported in patients with hepatocellular carcinoma (62.5%; 25/40) in tissue samples<sup>[8,31]</sup>.

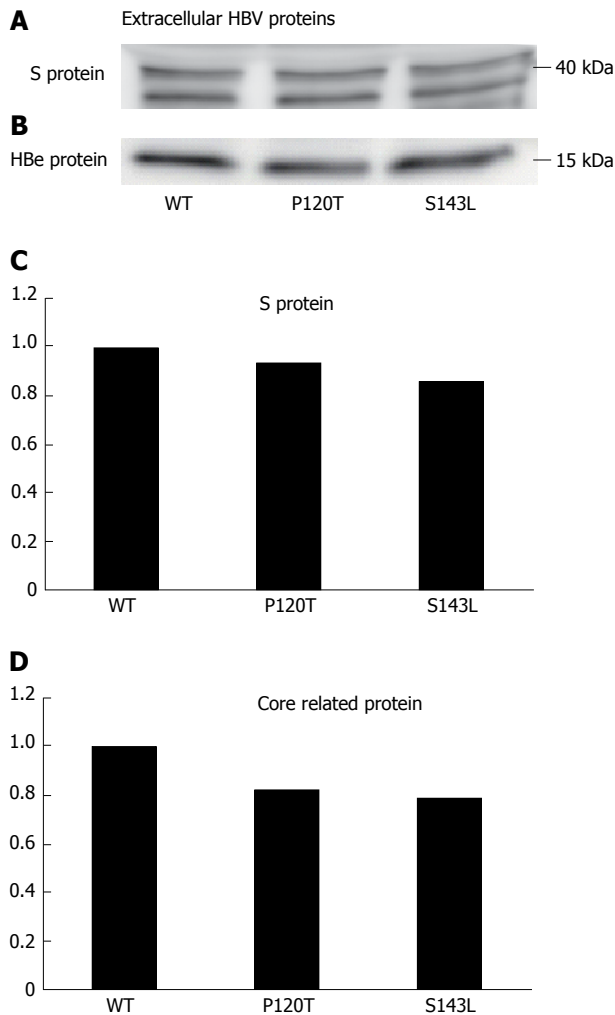
HCV infection was not uncommon in patients with occult HBV. In a similar Egyptian cohort (children with hematological malignancy), 38% (15/49) were HCV coinfecting patients<sup>[32]</sup>. HCV and HBV viruses shared many risk factors and routes of transmission, and more likely parenteral antischistosomal therapy was responsible for transmission of HBV and HCV in many Egyptians<sup>[33]</sup>.

The presence of anti-HBs in patients with occult HBV

was in line with a recent study conducted on patients treated for TB, which showed that half of patients with occult HBV infection had both anti-HBc and anti-HBs<sup>[34]</sup>. Occult hepatitis B was also detected in blood units from healthy volunteer blood donors showing an adequate level of anti-HBs<sup>[30]</sup>. Possible explanation for the presence of such cases (anti-HBc+/anti-HBs+/HBV DNA+) in HBsAg- individuals is that anti-HBs antibody is poorly neutralizing due to loss of recognition, allowing these mutant viruses to escape neutralization even when antibody is present at protective levels<sup>[35-37]</sup>.

Isolated anti-HBc positive individuals with undetectable anti-HBs or HBV DNA were observed in the studied cohort. In a cohort of blood donors with anti-HBc only, the observation of the anti-HBs kinetics after administration of Engerix HBV vaccine allowed the discrimination between the naïve HBV infection with likely false positive anti-HBc, subjects with resolved HBV infection, and subjects with persistent low level replication<sup>[38]</sup>. Coffin *et al.*<sup>[39]</sup> believed that the long-term presence of anticore antibodies alone is a consequence of sustained restimulation of the immune system by virus nucleocapsid produced during low-level hepadnaviral assembly.

Several studies reported the higher prevalence of mutations in the MHR region of the S gene product in HBV isolates retrieved from occult HBV cases compared to overt HBV cases<sup>[40]</sup>. In addition, Martin *et al.*<sup>[41]</sup> re-

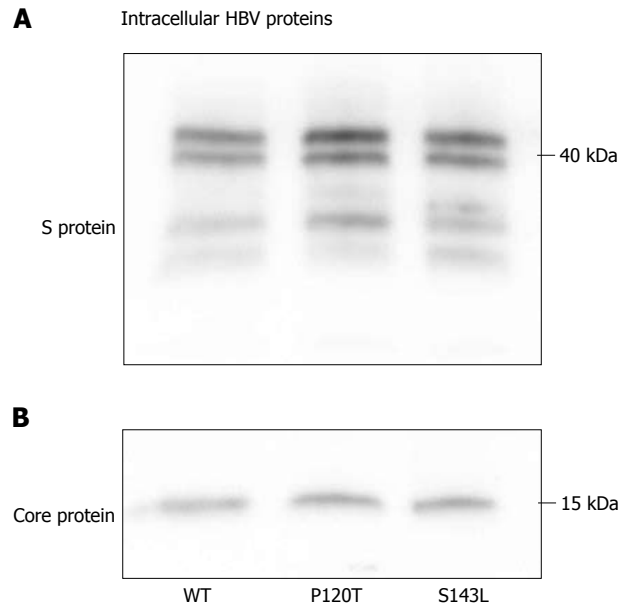


**Figure 3** Western blot analysis of hepatitis B surface antigen (A, C), hepatitis B virus core (B), core related proteins (D) expressed by hepatitis B virus genotype D clones (WT) and hepatitis B surface antigen mutant clones (120T and 143L) in the supernatant of Huh 7 cells. The results for the wild type clones in the three independent transfection experiments were similar and their mean value is set at 1.0. The hepatitis B surface antigen and hepatitis B virus core proteins levels are expressed relative to this value in (C) and (D). HBV: Hepatitis B virus.

ported the potential virological differences between chronic HBV and occult HBV in HIV coinfecting individuals and that positive selection immune pressures are acting against Pre-S and S regions in occult HBV, resulting in mutations that may adversely affect the production and/or detection of HBsAg. In concordance with previous findings, the high prevalence of mutations was observed in another immunocompromised group (patients with hematological malignancy).

The HBV complete genome was retrieved from 6 patients, and sequence and phylogenetic analysis revealed that they were of genotype D1, the prevalent HBV genotype in Egypt regardless of the studied cohort. Two amino acid substitutions, P120T and S143L, were found in HBsAg.

Inspection of 2846 HBV strains currently available in DDBJ, EMBL and GenBank genetic databases indicated that amino acid substitutions 120T and 143L are present in 0.5% (13/2846) and 0.4% (9/2846), respectively,

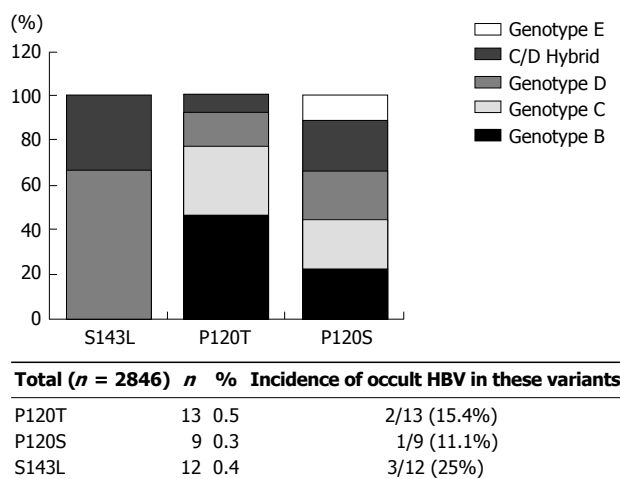


**Figure 4** Western blot analysis of hepatitis B surface antigen (A) and hepatitis B virus core (B) proteins expressed by hepatitis B virus genotype D clones (WT) and hepatitis B surface antigen mutant clones (120T and 143L) in the cell lysate of Huh 7 cells. HBV: hepatitis B virus.

of the total isolates examined. The 143L substitution is common in genotype D strains, while 120T is common in genotypes B, C, D, C/D hybrid, and E. Collectively, these results suggest that 143L specifically occurs in genotype D, while 120T is a genotype independent substitution (Figure 5). Interestingly, 15% (2/13) and 25% (3/12) of cases infected with T/S120 and 143L variants were occult HBV infection, respectively (Figure 5). In contrast, 129R was mainly found in the short sequences (low HBV DNA) in this study as well as genotypes A, B and C isolates as previously reported<sup>[26,42,43]</sup>. Taking previous data together, further *in vitro* analysis of 120T and 143L was applied to the current study.

Both mutations are located in the MHR that extends from amino acids 99 to 169 of the HBsAg. The MHR is exposed on the surface of the antigen and is the principal binding site of anti-HBs following natural infection, and after immunization<sup>[44]</sup>. In many cases, mutations in the MHR are frequently associated with occult HBV infection<sup>[45,46]</sup>, because they can change immunogenicity and render the HBsAg unrecognizable by commercially available detection assays. Clones with such mutations are often referred to as "diagnostic-escape" mutants<sup>[47]</sup>. Consistent with this, the results obtained by *in vitro* experiments showed that extracellular HBsAg with P120T was less detectable than wild type HBsAg or the mutant with S143L by CLEIA. The lower extracellular HBsAg levels observed were not due to reduced HBV replication or the intracellular retention of HBsAg. Rather, the P120T mutation appeared to reduce the antigenicity of HBsAg. A similar result was also reported by Yen *et al.*<sup>[48]</sup>, but their study did not address the impact of 120T mutation on HBV replication or HBsAg secretion. *In vitro* studies described the impairment of virion and/or S protein





**Figure 5** Genotype distribution of the detected S gene product mutation among the database reference sequences. HBV: Hepatitis B virus.

secretion in both Huh7 cells and hydrodynamic injected mice by Q129R MHR mutation<sup>[49]</sup>.

Our previous study demonstrated that HBV reactivation frequently occurs among patients with hematological malignancies under chemotherapy<sup>[50]</sup>. One important risk factor for the development of HBV reactivation in this critical group is the presence of occult HBV infection<sup>[50,51]</sup>. The present data suggest that patients with hematological malignancies should be screened and closely monitored for anti-HBc and HBV DNA.

In conclusion, the prevalence of occult hepatitis B was detected in patients with hematological malignancies in South Egypt in association with two mutations in the HBsAg, P120T and S143L. Neither of these mutations affected the replication activity, virion or S protein secretion but one of the mutations, P120T, interfered with detection by current commercial assays probably by inducing a conformational change. Our results highlight a challenge for detecting occult strains in developing countries.

## COMMENTS

### Background

Occult hepatitis B (OBI) is defined by the presence of hepatitis B virus (HBV) DNA in the serum or the liver in the absence of hepatitis B surface antigen (HBsAg) with or without anti-HBc or antibodies to HBsAg. Prevalence of OBI is different according to the endemicity of HBV. OBI is implicated in different clinical contexts including the progression of liver disease, the development of hepatocellular carcinoma, the risk for HBV reactivation, and the transmission of HBV infection. Both viral and host factors are implicated in the pathogenesis of OBI. Major hydrophilic region in genomic HBV extending from aa99 to aa169, clustered with a highly conformational epitope, is critical to the antigenicity of HBsAg and may affect the diagnosis of HBV in HBV screening tests.

### Research frontiers

In a cohort of 165 patients with hematological malignancies receiving cancer chemotherapy who were negative for HBsAg, 54 patients (35.5%) were serologically positive for antibodies to HBV core (anti-HBc). Occult HBV infection was determined in 42.6% (23/54) of patients with hematological malignancies who were negative for HBsAg, but positive for anti-HBc. The complete genome of HBV was successfully obtained from 6 cases with occult HBV infection and all were

of genotype D subtype D1. Two prevalent amino acid substitutions P120T and S143L were associated with OBI in the present study. *In vitro* analysis of these two amino acid mutations revealed that P120T mutation reduces the antigenicity of HBsAg *in vitro* without affecting the HBV DNA replication capacity.

### Innovations and breakthroughs

This study records the high prevalence of occult HBV infection in patients with hematological malignancies. Occult HBV infection is associated with 120T and 143L mutations, and 120T mutation might impair HBsAg detection by changing its conformation.

### Applications

The study strongly recommends mandatory serological screening for anti-HBc and HBV DNA in patients with hematological malignancies.

### Peer-review

In this manuscript, Abeer Elkady *et al* argue characteristics of escape mutations from occult hepatitis B virus infected patients with hematological malignancies in Egypt. The authors concluded that occult HBV infection is associated with P120T and S143L mutations and 120T mutation impairs the detection of HBsAg by chemiluminescence enzyme immunoassay. The aim of this study might be interesting and important.

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**S- Editor:** Gong ZM **L- Editor:** Wang TQ **E- Editor:** Li D



## Case of hepatocellular carcinoma in a patient with hereditary tyrosinemia in the post-newborn screening era

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### Abstract

Hereditary tyrosinemia type 1 (HT-1) is a metabolic disorder caused by a defect in tyrosine degradation. Without treatment, symptoms of hepatomegaly, renal tubular dysfunction, growth failure, neurologic crises resembling porphyrias, rickets and possible hepatocellular carcinoma can develop. The use of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione and early diagnosis through newborn screening initiatives have resulted in a sharp decline in morbidity and mortality associated with this disease. We present a case report of a 7-year-old patient with HT-1 who was born prior to the addition of tyrosinemia to the newborn screening in her birth area. At her time of diagnosis, the patient had developed many of the symptoms associated with her disease, including chronic kidney disease, rickets, and myopathy that left her non-ambulatory. During her initial evaluation, she was also noted to have hepatocellular carcinoma. With cadaveric liver transplantation and nutritional support, her symptoms all either resolved or stabilized. Her case illustrates the severity of the disease if left untreated, the need for vigilance in populations who do not routinely receive newborn screens, and the markedly improved outcomes in patients following transplant.

**Key words:** Tyrosinemia; Screening; Hepatocellular carcinoma; Liver transplantation

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**Core tip:** Hereditary tyrosinemia type 1 is a metabolic defect resulting in several disease manifestations including life threatening hepatorenal disease, neurologic disease, and rickets. Although neonatal screening for this disorder has allowed early identification and medical treatment with nitisinone, the need for recognition of this disorder in older individuals remains since aggressive intervention, including medical treatment and possible liver transplantation, may be lifesaving and have profound effects on morbidity and mortality.

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## INTRODUCTION

Hereditary tyrosinemia type 1 (HT-1) is a metabolic disorder caused by a defect in tyrosine degradation due to a deficiency of fumarylacetoacetate hydrolase (FAH). In 1956, Baber provided one of the earliest descriptions of the disorder in her report of a 9 mo old child with cirrhosis, renal tubular dysfunction, and rickets<sup>[1]</sup>. As a result of advancements in amino acid analysis, a number of reports followed over the next decade in which infants with similar findings were noted to have elevations of plasma tyrosine and aminoaciduria<sup>[2-4]</sup>. In 1977, Lindblad was able to identify decreased activity of FAH as the enzyme defect responsible for the disorder<sup>[5]</sup>.

The major disease manifestations of HT-1 are now well-defined and include hepatic dysfunction, renal tubular dysfunction, and peripheral nerve injury. The mechanism is most likely a result of increases in toxic metabolites, including fumarylacetoacetate, maleylacetoacetate, and succinoacetylacetate. Most affected children present in infancy with an acute form of the disease associated with failure to thrive, severe liver dysfunction, and death in infancy if untreated. A chronic form may also occur with hepatomegaly, renal tubular dysfunction, growth failure, neurologic crises resembling porphyrias, and rickets. Hepatocellular carcinoma appears to be a common finding in both forms of untreated disease and may be noted in up to 37% of children that survive beyond 2 years of age. The incidence of this tumor increases with age and is reported to be the cause of death in over 50% of untreated individuals<sup>[6]</sup>.

We report our recent experience in a 7-year-old female who underwent liver transplantation for advanced hepatocellular carcinoma in association with a delayed diagnosis of HT-1.

## CASE REPORT

A 7-year-old Hispanic female with an uneventful birth history presented to our referral institution for workup

and management of balance and gait disturbance and an inability to walk that was first noted at 3 ½ years of age. She was noted to have increasing “clumsiness” in the months leading to her inability to walk. She was also noted to have chronic renal disease and liver disease with hepatomegaly and enlarged kidneys in the first 4 years of life. Her inability to walk was previously attributed to severe pain throughout her body.

At 6 years of age, she developed bilateral lower extremity fractures and an upper extremity fracture while attempting to stand. She was noted to have bowing of her lower extremities and elevated alkaline phosphatase and was subsequently diagnosed with rickets that was believed to be the result of chronic kidney disease. Shortly thereafter, she was referred to a local medical center where she underwent liver biopsy, kidney biopsy, and muscle biopsy. Her liver biopsy revealed cirrhosis with minimal chronic inflammation and her kidney biopsy revealed nonspecific glomerular and tubular changes with some parenchymal fibrosis. Her muscle biopsies revealed severe myopathic changes with myofiber atrophy. She was noted to have mitochondrial DNA quantification on her muscle biopsy which was reduced and less than 29% of controls. She was subsequently diagnosed with mitochondrial depletion syndrome.

When her weakness and strength worsened further, she was referred to our institution's mitochondrial clinic where she underwent additional workup. Extensive metabolic evaluation revealed an elevated succinylacetone which led to a diagnosis of HT-1. Analysis of the *FAH* gene revealed a homozygous splice mutation known to be associated with HT-1. She ultimately underwent treatment with dietary modification and 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), but she was immediately referred for cadaveric liver transplantation after further workup revealed imaging findings consistent with hepatocellular carcinoma. Following this initial pre-transplant treatment, her condition improved substantially with significant improvement in her amino acid profile, improvement in her mental status, and reduction in her alpha-fetoprotein from 2320 ng/mL to 1585 ng/mL. Despite this metabolic improvement, she required liver transplantation as a result of her hepatocellular carcinoma. Since this lesion was felt to be adjacent to major blood vessels and liver transplantation was not ideal without improved nutrition and rehabilitation, she underwent transarterial chemoembolization for a 1.5 cm × 1.6 cm lesion in her liver (Figure 1) after which time she had further reduction in her alpha-fetoprotein to 511 ng/mL. After continued improvement in nutrition and physical conditioning, she ultimately underwent liver transplantation 4 mo after her diagnosis was made. Explant of her liver revealed a yellow-orange and nodular liver, and histology revealed cirrhosis and multinodular well-differentiated hepatocellular carcinoma with bile duct proliferation felt to be consistent with tyrosinemia (Figure 2).

Her immediate postoperative course was unremarkable and, following transplant, she had further improvements in her cognitive function, nutrition, and physical conditioning.



**Figure 1** Magnetic resonance imaging of the liver with a large 1.5 cm × 1.6 cm lesion with arterial enhancement characterizing hepatocellular carcinoma and requiring chemoembolization.

Within 6 mo, she was ambulating without assistance and attending regular school without difficulty. Her rickets had also improved and resolved with her bone mineral density Z-score in her hip improving from -4.1 prior to transplant to -1.1 fifteen months following transplant. Her renal function improved with her estimated GFR before and 6 mo after transplant noted to be 75 and 121 mL/min per 1.73 m<sup>2</sup> respectively. She had no recurrence of tumor on follow-up imaging obtained 2 years following her transplantation, and her alpha-fetoprotein was normal during this time.

## DISCUSSION

While HT-1 can result in a variety of multisystem and life-threatening complications, including hepatic and renal disease, recent approaches to management over the past few decades have resulted in a sharp decline in morbidity and mortality associated with this disease. Since Lindstedt first published his experience using NTBC in 5 patients with HT-1 in 1992, its incorporation into the standard treatment for HT-1 has resulted in improved effects on the long term outcome of individuals with this disease with improved metabolic control<sup>[7]</sup>. In addition, newborn screening for HT-1 is a common practice in most Western countries allowing early identification and treatment of affected individuals as early as the first month of life. In the large Quebec experience, infants beginning treatment with NTBC in the first month of life had no detectable liver lesions after more than 5 years of follow-up and no need for liver transplantation<sup>[8]</sup>. The combined effects of NTBC use and newborn screening have resulted in a significant reduction in the need for liver transplantation noted over the past decade<sup>[9]</sup>.

Since our patient was born in 2006, one year prior to initiation of newborn screening in her region, her diagnosis was not identified early in life. Her presenting symptoms of developmental delay and weakness which were first noted at 3 years of age led to an underlying diagnosis of mitochondrial depletion syndrome. Interestingly, electron microscopy of tissue from individuals with HT-1 has



**Figure 2** Explant of nodular and shrunken tyrosinemic liver obtained at the time of liver transplantation.

revealed mitochondrial abnormalities with a relative loss of matrinal bodies and decreased matrix density<sup>[10]</sup>. Like tyrosinemia, mitochondrial depletion syndromes may involve a variety of organs, including the liver, kidney, and peripheral nervous system. Her abnormal movements and unusual behavior could also be attributed to an underlying mitochondrial or primary neuromuscular disorder although these resolved after dietary and NTBC were started.

Marked elevations in alpha-fetoprotein were noted in our patient. Elevated alpha-fetoprotein is a typical finding in HT-1, even in the absence of hepatocellular carcinoma. Improvement in alpha-fetoprotein is also noted with improvement in metabolic control in HT-1 patients as we saw in our patient. Nevertheless, our patient required extensive workup to rule out hepatocellular carcinoma given the obvious risk of hepatocellular carcinoma in individuals with tyrosinemia, her continued markedly elevation alpha-fetoprotein, and her delayed age at diagnosis.

The presence of renal dysfunction in individuals with HT-1 requires consideration for combined liver-kidney transplantation in some individuals. Continued exposure to calcineurin inhibitors may cause significant deterioration in kidney function in some individuals receiving liver transplantation. In the Quebec experience, combined liver-kidney transplant may be warranted when the GFR < 40 mL/min per 1.73 m<sup>2</sup><sup>[11]</sup>. Despite her late age at diagnosis, our patient had a GFR which was slightly below normal but still sufficient enough to make isolated liver transplant a reasonable option.

This case highlights a number of important points. The presence of liver disease in association with other uncharacteristic organ pathology warrants consideration for an underlying metabolic disorder. Despite great advancement in newborn screening, metabolic disorders such as HT-1 are still possible due to imperfection of neonatal screening and potential for missed populations. Recent political and economic upheaval has introduced a large number of migrants into Western countries who often may not have had screening for a variety of metabolic or genetic disorders. In addition, children

receiving transplantation for metabolic liver disease have improved outcomes compared to children transplanted for other disorders, such as biliary atresia, with 1- and 5-year survival of 95% and 89%<sup>[12]</sup>. Finally, the presence of severe extrahepatic disease such as severe neuromuscular disease in our patient should not deter consideration for transplantation since liver transplantation can result in remarkable improvement and reversal in neurologic and cognitive dysfunction, particularly in children.

## COMMENTS

### Clinical diagnosis

The symptoms of hereditary tyrosinemia include hepatorenal dysfunction, neuromuscular symptoms, and rickets.

### Differential diagnosis

The differential diagnosis of hereditary tyrosinemia includes disorders of carbohydrate metabolism, mitochondrial disorders, and Wilson's disease.

### Laboratory diagnosis

Laboratory diagnosis of tyrosinemia includes confirmation by noting the presence of succinylacetone in urine specimens or body fluids, identification of causative mutations, or enzyme analysis revealing decreased fumarylacetoacetate hydrolase.

### Imaging diagnosis

Imaging in tyrosinemia may reveal complications of this disorder, including cirrhosis, hepatomegaly, hepatocellular carcinoma, renal abnormalities, and rickets.

### Pathological diagnosis

Pathological diagnosis of this disorder is obtained by laboratory methods described above.

### Treatment

Treatment of tyrosinemia involved use of nitisinone and a protein restricted diet in conjunction with long term management by a specialist. Liver transplantation may be required for severe disease or complications of this disorder.

### Experiences and lessons

A diagnosis of hereditary tyrosinemia should be considered in individuals with hepatorenal disease, rickets, and neuromuscular weakness since rapid initiation of aggressive treatment may be lifesaving.

### Peer-review

Imseis E *et al* reported about a case of hepatocellular carcinoma in a patient with hereditary tyrosinemia, a kind of rare lesion, in the post-newborn screening

era. This case report is very interesting.

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## Drug-induced liver injury: Do we know everything?

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### Abstract

Interest in drug-induced liver injury (DILI) has dramatically

increased over the past decade, and it has become a hot topic for clinicians, academics, pharmaceutical companies and regulatory bodies. By investigating the current state of the art, the latest scientific findings, controversies, and guidelines, this review will attempt to answer the question: Do we know everything? Since the first descriptions of hepatotoxicity over 70 years ago, more than 1000 drugs have been identified to date, however, much of our knowledge of diagnostic and pathophysiologic principles remains unchanged. Clinically ranging from asymptomatic transaminitis and acute or chronic hepatitis, to acute liver failure, DILI remains a leading causes of emergent liver transplant. The consumption of unregulated herbal and dietary supplements has introduced new challenges in epidemiological assessment and clinician management. As such, numerous registries have been created, including the United States Drug-Induced Liver Injury Network, to further our understanding of all aspects of DILI. The launch of LiverTox and other online hepatotoxicity resources has increased our awareness of DILI. In 2013, the first guidelines for the diagnosis and management of DILI, were offered by the Practice Parameters Committee of the American College of Gastroenterology, and along with the identification of risk factors and predictors of injury, novel mechanisms of injury, refined causality assessment tools, and targeted treatment options have come to define the current state of the art, however, gaps in our knowledge still undoubtedly remain.

**Key words:** Acute liver failure; Drug-induced liver injury; Hepatotoxicity; Acetaminophen toxicity; Cholestatic injury; Liver biopsy; Pharmacoepidemiology; Herbal-induced liver injury; Hy's law

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**Core tip:** Drug-induced liver injury has gained a great amount of interest in the past decade, raising the question of whether we know everything. Various global registries have been established and the first guidelines for diagnosis and management have come to define the



state of the art. The identification of risk factors and predictors of injury, novel mechanisms of injury, refined causality assessment tools, and targeted treatment options have amplified our understanding of the impact of drug-induced liver injury, however gaps in our knowledge still remain.

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## INTRODUCTION

Drug-induced liver injury (DILI) is a current hot topic for academics, clinicians, pharmaceutical companies and regulatory bodies, as seen by the increasing number of publications over the past fifteen years. Evidence to the fact is shown in the number of new monographs, revised chapters in textbooks, workshops and single-topic conferences specifically dedicated to DILI<sup>[1-5]</sup>. When DILI was the subject of a specific PubMed search, 44738 items were found in the past 5 and a half years (2010 through 2016), a number more than double the total number of items related to DILI published in the preceding decade (2000-2009).

This extensive body of new information leads us to a question that will be the focus of this review. By investigating the current state of the art of DILI, focusing on the latest scientific findings, controversies and guidelines, this review will take a clinician's point of view and attempt to find an answer to the question: Do we know everything?

## DILI: A BRIEF HISTORY

Iproniazid, cinchophen, and sulfonamides were amongst the first prototypical hepatotoxins to be identified, paving the way for future histological and clinical descriptions that followed the second world war<sup>[6,7]</sup>. By the mid-1960s, hepatotoxic agents including halothane, isoniazid (INH), carbamazepine, phenytoin and alpha methyl dopa were famously referred to by Popper *et al*<sup>[8]</sup> as "penalties for progress", and by the mid-1980s close to 1000 drugs were linked to hepatic injury<sup>[9]</sup>. Even though much of this early work<sup>[6,8]</sup> has remained the mainstay of diagnostic, and pathophysiologic principles even to this day, DILI remains a significant diagnostic challenge due to the fact that drugs can mirror acute and chronic hepatic diseases, and act through various mechanisms causing injury<sup>[10-15]</sup>.

## STATE OF THE ART OF DILI

Clinically, DILI ranges from asymptomatic transaminitis, acute or chronic hepatitis<sup>[16]</sup> to acute liver failure (ALF) or fulminant hepatic failure, defined as sudden and life-

threatening liver dysfunction leading to coagulopathy and hepatic encephalopathy within 26 wk of the onset of illness<sup>[17]</sup>. Although severe DILI is rare clinically, drugs have become the overall leading cause of ALF in the United States and other western countries<sup>[7]</sup>. In the United States, approximately 1600 to 2000 people per year develop ALF, with 30% of these patients receiving aggressive therapy including liver transplant<sup>[18]</sup>. Acetaminophen (paracetamol) is the offending drug in 40%-50% of these cases, with a further 11%-12% of ALF cases being caused by herbal compounds and dietary supplements (HDS), equalling the frequency of ALF due to acute viral hepatitis and greater than that seen with all other individually identifiable causes<sup>[7,19,20]</sup>. Indeed, due to this significant morbidity and mortality, DILI remains an important reason for drug withdrawal from the market, with most recent examples including, bromfenac and troglitazone<sup>[21]</sup>. Due to the significant time and expense involved in bringing a novel drug to market, it should come as no surprise, that identification of potential toxicities early in the development process is paramount<sup>[22]</sup>. However, compounds cannot be guaranteed to be totally free of the potential to cause harm and liver injury in preclinical stages of development, and as such, tremendous steps have been undertaken in regulatory science, so as to identify DILI in clinical and post-approval settings<sup>[23-25]</sup>. The creation of the Evaluation of Drug-Induced Serious Hepatotoxicity plot<sup>[26]</sup>, the "Rule-of-Two"<sup>[27,28]</sup>, FDA Adverse Event Reporting System<sup>[29]</sup>, the Sentinel projects<sup>[30]</sup>, and Liver Toxicity Knowledge Base<sup>[31]</sup> has empowered clinicians to detect and predict DILI as early and successfully as possible. Working in parallel at the bedside, new hepatotoxins have been uncovered including dronedarone<sup>[32]</sup>, ipilimumab<sup>[33,34]</sup>, and tolvaptan<sup>[35,36]</sup> and our further understanding of known hepatotoxins including azithromycin<sup>[37]</sup>, duloxetine<sup>[38]</sup>, fluoroquinolones<sup>[39]</sup>, statins<sup>[40]</sup>, telithromycin<sup>[41]</sup>, tyrosine kinase inhibitors<sup>[42]</sup> and others<sup>[43]</sup>, has broadened.

Additionally, the identification of risk factors, predictors and biomarkers of injury<sup>[44-52]</sup>, and novel mechanisms of injury<sup>[53-58]</sup>, along with refined causality assessment tools<sup>[59-61]</sup>, and targeted treatment options of hepatotoxicity<sup>[62-68]</sup>, have come to define the current state of the art.

## GUIDELINES AND REGISTRIES

Cumulatively, the aforementioned advances have led to the recent publication of the first guidelines for the diagnosis and management of DILI, offered by the Practice Parameters Committee of the American College of Gastroenterology<sup>[69]</sup>. The guidelines, as summarized in Table 1, provide key practical advice on all aspects and problems which may be faced in the work-up of a DILI case. This parallels the establishment of the United States DILI Network (US DILIN) in 2004<sup>[70,71]</sup>, a prospective study with a database containing > 1200 patients with acute DILI caused by approximately 200

**Table 1** Summary of drug-induced liver injury guidelines by the American College of Gastroenterology<sup>[7,69]</sup>

Elements necessary for the diagnostic evaluation of DILI
Known duration of exposure
Concomitant medications and diseases
Response to dechallenge (and rechallenge if performed)
Presence or absence of symptoms, rash, eosinophilia
Performing sufficient exclusionary tests (viral serology, imaging, <i>etc.</i> ) to reflect the injury pattern and acuteness of liver function tests ( <i>e.g.</i> , acute viral serology for A, B and C and autoimmune hepatitis when presenting with acute hepatocellular injury; routine testing for hepatitis E virus not recommended because of the problems with current commercial assays; Epstein-Barr virus, cytomegalovirus, and other viral serology if lymphadenopathy, atypical lymphocytosis present)
Sufficient time to determine clinical outcome - did the event resolve or become chronic?
Use of liver biopsy
Often not required if the acute injury resolves
Helpful in confirming clinical suspicion of DILI but rarely pathognomonic
Useful to differentiate between Drug-Induced autoimmune hepatitis and idiopathic autoimmune hepatitis
Useful to rule out underlying chronic viral hepatitis, non-alcoholic fatty liver disease, alcoholic liver disease, or other chronic liver disease
Used to exclude DILI where re-exposure or ongoing use of an agent is expected
Rechallenge: Generally best avoided, unless there is no alternative treatment
Use of Causality Assessment Methods
Roussel Uclaf Causality Assessment Method is best considered an adjunct to expert opinion (it should not be the sole diagnostic method)
Consensus opinion
Expert consultation
For patients with chronic viral hepatitis, DILI requires a high index of suspicion, knowledge of a stable clinical course before the new medication, and monitoring of viral loads to rule out flares of the underlying disease
Assigning causality to herbal compounds and dietary supplements can be especially difficult; require knowledge of all ingredients and their purity

DILI: Drug-induced liver injury.

agents other than acetaminophen, including HDS<sup>[72,73]</sup>. As of 2014, DILIN continued to publish analyses from the data in their registry, most notably defining clinical signatures of specific agents; chiefly, a new syndrome was identified to occur after a single intravenous dose of cefazolin, characterised by marked cholestasis and a self-limited moderate to severe clinical course, following a one to three week latency period<sup>[74]</sup>. Globally, numerous registries have been formed in the past decade, including those in Australia<sup>[75]</sup>, Spain<sup>[76]</sup>, Iceland<sup>[77,78]</sup>, India<sup>[79]</sup>, South Korea<sup>[80]</sup>, and Serbia<sup>[81]</sup>, amongst others<sup>[82-84]</sup>. In addition to DILIN and the other national databases, the United States National Institutes of Health and National Library of Medicine launched LiverTox<sup>[85]</sup> (<https://livertox.nlm.nih.gov/>) in April 2012. This comprehensive, up-to-date, interactive online resource, with over 650 agents currently listed, projects to expand its role into a virtual textbook on hepatotoxicity<sup>[7]</sup>. In the light of these collective efforts, gaps in our knowledge still undoubtedly remain<sup>[2,4,11]</sup>.

## EPIDEMIOLOGICAL ISSUES

One of the greatest challenges to furthering our understanding of the global epidemiology of DILI is the elusive nature of its clinical presentation. Illustration of the fact can be seen in several studies, which found that DILI is both under-recognized and under reported<sup>[83,86-89]</sup>. In one study<sup>[86]</sup>, around 50% of the suspected DILI cases investigated were found to be common hepatic disorders when assessed by specialists and DILI experts. In another study from France<sup>[83]</sup>, underreporting by clinicians untrained in the recognition of DILI was greater

by a factor of 16, when compared to those specifically trained to identify cases.

The fact remains, that acute DILI is a relatively rare clinical entity, and as such, determining the exact incidence from individual drugs is arduous. The estimated incidence of non-acetaminophen-related DILI, reported from a population-based Icelandic study, was found to be 19.1 cases per 100000 inhabitants<sup>[78]</sup>, similar to the 13.9 per 100000 found more than ten years prior, in France<sup>[83]</sup>. A higher incidence was found in Spain in 2005, with 34.2 per 100000 inhabitants per year, and 16.6 per 100000 inhabitants per year being serious life-threatening episodes<sup>[76]</sup>. In Great Britain, the estimated incidence per 100000 persons was 2.4 in 2004<sup>[86]</sup>, however more recent data is unavailable. In the United States, a retrospective cohort study determined an incidence rate of drug-induced ALF of 1.61 events per 1000000 person-years<sup>[90]</sup>. By using population-based epidemiological data within the paediatric population, the incidence of acute liver injury was found to be comparable to that of the adult population, with higher incidence in Italy, when compared to the Netherlands (73 and 21 per 100000, respectively)<sup>[91]</sup>. Antibiotics were the most frequent offending drugs in this study and others, as comprehensively discussed by Björnsson<sup>[89]</sup>, stating that amoxicillin-clavulanic acid and INH in particular, along with other antibiotics and antiepileptics are the most common agents linked to hepatotoxicity. If one takes into account data from the United States Acute Liver Failure Study Group, acetaminophen is the most common overall causative agent for ALF with 45.8%, followed by non-acetaminophen DILI with 11%<sup>[19]</sup>, and INH the leading cause of DILI thereafter with 18.8%<sup>[20]</sup>.

These findings come from large cohorts, however the vast majority of DILI research comes in the form of numerous case reports identifying novel hepatotoxic agents; the most recent example from 2016, being hepatotoxicity in HIV/HCV infected patients receiving ledipasvir/sofosbuvir with or without ribavirin<sup>[92,93]</sup>.

Herbals pose yet another obstacle to our understanding of the epidemiology of DILI. Currently, the absence of regulatory guidelines for the production and sale of herbal compounds, means that the calculation of the true incidence of herbal-induced liver injury (HILI) becomes very difficult. Evidence is emerging from Asia, in particular China, where in a cohort of 21789 patients with DILI found that alternative medicines were one of the two most common etiologies reported<sup>[94]</sup>. It is estimated that 15% of DILI cases may be attributed to herbs and other traditional Chinese medicines<sup>[95]</sup>. In South Korea, DILI incidence was 12 per 100000 persons, with 70% due to herbal and folk remedies<sup>[80,96]</sup>. According to the DILIN registry, HDS were responsible for DILI in 16% of cases, second only to antimicrobials<sup>[72]</sup>. What is potentially worrying is that patients with chronic liver disease (CLD) have been increasingly using HDS<sup>[97]</sup>, leading to an increase in safety alerts from the FDA and other regulatory bodies<sup>[43,73]</sup>. The most recent HDS to receive hepatotoxicity warning labels were the muscle building, fat burning product OxyELITE Pro<sup>[97]</sup> (USP Labs LLC, Dallas, Texas) and the weight loss supplement Herbalife<sup>[98]</sup>. Other causes of HILI include anabolic steroids, black cohosh, green tea, Hydroxycut (Iovate Health Sciences Inc, Oakville, Ontario, Canada), and kava<sup>[99]</sup>, and therefore HDS should also be on one's mind in any case of suspected liver injury.

## DEFINING, RECOGNISING AND PREDICTING DILI

At this stage, it may be helpful to remind one that DILI is initially defined as either intrinsic (predictable, dose-dependent) or idiosyncratic (unpredictable and non-dose dependent). By far the most common intrinsic cause of DILI is acetaminophen<sup>[19]</sup>. Twenty billion doses of non-prescription acetaminophen are sold annually in the United States, with \$87 million dollars spent treating complications of overdose<sup>[100,101]</sup>. The intrinsic nature of acetaminophen hepatotoxicity stems from the production of N-acetyl-p-benzoquinone imine; excessive accumulation of this reactive metabolite leads to a depletion of intracellular glutathione, in turn leading to zone 3 centrilobular necrosis of the hepatocytes<sup>[102,103]</sup>. This predictable course of acetaminophen toxicity led to the introduction of N-acetylcysteine (NAC) as an antidote in 1977<sup>[104]</sup>, remaining the drug of choice for overdose treatment today<sup>[100]</sup>.

The mechanisms of idiosyncratic DILI on the other hand, have a far more complex nature and are the focus of the majority of current research. Broadly speaking they may be divided into two categories, hypersensitivity-type

reactions (also known as immunologic), and metabolic types of injuries<sup>[10]</sup>. Hypersensitivity-type reactions, occurring due to reactive metabolites covalently binding proteins, forming drug-protein adducts, and thus triggering immune-mediated reactions or direct hepatic toxicity<sup>[12]</sup>, account for 23%-37% of all idiosyncratic DILI cases<sup>[10]</sup>. In addition, lipophilicity combined with dose, also known as the "rule-of-two"<sup>[27,28]</sup>, is known to enhance the risk of developing DILI, due to increased blood uptake into hepatocytes, forming greater amounts of reactive metabolites and subsequently interacting with hepatocanalicular transport and mitochondrial membranes<sup>[12]</sup>. As such, metabolic mechanisms include oxidative stress, mitochondrial liability and inhibition of hepatobiliary transporters<sup>[12]</sup>. In the case of INH induced DILI, hepatocellular injury may result from the creation of covalent drug-protein adducts, leading to hapten formation and an immune response, and/or through direct mitochondrial injury by INH or its metabolites, leading to mitochondrial oxidant stress and energy homeostasis impairment<sup>[54]</sup>. If such mitochondrial deficiencies are already present, even non-toxic concentrations of INH, may trigger marked hepatocellular injury, due to underlying impairment of complex I function<sup>[54]</sup>. Other examples of mitochondrial injury include: Impaired beta-oxidation, and mitochondrial respiration, membrane disruption and mtDNA damage, usually caused by tamoxifen, valproic acid, diclofenac and tacrine, respectively<sup>[12]</sup>.

Indeed, hundreds of offending drugs have been identified thus far, with the list constantly growing. However, according to the DILIN registry<sup>[72]</sup>, the top 10 drugs account for greater than one-third of all idiosyncratic DILI cases. The most common causative agents and drug classes, according to various registries, are summarized in Table 2. The lists are rather heterogenic, however, antibiotics amoxicillin-clavulanate and INH top most registries as individual agents. Unsurprisingly, antituberculous agents top the list of severe and often fatal DILI in India, where acetaminophen use is rare and tuberculosis is prevalent<sup>[79]</sup>. Of the drug classes, antibiotics are the most common agents amongst the registries investigated with the exceptions of Spain and Sweden, where "other" drugs are most common with 44% and 69%, respectively. Collectively, these data illustrate that DILI cases and the drugs responsible vary from country to country, based on the overall prevalence of certain diseases within each healthcare system.

Due to the large number of different causative agents, further division of idiosyncratic DILI is classically determined on three biochemical patterns of liver injury: Hepatocellular, cholestatic and mixed, and based on the ratio of alanine aminotransferase (ALT) to alkaline phosphatase (ALP) defined as an *R* value<sup>[105]</sup> (Table 3). The prognosis of each case is greatly dependant on which pattern of injury has occurred, and although bilirubin is not incorporated into the *R* value, it remains a central prognostic marker in calculating the Model for End-Stage Liver Disease score along with defining Hy's law<sup>[7]</sup>.

**Table 2** The most common individual drugs and classes responsible for idiosyncratic drug-induced liver injury according to various Global Registries

	Iceland <sup>[78]</sup> , <i>n</i> = 96	India <sup>[79]</sup> , <i>n</i> = 313	Spain <sup>[76]</sup> , <i>n</i> = 446	Sweden <sup>[77]</sup> , <i>n</i> = 784	United States DILIN <sup>[72]</sup> , <i>n</i> = 899
Individual drugs (%)					
Amoxicillin-clavulanate	22.9	INH + anti-TB 57.8	Amoxicillin-clavulanate 13.2	Flucloxacillin 16.5	Amoxicillin-clavulanate 10%
Diclofenac	6.3	Phenytoin 6.7	INH + anti-TB 6.9	Erythromycin 5.4	INH 5.3%
Nitrofurantoin	4.2	Dapsone 5.4	Ebrotidine 4.9	Disulfiram 3.4	Nitrofurantoin 4.7%
Infliximab	4.2	Olanzapine 5.4	Ibuprofen 4	TMP-SMX 2.7	SMX-TMP 3.4%
Azathioprine	4.2	Carbamazine 2.9	Flutamide 3.8	Diclofenac 2.6	Minocycline 3.1%
Isotretinoin	3.1	Cotrimoxazole 2.2	Ticlopidine 2.9	Carbamazepine 2.2	Cefazolin 2.2%
Atorvastatin	2.1	Atorvastatin 1.6	Diclofenac 2.7	Halothane 1.9	Azithromycin 2%
Doxycycline	2.1	Leflunamide 1.3	Nimesulide 2	Naproxen 1.4	Ciprofloxacin 1.8%
		Ayurvedic 1.3	Carbamazepine 1.8	Ranitidine 1.3	Levofloxacin 1.4%
Drug classes (%)					
Antibiotics	37	65	32	27	45.4
HDS	16	1.3	2	NS	16.1
CNS	7	12	17	3	9.8
Hypolipidemic	3.1	1.6	5	1	3.7
Others	37	20	44	69	25.7

United States DILIN: United States Drug-Induced Liver Injury Network; INH: Isoniazid; SMX: Sulfamethoxazole; TMP: Trimethoprim; TB: Tuberculosis; HDS: Herbal and dietary supplements; CNS: Central nervous system; NS: Not specified.

**Table 3** *R* values<sup>[105]</sup>

Calculation of <i>R</i> value
ALT/AST value divided by its ULN = fold elevation/fold elevation above ULN for alkaline phosphatase
Definitions
Hepatocellular injury = $R > 5$
Cholestatic injury = $R < 2$
Mixed injury = $R > 2 < 5$

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ULN: Upper limit of normal.

The cornerstone of any liver assessment rests on ALT and aspartate aminotransferase (AST) elevations indicating hepatocellular injury, however in the case of DILI, these indicators are neither sensitive nor specific and cannot predict the pattern of injury because they are elevated after injury has already occurred<sup>[22,105,106]</sup>. This brings into question the role of liver biopsy. The United States DILIN has recognized 18 distinct histological categories of damage: Acute hepatitis, chronic hepatitis, acute cholestatic, chronic cholestatic, cholestatic-hepatitic, granulomatous, macrovesicular steatotic, microvesicular steatotic, steatohepatitic, zonal necrosis, nonzonal necrosis, vascular injury, hepatocellular alteration, nodular regenerative hyperplasia, mixed or unclassified injury, minimal nonspecific changes, absolutely normal, and massive necrosis<sup>[107-109]</sup>. The most common of these are acute and chronic hepatitic, acute and chronic cholestatic, and mixed hepatitis-cholestatic<sup>[107]</sup>, and are most often associated with fluoroquinolones, nitrofurantoin, methyl dopa, and amoxicillin-clavulanate, respectively<sup>[10]</sup>. Although useful in narrowing the differential diagnosis to a specific drug or class, liver biopsy is not required for the clinical evaluation and diagnosis of idiosyncratic DILI, and is performed in less than half of suspected cases<sup>[76]</sup>. Testament to this reasoning is the fact that the histological

patterns of DILI are neither pathognomonic nor do they perfectly correlate with the biochemical patterns<sup>[10,107]</sup>. Indeed, biochemical parameters underestimate the degree of cholestasis and bile duct injury<sup>[107]</sup>, and although hepatocellular damage correlates better, the mixed biochemical pattern overestimates the degree of cholestasis compared to hepatocellular damage<sup>[107]</sup>. With this in mind, according to the first guidelines for DILI diagnosis and management<sup>[69]</sup>, liver biopsy is integral in differentiating drug-induced autoimmune hepatitis (DI-AIH) from idiopathic autoimmune hepatitis (AIH) (Table 1). Histopathological evidence of portal neutrophils, and intracellular cholestasis, favours the diagnosis of DI-AIH over AIH<sup>[7,69]</sup>, and therefore one may employ biopsy in such cases.

The clinician is therefore left with their experience and knowledge of mimickers of DILI, when distinguishing between drug and non-drug causes of hepatic injury. Employing *R* values and the absolute height of liver enzymes are helpful in ruling DILI in or out. In the latest DILIN series, the mean values of ALT were 825 IU/L overall, approximately 20 × the upper limit of normal (ULN), with mean peaks of 1510 IU/L<sup>[72]</sup>. For cholestatic DILI the mean peak of ALP was 682 IU/L (6 × ULN)<sup>[72]</sup>. For idiosyncratic drug-induced ALF the median peak values of ALT were around 500 IU/L<sup>[19]</sup>, incomparable with the record elevations seen in acetaminophen injury<sup>[6]</sup>. Simply put, for values of ALT or AST > 7500 IU/L, the differential diagnosis is essentially shock liver, toxic mushroom or other chemical poisoning, and acetaminophen overdose, and not idiosyncratic DILI<sup>[6]</sup>. Similarly, the enzyme elevations of acute idiosyncratic DILI are different from those found in alcoholic liver disease<sup>[6,7]</sup>. With our growing clinical expertise, newly identified viral causes, including hepatitis E virus (HEV), have made clear recognition even more arduous<sup>[7]</sup>. Mimicry by HEV should therefore be on the clinician's mind when forming a differential diagnosis



**Table 4 Classic Clinical Syndromes of drug-induced liver injury and the drugs most commonly associated<sup>[6,7,117]</sup>**

Acute viral hepatitis-like: <i>e.g.</i> , INH: Absence of hypersensitivity symptoms; present with malaise, fatigue, anorexia, nausea, vomiting, right upper quadrant pain
Hypersensitivity syndrome: Fever, rash, and/or eosinophilia seen in 25%-30% of DILI cases, usually with short latency and prompt rechallenge response ( <i>e.g.</i> , amoxicillin-clavulanate, phenytoin, carbamazepine, SMX-TMP, halothane)
Sulfone syndrome: <i>e.g.</i> , dapsone: Fever, exfoliative dermatitis, lymphadenopathy, atypical lymphocytosis, eosinophilia, hemolytic anemia, methemoglobinemia
Pseudomononucleosis syndrome: <i>e.g.</i> , phenytoin, dapsone, sulfonamides: Hypersensitivity syndrome with atypical lymphocytosis, lymphadenopathy, and splenomegaly
DILI associated with severe skin injury: Stevens-Johnson syndrome, toxic epidermal necrolysis, <i>e.g.</i> , beta-lactam antibiotics, allopurinol, carbamazepine
Autoimmune hepatitis associated with positive autoantibodies: <i>e.g.</i> , nitrofurantoin, minocycline, methyl dopa
Immune-mediated colitis with autoimmune hepatitis: <i>e.g.</i> , ipilimumab
Acute cholecystitis-like: <i>e.g.</i> , erythromycin estolate
Reye syndrome-like: <i>e.g.</i> , valproic acid: Hepatocellular injury, acidosis, hyperammonemia, encephalopathy, abdominal pain, nausea, vomiting, paradoxical worsening of seizure activity, microvesicular steatosis on biopsy

INH: Isoniazid; SMX: Sulfamethoxazole; TMP: Trimethoprim; DILI: Drug-induced liver injury.

of DILI<sup>[7,60]</sup>.

As early as 1978, Hyman Zimmerman stated that drugs causing acute hepatocellular injury with jaundice were associated with a case-fatality rate of 10% or higher<sup>[7,110]</sup>, a statement that was termed "Hy's Law" by Robert Temple at the FDA<sup>[7,110]</sup>. The current, modified definition of Hy's Law<sup>[35,110,111]</sup> consists of ALT/AST > 3 × ULN in addition to total bilirubin > 2x ULN in the absence of cholestatic injury (ALP < 2 × ULN), with no other identifiable cause<sup>[69,111]</sup>. Of such importance is this law that it remains a key element in determining whether DILI is present or not, and may in fact be the sole reason for abandonment of a drug's development<sup>[112]</sup>.

## BIOMARKERS

In the light of such difficulty in distinguishing DILI from other causes of hepatic injury, researchers have begun investigating potential biomarkers in an attempt at earlier identification<sup>[113]</sup>. Many possible genetic associations between individual human leukocyte antigens and the potential for DILI have been explored<sup>[114-116]</sup>, however no definitive biomarker has yet been found. Of promise, are microRNAs, cytokeratin-18, and high mobility group box protein 1<sup>[113]</sup>.

## DIAGNOSING, AND ESTABLISHING CAUSALITY

So, with no particularly sensitive or specific biomarker, and little use of liver biopsy, DILI essentially remains a diagnosis of exclusion<sup>[49,69,107]</sup>. Recognising the clinical picture of DILI is therefore paramount<sup>[6,117]</sup> (Table 4). With such diverse presentation and because many individual cases of DILI are presented as case reports or case series, it is essential for the clinician to establish solid causality when suspecting DILI. Nearly 25 years ago, an international meeting of hepatologists convened in an attempt to create an objective causality assessment tool for DILI<sup>[7,105]</sup>. Although not quite user-friendly, the Roussel Uclaf Causality Assessment Method (RUCAM)

remains in widespread use today<sup>[60]</sup>. It is based on expert consensus, and thus scoring requires extensive knowledge, and along with its many omissions, RUCAM is under much scrutiny in clinical practice, with a re-evaluation and revision far overdue<sup>[60]</sup>. As such, it is not the only causality tool employed by the DILIN, which has created its own additional criteria based on expert opinion incorporated into RUCAM, as illustrated in Table 5<sup>[59,118]</sup>. Even with more accurate causality tools, the clinical problems in diagnosing DILI in the setting of underlying CLD<sup>[69,72]</sup>, malignancy<sup>[119]</sup>, or congestive heart failure<sup>[120]</sup> still rests heavily on physician's expertise which cannot easily be substituted by scoring systems<sup>[60,69]</sup>, a fact which is even more relevant in the face of HILI, because of the unknown and unregulated ingredients often incorporated into HDS<sup>[73]</sup>, again indicating the need for future research in this field<sup>[121]</sup>.

## RISK FACTORS AND NATURAL PROGRESSION OF DILI

With the difficulty of establishing diagnosis and causality, an important point to remember is who is at the greatest risk for DILI. The exact pathogenesis of idiosyncratic DILI and HILI is poorly understood, and the risk factors arise from three diverse aspects: (1) clinical host-related; (2) environmental; and (3) drug-related. Non-modifiable risk factors include age and gender<sup>[122]</sup>; however one must remember discrepancies in DILI reporting when citing one particular age or gender at greatest risk, for example, males have been indicated as high risk patients for DILI associated with systemic antivirals, whereas liver injury and ALF has been reported with higher frequency in children<sup>[81,123]</sup>. In any case, females have been predominately identified in many registries<sup>[71,76-79]</sup>. As mentioned above, much research has focused on genome-wide studies<sup>[114-116,124]</sup>, and this is an area where we should be focusing our future attention. Environmental factors are poorly understood, with no definitive studies linking diet, or alcohol and coffee consumption to increased DILI risk, again illustrating a need for answers. The "Rule-of-Two",

**Table 5** Drug-induced liver injury network scoring criteria<sup>[59,118]</sup>

Causal relationship	Percentage of likelihood	Definition
Unlikely	< 25	Clear evidence that an etiology other than the drug is responsible
Possible	25-49	Evidence for the drug is present but equivocal
Probable	50-75	Preponderance of the evidence links the drug to the injury
Highly likely	75-95	Evidence for the drug causing injury is clear and convincing but not definite
Definite	< 95	Evidence of the drug being causal is beyond any reasonable doubt

defined as increased DILI risk with higher lipophilicity and drug dose or greater degrees of hepatic metabolism<sup>[27,28]</sup>, is a known risk factor. It accurately predicted liver injury in 14 of 15 drugs withdrawn due to hepatotoxicity, with a warning affixed to the final drug, and successfully predicted hepatotoxicity in multidrug regimens<sup>[7]</sup>. In spite of this success, upon multivariate logistic regression analysis, high lipophilicity was not a significant factor<sup>[27]</sup>, suggesting a redefinition may be necessary.

If a drug causes acute DILI, it is generally accepted that discontinuation will lead to a resolution of any injury within a few weeks<sup>[125]</sup>, and this is definitely true for hepatocellular injury<sup>[76,126]</sup>. In the case of cholestatic injury, often caused by antimicrobials, this process of resolution may take months, and can even persist after drug discontinuation<sup>[126]</sup>; in fact mimicry of primary biliary cholangitis and the development of portal hypertension has occurred<sup>[127]</sup>. Chronically administered drugs such as methyldopa, minocycline and nitrofurantoin have been associated with an insidious and self-limited autoimmune hepatitis, which resolves after discontinuation of the offender<sup>[128]</sup>. As such, the United States DILIN follows patients for a minimum of 6 mo after any case of DILI<sup>[72]</sup>. However, as of August 2016, Medina-Caliz *et al.*<sup>[129]</sup>, on behalf of the Spanish DILI registry, defined a new cut-off for chronic DILI of 1 year, suggesting that ALP and total bilirubin measurements in the second month after acute injury may help predict chronicity. Furthermore statins were implicated as distinctly related to chronicity<sup>[129]</sup>. Therefore, it is prudent to consider acute DILI transforming into chronic DILI in certain patients.

## PREVENTION AND TREATMENT OPTIONS

The saying goes, the best treatment is prevention, and in the case of DILI this sentiment holds true. Liver injury may be caused by most drugs, and labels often carry a warning to lower the dose in the setting of CLD<sup>[124]</sup>, however, there is little evidence to support this reducing the risk for DILI<sup>[130]</sup>. As such, liver enzyme monitoring has been proposed as an option in all drugs with a high risk of hepatotoxicity<sup>[131]</sup>. An example is bosentan, however, even after stringent risk evaluation, adherence remained an issue<sup>[132]</sup>, and therefore, testing for CYP2C9 prior to administration may prove effective<sup>[133]</sup>. Similarly, statins were recommended to be followed with regular enzyme monitoring based on animal toxicity<sup>[134]</sup>, however again compliance was sub-optimal<sup>[135]</sup> and hence, ALT monitoring was dropped by the FDA<sup>[134]</sup>. Nevertheless,

in CLD patients ALT monitoring of patients receiving statins in the first months is sensible, given the fact that potential benefits may outweigh risks<sup>[134]</sup>. The fact that INH remains a major cause of DILI and drug-induced ALF, illustrates that monitoring is not as effective as one would hope<sup>[79]</sup>. Whether ALT finger stick testing, such as in the case of glucose, could become a global standard practice and positively influence monitoring regimens, remains to be answered in the not too distant future<sup>[136,137]</sup>.

A rather controversial issue is that of desensitization-rechallenge. Generally it is discouraged<sup>[69,131]</sup> for fear of an even more severe reaction or ALF, and death<sup>[138]</sup>. Nevertheless, for life-threatening diseases including active tuberculosis where no other therapy is adequate, rechallenge has been successfully carried out<sup>[139]</sup>. Studies investigating the effects of switching drugs within one class or between different classes with similar effects are sparse<sup>[7]</sup>, yet drug substitutions have been reported with non-estolate salts of erythromycin<sup>[127]</sup>, statins<sup>[140]</sup>, and thiazolidinediones<sup>[141]</sup>. Albeit more likely to cause liver injury, cephalosporins are good substitutes for penicillin<sup>[142]</sup>, though it should go without saying that if the benefits do not outweigh the risks, desensitization-rechallenge ought to be avoided.

Even though our ability to detect, diagnose and prevent acute idiosyncratic DILI has had many advances, treatment has largely remained unchanged, with removal of the offending drug as soon as possible being the only undisputable option<sup>[6,43,69,125]</sup>. This may at times place the patient at risk for not receiving efficacious and essential medications, and hence, alternatives and adjuvants to the removal of responsible agents have been investigated. Circumstantial success has been achieved in some patients with cholestatic DILI with the use of ursodesoxycholic acid and steroids<sup>[66]</sup>, however a targeted treatment for hepatocellular idiosyncratic DILI remains to be found. In the case of intrinsic DILI, acetaminophen overdose is and has been prevented and managed with NAC for decades<sup>[100,104,143]</sup> with the identification of patients at high risk for anaphylactoid reactions to NAC being essential for optimal treatment<sup>[144]</sup>. For non acetaminophen drug-induced ALF, NAC has been shown to be of benefit in adults in the early stages of disease, however, once liver coma sets in, the use of NAC is futile<sup>[67]</sup>; and it is virtually useless in children with ALF<sup>[68]</sup>. Other treatments have shown some benefits for specific agents including: Folic acid in the case of methotrexate toxicity<sup>[145]</sup>, carnitine supplementation in children for

valproic acid related liver injury<sup>[146]</sup>, and increasing hepatic clearance with an enterohepatic washout regimen of cholestyramine for leflunomide associated injury<sup>[147]</sup>. Plasma exchange and bioartificial liver assist devices such as molecular absorbant recirculating systems have proven to successfully bridge certain patients to liver transplant, which remains the best therapy for irreversible ALF<sup>[20,64,65,148]</sup>. The search for novel treatment options broadly ranges from the use of nanotechnology to deliver hepatoprotective agents directly to the liver<sup>[63]</sup>, to the humble milk thistle<sup>[149]</sup>. So one can see that apart from some anecdotal treatment options and of course removal of the offender, we are mostly alone in the dark and in need of further advances.

## CONCLUSION

Our knowledge of DILI has come a long way in the past 60 years. We have an extensive amount of knowledge about which drugs are responsible and how to detect them, our understanding of the various mechanisms involved is constantly expanding, and we are identifying which patients are most at risk, however our knowledge is far from complete. In keeping with our oath, *Primum non nocere*, the quintessential question should not be “do we know everything?”, but rather, do we know enough to successfully prevent, accurately diagnose, and safely treat all of our patients.

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## Case Control Study

## Egg consumption and risk of non-alcoholic fatty liver disease

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**Institutional review board statement:** The study was approved by the ethics committee of National Nutrition and Food Technology Research Institute, Tehran, Iran.

**Informed consent statement:** All patients signed the informed consent form.

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### Abstract

#### AIM

To evaluate the association between egg consumption and risk of non-alcoholic fatty liver disease (NAFLD) development.

#### METHODS

This case-control study was conducted on individuals who were referred to two hepatology clinics in Tehran, Iran in 2015. The study included 169 patients with NAFLD and 782 controls. Egg consumption was estimated using a validated food frequency questionnaire. The participants were categorized according to the frequency of their egg consumption during the previous year: Less than two eggs per week, two to three eggs per week, and four or more eggs per week.

#### RESULTS

In the crude model, participants who consumed 2 to 3 eggs per week, were 3.56 times more likely to have NAFLD in comparison to those who consumed less than 2 eggs per week (OR: 3.56; 95%CI: 2.35-5.31). Adjustment for known risk factors of NAFLD strengthened



this significant association so that individuals have consumed two to three eggs per week had 3.71 times higher risk of NAFLD than those who have eaten less than two eggs per week (OR: 3.71; 95%CI: 1.91, 7.75).

## CONCLUSION

Our data indicate that higher egg consumption in common amount of usage is associated with higher risk of NAFLD.

**Key words:** Egg; Diet; Non-alcoholic fatty liver disease; Dietary cholesterol

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**Core tip:** The data indicate that egg consumption in common amount of usage is associated with risk of non-alcoholic fatty liver disease. According to the case-control design of this study, it can not show the causality effect; thus, these findings should be confirmed in future prospective studies with separate parts of eggs to find the etiological relationships.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of liver disorders from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and even hepatocellular carcinoma<sup>[1]</sup>. NAFLD is the most common cause of chronic liver diseases around the world<sup>[2]</sup> and may be considered as hepatic manifestation of metabolic syndrome<sup>[3]</sup>. The increasing prevalence of obesity, together with insulin resistance, hypertension, dyslipidemia, and eventually the metabolic syndrome dispose many people to the risk of NAFLD development in the future years<sup>[4]</sup>.

Increasing evidence showed that dietary factors contribute to the pathophysiology and treatment of NAFLD<sup>[5-7]</sup>. Among the known dietary factors that involved in the development of NAFLD, dietary cholesterol has drawn a great deal of attention. Current studies of animal models propose that excess dietary cholesterol is regarded as the key factor related to the risk of steatohepatitis and hepatic inflammation<sup>[8-10]</sup>. Addition of cholesterol to the diet of obese, diabetic mice increased the accumulation of hepatic free cholesterol, hepatocyte apoptosis, and liver fibrosis<sup>[11]</sup>. Moreover, an association between raised cholesterol intake and the risk or severity of NAFLD has been addressed by epidemiological studies<sup>[12-14]</sup>.

Among single foods, eggs are regarded as a main

source of dietary cholesterol, with one large egg containing almost 210 mg of cholesterol; on the other hand, eggs are rich in proteins, and other nutrients<sup>[15]</sup>, which can improve human health. There is limited evidence on the relationship between egg consumption and NAFLD and its risk factors with controversial results<sup>[16-18]</sup>. Therefore, the present study was designed to examine the association between egg consumption and risk of NAFLD development.

## MATERIALS AND METHODS

### Participants

The present case-control study was conducted on individuals who were undertaken a liver Ultrasound, and were referred to two Hepatology clinics in Tehran, Iran in 2015. The study included 169 patients with NAFLD and 782 controls. The cases were patients with NAFLD, which was diagnosed by a gastroenterologist according to the presence of hepatic steatosis in Ultrasound exam within previous month, and referred to our clinics to be examined by Fibroscan<sup>®</sup>, and the Fibroscan result showed a Controlled Attenuation Parameter score of more than 263, and fibrosis score of more than 7. These patients were selected with the convenience-sampling procedure. Controls were randomly selected age- and sex-matched subjects from the same clinic among patients with pancreatobiliary disorders who had been undertaken an Ultrasound showing no hepatic steatosis. The age ranges for matching were 20-40, 40-60 and > 60 years old. Data on each pair of cases and controls were collected at the same time. The participation rate in the study was 94% for cases and 98% for controls. Written informed consent was obtained from all the participants. The study protocol was approved by the local Ethics Review Committee.

### Assessment of dietary intake

Dietary intake of patients was assessed using a valid and reliable semi-quantitative food frequency questionnaire (FFQ), which included 168 items of foods with standard servingsizes, as commonly consumed by Iranians<sup>[19]</sup>. The consumption frequency of each food item was questioned on a daily, weekly or monthly basis and converted to daily intakes. In the case of egg consumption, the participants were categorized according to the frequency of their egg consumption during the previous year: Less than two eggs per week, two to three eggs per week, and four or more eggs per week. Dietary nutrients intakes were calculated using NUTRITIONIST V (First Databank, Hearst Corp, San Bruno, CA, United States). The patients who had completed less than 90% of dietary questionnaires and subjects who reported extremely low or high energy intakes (< 500 or > 5000 kcal/d) were excluded from the study<sup>[20]</sup>.

### Assessment of other variables

Physical activity was evaluated using the metabolic

**Table 1** Baseline characteristics, biochemical parameters and dietary intakes of study participants based on the patients with non-alcoholic fatty liver disease and control group

	Cases ( <i>n</i> = 169)	Controls ( <i>n</i> = 782)	<i>P</i> value <sup>a</sup>
Age (yr), mean ± SD	42.65 ± 12.21	43.71 ± 14.52	0.373
Male <i>n</i> (%)	81 (47.9)	314 (40.2)	0.063
BMI (kg/m <sup>2</sup> ), mean ± SD	33.19 ± 8.71	27.74 ± 4.495	< 0.001
Physical activity (MET), mean ± SD	31.89 ± 3.15	34.33 ± 2.85	< 0.001
Current smokers, <i>n</i> (%)	151 (89.9)	145 (18.5)	< 0.001
Drank alcohol in past year, <i>n</i> (%)	22 (13.1)	68 (8.7)	0.077
Diabetes type 2, <i>n</i> (%)	26 (15.6)	53 (6.8)	< 0.001
FBS (mg/dL), mean ± SD	109.29 ± 39.39	90.09 ± 29.24	< 0.001
Total cholesterol (mg/dL), mean ± SD	184.79 ± 54.94	177.72 ± 38.74	0.221
LDL (mg/dL), mean ± SD	121.17 ± 43.04	104.26 ± 31.65	< 0.001
HDL (mg/dL), mean ± SD	41.26 ± 16.72	47.72 ± 10.51	0.001
Triglycerides (mg/dL), mean ± SD	180.40 ± 123.81	131.97 ± 81.59	< 0.001
Total energy (kcal), mean ± SEM	2627.67 ± 61.39	2746.69 ± 27.23	0.068
Carbohydrate (% of total energy), mean ± SEM	58.12 ± 0.95	59.82 ± 0.44	0.001
Protein (% of total energy), mean ± SEM	15.84 ± 0.18	14.07 ± 0.08	< 0.001
Fat (% of total energy), mean ± SEM	29.23 ± 0.30	33.78 ± 0.20	< 0.001
Dietary cholesterol (mg/d), mean ± SEM	315.31 ± 11.50	263.41 ± 5.35	< 0.001
Saturated fat (g/d), mean ± SEM	30.62 ± 5.72	62.67 ± 2.67	< 0.001
Monounsaturated fat (g/d) (mg/d), mean ± SEM	29.85 ± 0.48	32.00 ± 0.23	< 0.001
Polyunsaturated fat (g/d) (mg/d), mean ± SEM	18.51 ± 5.74	59.58 ± 2.67	< 0.001
Dietary fiber (g/d), mean ± SEM	19.21 ± 0.50	14.68 ± 0.23	< 0.001
Red/processed meats (g/d), mean ± SEM	70.95 ± 2.66	36.00 ± 1.24	< 0.001

<sup>a</sup>Independent *t*-test for quantitative variables and  $\chi^2$  test for qualitative variables. Dietary intakes (except total energy) were adjusted for age and total energy intake. BMI: Body mass index; MET: Metabolic equivalent task; FBS: Fasting blood sugar; LDL: Low-density lipoprotein cholesterol; HDL: High density lipoprotein cholesterol.

equivalent task (MET) questionnaire<sup>[21,22]</sup>. Other covariate information including age, gender, smoking habits, alcohol consumption, medical history, and current use of medications were assessed using questionnaires. Weight and height of all participants were measured.

### Statistical analysis

Baseline characteristics and dietary intakes were compared between cases and controls using *t*-test for continuous variables and  $\chi^2$  for categorical variables. Egg consumption was divided into three ascending categories on an ordinal scale. Mean or prevalence of baseline characteristics was computed for each category. Baseline characteristics were also compared using ANOVA for continuous variables and  $\chi^2$  for categorical variables. The relationship between NAFLD and egg consumption was assessed using multiple regression analysis. Estimates were presented in three models; the first model was adjusted for age (continuous), and total energy intake (kcal/d). In the second model, we further controlled for body mass index (BMI), history of diabetes and smoking (non-smoker, current smoker). Finally, we further adjusted for physical activity (MET) and gender. All models were conducted by treating the first category of egg consumption (< 2/wk) as a reference. All the statistical analyses were done using SPSS for Windows (version 19; SPSS Inc., Chicago, IL).

## RESULTS

Baseline characteristics, biochemical parameters and

dietary intakes of the cases and controls are shown in Table 1. Mean age of the total study population was 43.54 ± 14.13 years and 41.5% (395) of participants were male. By design, cases and controls had the similar age and sex distribution. Patients with NAFLD had significantly more BMI, lower physical activity, lower consumption of alcohol, and were more likely to be smoker, and have diabetes in comparison to controls. Furthermore, the cases had elevated fasting blood glucose (FBS), low-density lipoprotein cholesterol (LDL), Triglycerides, and reduced high density lipoprotein cholesterol (HDL) levels and increased intake of protein, cholesterol, fiber and red/processed meats compared with the controls (Table 1).

Basic characteristics and dietary intakes of the studied participants by categories of egg consumption are presented in Table 2. Compared to egg consumption of lower than two per week, higher egg consumption was associated with a lower average age, male sex, current smoking, higher energy intake, lower percent of total energy from carbohydrate and fat. Additionally, the subjects with higher egg consumption tended to consume more protein, cholesterol, monounsaturated fat and red/processed meats, but less saturated and polyunsaturated fatty acids (Table 2).

In secondary analysis, there was a similar egg-NAFLD association in women (*P*-trend 0.001) and men (*P*-trend 0.048) (Table 3).

Multivariate adjusted odds ratios for NAFLD based on egg consumption categories are indicated in Figure 1. In the crude model, participants that consumed 2 to 3 eggs per week, were 3.56 times more likely to have NAFLD in

**Table 2 Basic characteristics and dietary intakes of study participants by frequency of egg consumption *n* (%)**

	Egg consumption categories			<i>P</i> value <sup>a</sup>
	< 2/wk ( <i>n</i> = 589)	2-3/wk ( <i>n</i> = 142)	≥ 4/wk ( <i>n</i> = 220)	
Age (yr)	45.65 ± 12.26	39.73 ± 13.18	40.35 ± 13.30	< 0.001
Male gender	218 (37.0)	56 (39.4)	121 (55)	< 0.001
BMI (kg/m <sup>2</sup> ), mean ± SD	28.58 ± 5.44	29.60 ± 7.34	28.51 ± 5.87	0.150
Physical activity (MET), mean ± SD	33.99 ± 3.05	33.42 ± 3.21	33.94 ± 2.95	0.136
Current smokers	155 (26.3)	59 (41.8)	82 (37.3)	< 0.001
Total energy (kcal), mean ± SEM	2580.59 ± 30.68	2744.94 ± 57.45	3101.07 ± 51.20	< 0.001
Carbohydrate (% of total energy), mean ± SEM	60.44 ± 0.67	59.48 ± 0.63	58.14 ± 0.85	0.001
Protein (% of total energy), mean ± SEM	14.09 ± 0.10	14.71 ± 0.20	14.95 ± 0.17	0.001
Fat (% of total energy), mean ± SEM	33.06 ± 0.24	32.56 ± 0.49	32.97 ± 0.40	< 0.001
Dietary cholesterol (mg/d)	226.40 ± 5.75	291.95 ± 11.60	383.90 ± 9.53	< 0.001
Saturated fat (g/d)	56.70 ± 3.16	64.70 ± 6.38	52.57 ± 5.24	< 0.001
Monounsaturated fat (g/d) (mg/d), mean ± SEM	31.20 ± 0.26	31.32 ± 0.53	32.91 ± 0.44	< 0.001
Polyunsaturated fat (g/d) (mg/d), mean ± SEM	53.10 ± 3.20	57.26 ± 6.45	46.71 ± 5.30	< 0.001
Dietary fiber (g/d)	15.65 ± 0.28	16.25 ± 0.57	14.60 ± 0.47	< 0.001
Red/processed meats (g/d)	37.76 ± 1.53	47.79 ± 3.10	50.51 ± 2.54	< 0.001

<sup>a</sup>Dietary intakes (except total energy) were adjusted for age and total energy intake. BMI: Body mass index; MET: Metabolic equivalent task.

**Table 3 Odds ratio for non-alcoholic fatty liver disease according to egg consumption stratified by gender**

Egg consumption	Multivariate adjusted model <sup>a</sup>	
	Female	Male
< 2/wk	1.00	1.00
2-3/wk	5.55 (2.30-13.37)	1.90 (0.50-7.16)
≥ 4/wk	1.67 (0.68-4.10)	0.25 (0.06-1.01)
<i>P</i> for trend	0.001	0.048

<sup>a</sup>Adjusted for age, energy intake, body mass index, history of diabetes, smoking, and physical activity.

comparison to those who consumed less than 2 eggs per week (OR: 3.56; 95%CI: 2.35-5.31). After controlling for age and total energy intake, consuming 2 to 3 eggs per week was positively associated with the risk of NAFLD (OR: 3.83; 95%CI: 2.49-5.89). These associations remained significant even after additionally controlling for BMI, history of diabetes and smoking (OR: 3.57; 95%CI: 1.89-6.75). Further adjustment for physical activity, and gender strengthened this significant association so that individuals who have consumed two to three eggs per week had 3.71 times higher risk of NAFLD than those who have eaten less than two eggs per week (OR: 3.71; 95%CI: 1.91-7.75). Egg consumption more than four per week was not significantly associated with the NAFLD risk.

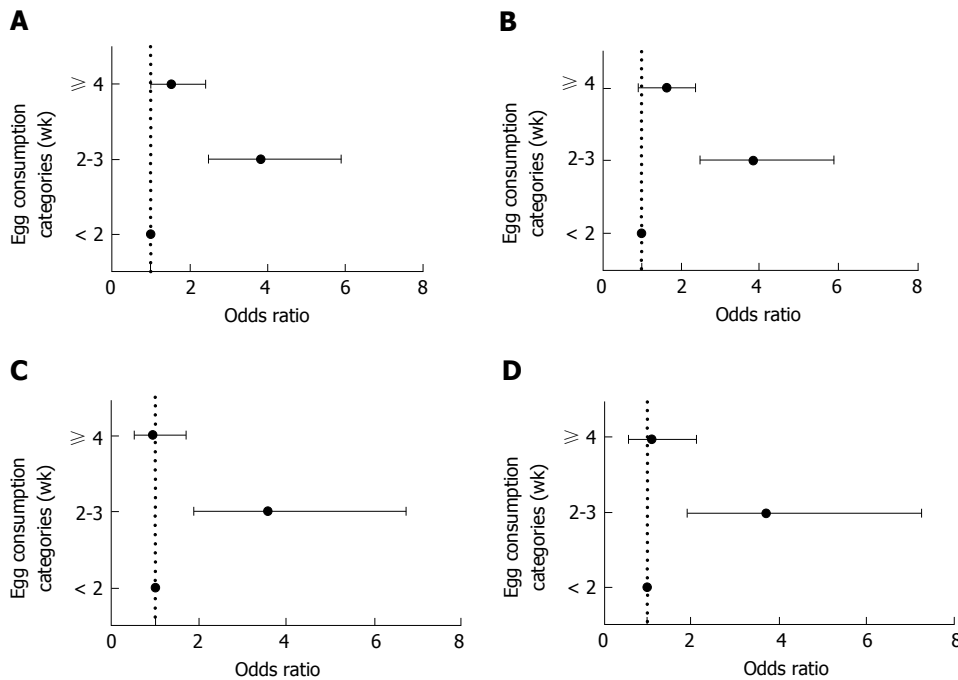
## DISCUSSION

The results of the present study showed that the egg consumption increases the risk of NAFLD in common range of its consumption (two to three eggs per week). This relationship was also significant after adjustment for age, gender, BMI, history of diabetes, smoking, and physical activity.

The role of diet and dietary supplements on the pathogenesis of NAFLD have been shown previously<sup>[23-36]</sup>; however, to our knowledge, no study has yet evaluated the

association of egg consumption and NAFLD risk. It is well established that eggs contain a wide variety of essential nutrients and bioactive compounds that can affect human health. Their high quality protein, fats and micronutrients and low price make them an important part of many people's diet<sup>[37]</sup>; despite the nutritional benefits of egg consumption, there are concerns about their high content of cholesterol and saturated fat and their influences on metabolic disorders<sup>[38]</sup>. Thus, one possible explanation for the inverse association between egg consumption and risk of NAFLD development may be due to the high cholesterol content of egg. Previous studies have shown that a higher consumption of cholesterol is associated with NAFLD and its exacerbation<sup>[12,13,39,40]</sup>. In addition, the presence of high amount of cholesterol in diet is necessary for development of NAFLD<sup>[41]</sup>. Baumgartner *et al.*<sup>[39]</sup> have shown that daily egg consumption increases serum cholesterol and LDL-C concentrations in women; however, there was no effects on markers for inflammation, endothelial activity, and liver function. Interestingly, the consumption of egg white hydrolyzed with pepsin considerably improved hepatic steatosis<sup>[42]</sup>. Thus, it seems that the association between egg consumption and NAFLD is mainly due to high cholesterol content of it, and might not be seen when people consume only the white part of it. Therefore, more studies are recommended to evaluate the effects of consumption of different parts of egg on NAFLD risk<sup>[13]</sup>.

An unexpected finding of the present study was that more than 4 eggs consumption per week was not significantly associated with risk of NAFLD. This may be explained by the fact that nutritional factors are correlated with each other, and determining of the effect of particular nutrients or particular foods on a risk factor is difficult. The effects of egg cholesterol on serum cholesterol concentrations depends on the content of individuals' diet specially the fiber content of it<sup>[43,44]</sup>. It is possible that those who ate more than 4 eggs per week, consumed it in mixed dishes containing vegetables, which reduces the absorption of cholesterol. Thus, we



**Figure 1 Multivariate-adjusted odds ratio for non-alcoholic fatty liver disease according to egg consumption.** A: Crude model; B: Model 2, multivariate adjusted for age and energy intake; C: Model 3, further controlled for, body mass index, history of diabetes and smoking; D: Model 4, additionally adjusted for physical activity, and gender. Data are presented as the odds ratio (95%CI).

suggest that future studies assess the type of dishes with egg to find the possible interactions of different constituent of them.

It has been reported that dietary intake of patients with NAFLD was richer in saturated fat, cholesterol and was poorer in polyunsaturated fat<sup>[12]</sup>. Subramanian *et al.*<sup>[40]</sup> have concluded that dietary cholesterol confers in progression of NAFLD to NASH. Furthermore, Zelber-Sagi *et al.*<sup>[18]</sup> found that NAFLD patients have a higher intake of meat, which is another source of dietary cholesterol; however, some other studies only found a significant association between NAFLD and high dietary intake of carbohydrate and simple sugars<sup>[45,46]</sup>, and some studies did find an association only between NAFLD and low intake of n-3 fatty acids and some antioxidants<sup>[16]</sup>. These dietary habits may accelerate the development of NAFLD by directly affecting steatosis of liver and oxidative injury<sup>[12]</sup>.

This study was the first study that examined the relationship between egg consumption and risk of NAFLD in newly diagnosed patients who have not probably changed their diet due to the disease diagnosis; other strengths of this study includes its relatively large sample size, the high participation rate of participants, and socioeconomic differences of participants, which affects their dietary intakes.

Although we used a validated FFQ for measurement of dietary intakes, measurement error, and recall bias are unavoidable errors. Moreover, there might be some unknown risk factors that affect our results. Therefore, we recommend this analysis to be done in other populations.

In conclusion, our data indicate that egg consumption

in common amount of usage is associated with risk of NAFLD. According to the case-control design of this study, it can not show the causality effect; thus, these findings should be confirmed in future prospective studies with separate parts of eggs to find the etiological associations.

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## COMMENTS

### Background

Among the known dietary factors that affect the pathogenesis of non-alcoholic fatty liver disease (NAFLD), dietary cholesterol has drawn a great deal of attention. Current studies propose that excess dietary cholesterol is regarded as the key factor related to the risk of steatohepatitis and hepatic inflammation. Among individual foods, eggs are regarded as a main source of dietary cholesterol; on the other hand, eggs are rich in proteins, and other nutrients. Limited research has assessed the relationship between egg consumption and risk of (NAFLD) development.

### Research frontiers

Understanding of the association between egg consumption and risk of NAFLD development can contribute to clarify how intake of special food groups correlate with the disease and could lead to more particular guidelines for NAFLD prevention.

### Innovations and breakthroughs

This study showed that egg consumption in common amount of usage is associated with risk of NAFLD. It seems that this association is mainly due to



high cholesterol content of it, and might not be seen when people consume only the white part of it.

### Applications

According to the results of this study, the authors recommend low intake of eggs specially the yolk part of it for prevention of NAFLD; however, further studies are recommended to reach to a consensus in this regard.

### Peer-review

This is an interesting paper evaluating the association between egg consumption and NAFLD.

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Retrospective Study

## Serum 25-hydroxyvitamin D deficiency and hepatic encephalopathy in chronic liver disease

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**Institutional review board statement:** The study has approved by the Ethics Review Committee (RPAH Zone).

**Informed consent statement:** Informed consent was not necessary due to the retrospective nature of this investigation.

**Conflict-of-interest statement:** There were no conflicts of interests for any of the investigators.

**Data sharing statement:** Technical appendix, statistical code and dataset are available from the corresponding author at [helen.vidot@sswahs.nsw.gov.au](mailto:helen.vidot@sswahs.nsw.gov.au). Informed consent was not sought as all data was collected as part of standard care and all data is anonymised and risk of identification is low. No additional data is available.

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### Abstract

#### AIM

To investigate the relationship between 25-hydroxyvitamin D (25-OHD) deficiency and hepatic encephalopathy (HE) in patients with chronic liver disease (CLD).

#### METHODS

A retrospective analysis of the results of 392 adult patients with chronic liver disease who were assessed for liver transplantation between 2006 and 2010 was undertaken. HE, severity of CLD, nutritional status and 25-OHD were analysed in patients assessed for liver transplantation between 2006 and 2010. Patients who presented with acute, fulminant or subacute disease, with a primary diagnosis of liver cancer, were assessed for re-transplantation or who did not have a 25-OHD

measurement were excluded from the analysis.

## RESULTS

One hundred and sixty-five patients were included in this analysis. The mean age of all patients was  $53 \pm 8$  years. Moderate to severe 25-OHD deficiency was identified in 49 patients of whom 36 had grade 2-3 HE compared with 13 patients who were not encephalopathic ( $P \leq 0.0001$ ). Mild 25-OHD deficiency was not associated with HE. There was a significant correlation between the severity of 25-OHD deficiency and the severity of liver disease ( $r = 0.39$ ,  $P \leq 0.0001$ ) and disease severity and the presence of HE ( $P \leq 0.0001$ ). Importantly, individuals with 25-OHD deficiency were more likely to have a diagnosis of overt HE (OHE) at a significantly lower model for end stage liver disease (MELD) score than individuals without OHE ( $P \leq 0.0001$ ). This significant difference was observed with MELD scores from 10 to 38.

## CONCLUSION

25-OHD deficiency was observed in the majority of patients with CLD and for the first time was found to be significantly worse in patients with OHE.

**Key words:** Vitamin D; Chronic liver disease; Hepatic encephalopathy; Model For End Stage Liver Disease; Dementia; Malnutrition; Cognitive function

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**Core tip:** A strong association between vitamin D deficiency and deteriorating liver disease is identified in this investigation which supports previous reported findings. The novel finding in this investigation is the relationship between vitamin D deficiency and overt hepatic encephalopathy (OHE) in patients with chronic liver disease (CLD) which is independent of renal impairment and nutritional status. As repeated episodes of OHE may result in some residual neuropsychiatric alterations, maintenance of vitamin D levels within normal range in patients with CLD should be considered in clinical management. These results provide a strong rationale for future intervention studies in this group.

Vidot H, Potter A, Cheng R, Allman-Farinelli M, Shackel N. Serum 25-hydroxyvitamin D deficiency and hepatic encephalopathy in chronic liver disease. *World J Hepatol* 2017; 9(10): 510-518 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i10/510.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i10.510>

## INTRODUCTION

Hepatic encephalopathy (HE) describes a complex collection of neuropsychiatric symptoms ranging from sub-clinical neuropsychiatric changes to coma<sup>[1]</sup> and has been identified in up to 55% of patients with chronic liver disease<sup>[2]</sup>. Symptoms include impaired cognition

and motor function and reduced energy levels<sup>[3]</sup> HE can be classified as covert or overt HE (OHE)<sup>[1]</sup>. Features of HE can be likened to symptoms seen in patients with dementia<sup>[4]</sup>.

The aetiology of HE is complex and multifactorial, and includes abnormal ammonia metabolism, dysbiosis which promotes inflammation in the gut and liver<sup>[5]</sup>, low levels of circulating branched chain amino acids<sup>[6]</sup>, electrolyte abnormalities<sup>[7]</sup> and alterations in zinc and manganese levels<sup>[8]</sup>. Importantly, the features of HE can often be significantly reversed following treatment consistent with a largely functional not a structural cause of cognitive impairment<sup>[9]</sup>.

The development of HE presents significant challenges to patients and their carers<sup>[10]</sup>. Until recently, lactulose was the major cornerstone in the management of HE and continues to be used as a first line management for the control of the symptoms of chronic HE and for the reversal of the symptoms of acute episodes of HE<sup>[11]</sup>. The introduction of Rifaximin has reduced the rate of OHE and the frequency of hospital admissions due to OHE and improved the quality of life for the patient and their carers<sup>[12]</sup>.

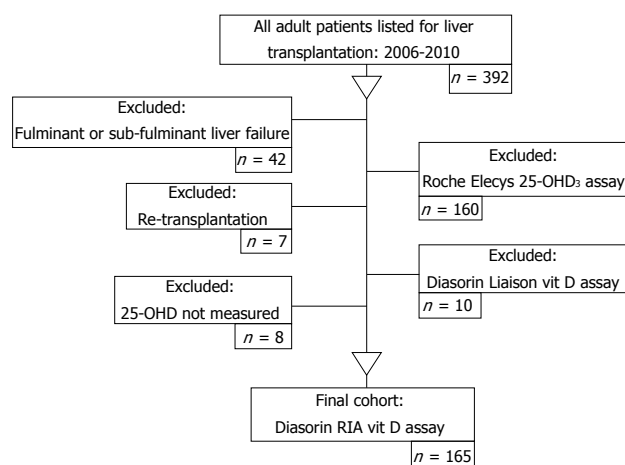
Patients who experience repeated episodes of OHE can have persistent and cumulative deficits in working memory, response inhibition and learning<sup>[13]</sup>. There is growing evidence that some deficit in cognitive function remains in liver transplant recipients who were severely encephalopathic or who experienced multiple episodes of OHE prior to liver transplantation<sup>[9]</sup>. Therefore, the prevention of OHE is paramount to the preservation of mental integrity in patients with cirrhosis.

Vitamin D is a multifunctional steroid hormone with diverse actions that are only partially understood. It is increasingly apparent that vitamin D is not just involved in calcium homeostasis and bone metabolism but has multiple biological targets mediated by vitamin D receptors (VDR)<sup>[14]</sup> which are present in more than 30 tissues<sup>[15]</sup> including the brain, kidneys, intestine, parathyroid gland, pituitary, prostate, mammary glands, cardiac and skeletal muscle, non-parenchymal liver cells, endothelial cells and the immune system<sup>[16-20]</sup>.

Vitamin D is obtained from dietary sources and ultraviolet light exposure. The first step in the activation of vitamin D is the hydroxylation of cholecalciferol to the active metabolite 25-hydroxyvitamin D (25-OHD) which occurs in the liver<sup>[21]</sup>. This is the major circulating metabolite of vitamin D, bound to the carrier protein vitamin D binding protein (DBP) with a half-life of 15-21 d<sup>[21,22]</sup>. The second activation process to 1,25 dihydroxy vitamin D occurs predominantly in the kidney<sup>[21]</sup> and to a lesser extent in a range of other tissues including bone, breast, brain, monocytes, parathyroid gland and placenta<sup>[21]</sup>. This active metabolite has a shorter half-life of 10-20 h<sup>[22]</sup>. Consequently, vitamin D status is commonly assessed by measuring circulating levels of 25-OHD<sup>[22]</sup>.

Vitamin D has been identified as an immune modulator and anti-infective agent<sup>[23]</sup> and an association





**Figure 1 Study design.** The selection and application of inclusion/exclusion criteria for inclusion in the final analysis. 25-OHD: 25-hydroxyvitamin D; RIA: Radioimmunoassay.

between vitamin D deficiency and the progression of liver disease has been identified in hepatitis C virus (HCV)<sup>[24]</sup>, alcoholic liver disease (ALD)<sup>[25]</sup> and non-alcoholic fatty liver disease (NAFLD)<sup>[24]</sup>.

There is growing evidence of clinical associations between vitamin D status and global and specific areas of cognitive function<sup>[26]</sup> and that vitamin D deficiency may be associated with both depression and schizophrenia<sup>[27]</sup>. Further, vitamin D deficiency is associated with low mood and impairment in some areas of cognitive functioning without any impairment in physical performance<sup>[28]</sup> and with an accelerated decline in cognitive function<sup>[29]</sup>.

VDR protein is present in most neurons and the glia in the human brain<sup>[30]</sup>. The hypothalamus and the cortex of the human brain are key areas in cognition<sup>[31]</sup>. The presence of both VDR protein and vitamin D metabolites in these areas of the brain are an indication that the vitamin D system is involved in the normal functioning of the human brain<sup>[32]</sup>.

As the first step in the hydroxylation of vitamin D occurs within the liver 25-OHD, levels decrease with progressive liver dysfunction. Vitamin D deficiency has been reported in up to 92% of patients with chronic liver disease and at least one third of these have severe 25-OHD deficiency<sup>[33]</sup>. 25-OHD deficiency is associated with increasing Child-Pugh classification rather disease aetiology<sup>[34]</sup> and is more prevalent in patients with cirrhosis than those who are not cirrhotic<sup>[33]</sup>. Increased all-cause mortality is associated with 25-OHD deficiency and specifically with increased mortality in patients with cirrhosis<sup>[35]</sup>. To date, an association between 25-OHD deficiency and HE has not been described. Therefore, we aimed to investigate the relationship between 25-OHD, cirrhosis and HE.

plantation routinely undergo a comprehensive series of tests and examinations prior to consideration of suitability for transplantation. A retrospective analysis of the results of 392 adult patients with chronic liver disease who were assessed for liver transplantation between 2006 and 2010 was undertaken. Approval to access medical records was granted by the Sydney Local Health District Ethics Review Committee (RPAH X15-0209).

### Data collection

Data collated included primary diagnosis, demographic information, standard biochemical markers of liver function, disease severity scores model for end stage liver disease (MELD)<sup>[36]</sup> and Child Turcotte Pugh (CTP)<sup>[37]</sup>, subjective nutritional assessment (SGA) scores<sup>[38]</sup>, 25-OHD levels and the presence of HE assessed using the West Haven criteria<sup>[39]</sup>. Patients who presented with acute, fulminant or subacute disease were excluded from the analysis due to the acute nature of their illness. Patients with a primary diagnosis of liver cancer or who were undergoing assessment for re-transplantation or who did not have a 25-OHD measurement were also excluded from the analysis.

25-OH vitamin D status was defined as sufficient (> 75 nmol/L), insufficient (50-75 nmol/L), mild deficiency (25-50 nmol/L), moderate deficiency (12.5-25 nmol/L) and severe deficiency (< 12.5 nmol/L)<sup>[40,41]</sup>.

During the study period, three different 25-OH D assay methods were used: (1) radioimmunoassay (RIA), referred to as the Diasorin-RIA<sup>®</sup> assay; (2) the electrochemiluminescence immunoassay, referred to as the Roche Elecys<sup>®</sup> vitamin D<sub>3</sub> assay; and (3) the Liaison total automated direct competitive chemiluminescence immunoassay, referred to as the Diasorin Liaison<sup>®</sup> 25-OH vitamin D assay. 25-OHD levels are frequently overestimated with greater intra-assay variation when assessed by more recent methodologies<sup>[42]</sup>. As the Diasorin RIA<sup>®</sup> assay was regarded as the most accurate measure of 25-OHD<sup>[43]</sup> at the time of this investigation, the final analysis included only patients with 25-OHD levels measured using the Diasorin RIA<sup>®</sup> assay technique (Figure 1).

### Statistical analysis

Results were analysed using Prism 6 for Mac (GraphPad Software Inc, La Jolla, CA, United States). Categorical values were analysed using  $\chi^2$  and quantitative continuous results were compared using the Mann-Whitney *U* test. Relationships between quantitative variables were assessed using Spearman correlation analysis. Multiple comparisons were made using One-way ANOVA, Kruskal-Wallis test and Dunn's multiple comparison test. The threshold for statistical significance is  $P < 0.05$ .

## MATERIALS AND METHODS

### Patient selection

All patients who present for assessment for liver trans-

## RESULTS

The patient selection process is outlined in Figure 1. After the exclusion criteria were applied 165 patients remained

**Table 1** Population characteristics

	Total cohort ( <i>n</i> )	Overt HE <sup>1</sup> ( <i>n</i> )	No HE <sup>1</sup> ( <i>n</i> )
Demographics	165	88	77
Gender			
Male	119	68	51
Female	46	20	26
Mean age (years ± SD)	53 ± 8	52 ± 7	54 ± 8
Primary indication for liver transplantation			
Viral hepatitis	91	52	39
Alcoholic cirrhosis	23	18	5
Cholestatic disease (PBC, PSC, autoimmune)	30	10	20
Non-alcoholic steatohepatitis	7	6	1
Other	14	2	12
Ethnicity			
Caucasian	130	75	55
Asian	22	6	16
Middle Eastern	8	4	4
Other	5	3	2
Clinical characteristics			
CTP score mean ± SD	9 ± 2.5	11 ± 1.7 <sup>a</sup>	7 ± 2 <sup>a</sup>
CTP stage			
A	41	0 <sup>a</sup>	41 <sup>a</sup>
B	47	22	25
C	77	66 <sup>a</sup>	11 <sup>a</sup>
Ascites			
None	51	7 <sup>a</sup>	44 <sup>a</sup>
Medically controlled	54	36 <sup>a</sup>	18 <sup>a</sup>
Poorly controlled	60	45 <sup>a</sup>	15 <sup>a</sup>
BMI (kg/m <sup>2</sup> ± SD)	27.4 ± 5.2	28.7 ± 5.4 <sup>a</sup>	25.7 ± 4.2 <sup>a</sup>
Nutritional status	104	65	39
SGA: A (well nourished)	12	9	3
SGA: B (moderately malnourished)	65	40	25
SGA: C (severely malnourished)	27	16	11
Biochemical characteristics			
25-OHD (vitamin D) (nmol/L) mean ± SD	36 ± 15	30 ± 13 <sup>a</sup>	42 ± 16 <sup>a</sup>
MELD score mean ± SD	17.1 ± 6.8	19.9 ± 6.5 <sup>a</sup>	13.9 ± 5.7 <sup>a</sup>
Bilirubin (μmol/L) mean ± SD	114 ± 152	141 ± 167 <sup>a</sup>	83 ± 128 <sup>a</sup>
Creatinine (μmol/L) mean ± SD	84 ± 50	85 ± 35 <sup>a</sup>	83 ± 63
Albumin (g/L) mean ± SD	33 ± 6	31 ± 5 <sup>a</sup>	35 ± 6 <sup>a</sup>
INR mean ± SD	1.6 ± 0.5	1.8 ± 0.6 <sup>a</sup>	1.3 ± 0.3 <sup>a</sup>
Sodium (mmol/L) mean ± SD	136 ± 5	135 ± 5 <sup>a</sup>	138 ± 4 <sup>a</sup>
Zinc (μmol/L) mean ± SD	8 ± 4	8 ± 3 <sup>a</sup>	10 ± 5 <sup>a</sup>

<sup>a</sup>*P* < 0.05. <sup>1</sup>Unless otherwise indicated there is no significant difference between HE and no HE. 25-OHD: 25-hydroxyvitamin D; HE: Hepatic encephalopathy; CTP: Child Turcotte Pugh; BMI: Body mass index; SGA: Subjective nutritional assessment; MELD: Model for end stage liver disease; INR: International normalized ratio.

in the study. Table 1 identifies the primary disease aetiology for liver transplantation assessment and the patient physical and biochemical characteristics.

The group was predominantly male with a mean age of 53.0 ± 8 years. Seventy nine percent of the group was Caucasian and there was no significant difference in 25-OHD levels identified across the different ethnic groups. The major cause of listing for liver transplantation was decompensated cirrhosis secondary to viral hepatitis. All patients had advanced disease as demonstrated by the CTP and MELD scores. OHE was present in 53% of patients. The majority of the cohort (69%) had ascites which was defined as grade 3-4 in 53% of patients<sup>[44]</sup>. Patients with OHE had a higher body mass index (BMI) (*P* < 0.001) with an associated significantly increased incidence of medically controlled and poorly controlled ascites (*P* < 0.0001) and significantly lower serum albumin levels (*P* < 0.0005).

Our results showed a strongly negative correlation between MELD score and 25-OHD levels (*P* < 0.0001) in all patients (Figure 2). Patients with OHE had significantly worse liver disease with a MELD score of 19.9 ± 6.5 whilst those who were not encephalopathic had significantly lower MELD score of 13.9 ± 5.7 (*P* < 0.0001) (Figure 3A). 25-OHD levels were lower in patients with OHE (*P* < 0.0001) (Figure 3B).

SGA of nutritional status was available for 104 patients. The majority of patients were either moderately or significantly malnourished (88%) and there was no significant correlation between nutritional status and 25-OHD levels (Figure 4). There is trend towards a higher incidence and increased severity of malnutrition in patients with OHE but this did not reach statistical significance. The correlation between RIA 25-OHD and the biochemical and physical parameters of the group are further outlined in Table 2.

**Table 2** 25-hydroxyvitamin D categories in patients assessed for liver transplantation

	25-OHD (nmol/L)	Total number	Overt HE <sup>1</sup> (n = 88)	No overt HE <sup>1</sup> (n = 77)
Sufficient	> 75	2	0	2
Insufficient	50-75	27	6	21
Mildly deficient	25-50	87	46	41
Moderately deficient	12.5-25	42	30 <sup>a</sup>	12 <sup>a</sup>
Severely deficient	< 12.5	7	6	1

<sup>a</sup>P < 0.05. <sup>1</sup>Unless otherwise indicated there were no significant differences between the overt HE and no-overt HE groups. 25-OHD: 25-hydroxyvitamin D; HE: Hepatic encephalopathy.

**Table 3** Univariate 25-hydroxyvitamin D correlations with physical and biochemical markers determined by Spearman correlation

	Spearman r	P value	Significance
Age	0.1110	0.16	ns
Total bilirubin	-0.3493	< 0.0001	f
Albumin	0.3153	< 0.0001	f
ALP	0.0203	0.80	ns
GGT	0.2055	0.0081	b
ALT	0.0246	0.75	ns
AST	-0.1741	0.0253	a
Creatinine	-0.0687	0.38	ns
Na <sup>+</sup>	0.2666	0.0005	e
Zn <sup>+</sup>	0.2790	0.0004	e
RBP	0.2913	0.0002	e
Transferrin	0.3568	< 0.0001	f
INR	-0.4232	< 0.0001	f
Ca <sup>2+</sup>	0.2370	0.0022	b
Ca <sup>2+</sup> corrected	-0.1531	0.08	ns
PTH	-0.1824	0.0205	a
BMI	-0.2244	0.0055	b

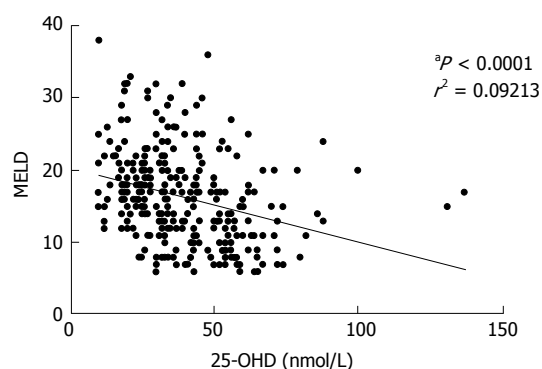
<sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01; <sup>c</sup>P < 0.001; <sup>f</sup>P < 0.0001. ns: Not significant; ALP: Alkalinephosphatase; GGT: Glutamyl transpeptidase; ALT: Alanine transaminase; AST: Aspartate transaminase; RBP: Retinol binding protein; INR: International normalized ratio; PTH: Parathyroid hormone; BMI: Body mass index.

Mild 25-OHD deficiency was not associated with an increase in OHE (Table 3). However, moderate and severe 25-OHD deficiency was significantly associated with the development of OHE ( $P < 0.0001$ ). The relationship between 25-OH vitamin D and OHE is outlined in Figure 3B.

Using  $\chi^2$  analysis lower 25-OHD levels were associated with a significant trend towards increasing levels of OHE ( $P < 0.0001$ ). A significant difference between 25-OHD levels in patients with OHE and those without OHE was identified ( $P < 0.0001$ ) and is demonstrated in Figure 5. Furthermore, there was a significant correlation between increasing OHE in patients with lower 25-OHD levels at the same level of disease severity as measured by the MELD scores.

## DISCUSSION

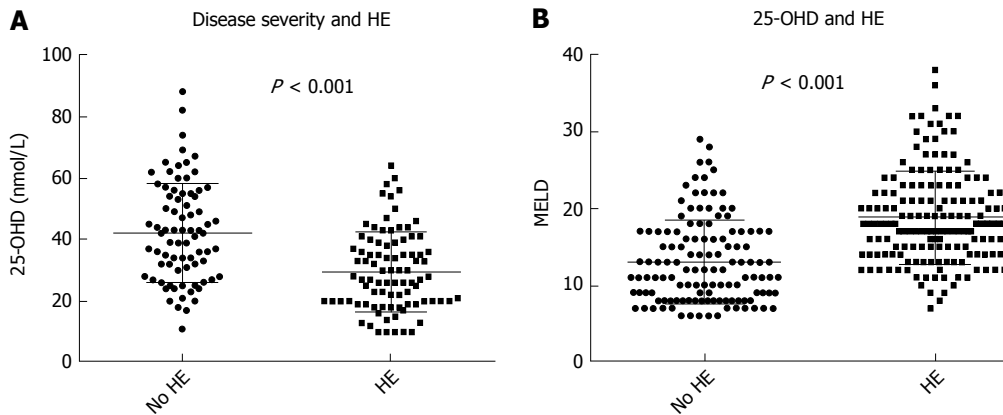
Our results demonstrate that at the same level of disease severity patients with OHE have significantly lower 25-OHD levels than those who do not have OHE. This is the first description of this association.

**Figure 2** Vitamin D and severity of liver disease. 25-OHD levels fall with worsening liver disease. 25-OHD: 25-hydroxyvitamin D; MELD: Model for end stage liver disease.

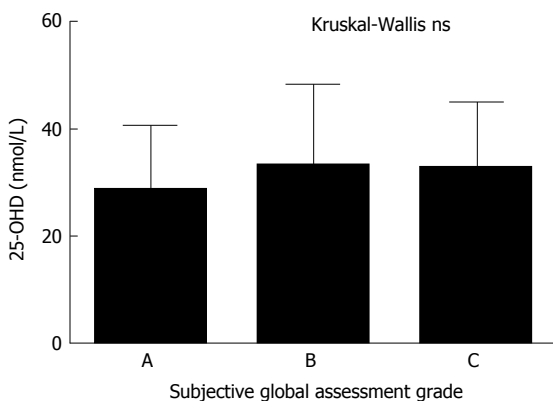
When stratified into patients who have OHE and those who do not, our results show that, in patients assessed for liver transplantation, there is a statistically significant relationship between low 25-OHD levels and OHE. Importantly, a significantly higher incidence of OHE was observed in individuals with low 25-OHD levels at similar levels of disease severity. This association raises the possibility that vitamin D deficiency has an effect on the manifestation of HE or is associated with other unrecognised factors. Importantly, OHE rarely occurred with normal Vitamin D levels but individuals could have low vitamin D and not have OHE. This is a similar relationship to the association of elevated serum ammonia with the development of OHE<sup>[45]</sup>. Consequently, the presence of moderate to severe 25-OHD deficiency means OHE is more likely in patients with ESLD.

It was beyond the scope of this investigation to explore the relationship between covert HE and 25-OHD deficiency. A large proportion of patients with insufficient (78%) or mild 25-OHD deficiency (47%) were not diagnosed with OHE. It possible that in a proportion of patients with insufficient or mild 25-OHD deficiency reduced levels of 25-OHD could be associated with the development or presence of covert HE. This is analogous to ammonia levels in ESLD which are elevated in HE but elevation does imply the presence of HE either OHE or covert HE.

There is a significant relationship between worsening liver function and 25-OHD deficiency. This is consistent with the growing awareness of the association between



**Figure 3** Disease severity, overt hepatic encephalopathy and vitamin D levels. A: Patients with overt HE have significantly higher MELD than patients without overt HE; B: 25-OHD levels measured by Diasorin-RIA and HE. Patients with overt HE have significantly lower 25-OHD levels than those without overt HE. 25-OHD: 25-hydroxyvitamin D; HE: Hepatic encephalopathy; MELD: Model for end stage liver disease.



**Figure 4** 25-hydroxyvitamin D and nutritional status. 25-OHD levels are independent of nutritional status. 25-OHD: 25-hydroxyvitamin D; ns: Not significant.

disease severity and reduced levels of 25-OHD in patients with cirrhosis<sup>[25]</sup>. Less than 2% of patients assessed for liver transplantation in this group had adequate levels of 25-OHD which is comparable to a previous study which identified vitamin D deficiency in 96% of patients waiting for liver transplantation<sup>[46]</sup>.

As liver disease progresses, patients become increasingly malnourished with an associated increase in HE<sup>[8]</sup>. Alterations in macronutrient requirements and reductions in oral intake are a feature of decompensated cirrhosis<sup>[8]</sup>. However, our results did not show a significant association between malnutrition and 25-OHD levels. This is consistent with changes in 25-OHD metabolism being a determinant of deficiency in CLD.

Vitamin D supplementation is now recognised as an important component of the management of patients with cirrhosis. Routine vitamin D supplementation for patients with chronic liver disease and insufficient levels of 25-OHD has become the standard of care in hepatology clinics to treat or prevent osteoporosis in CLD<sup>[47]</sup>. These results suggest a role for vitamin D supplementation in patients with CLD and reduced levels of 25-OHD.

There is growing evidence for an association between

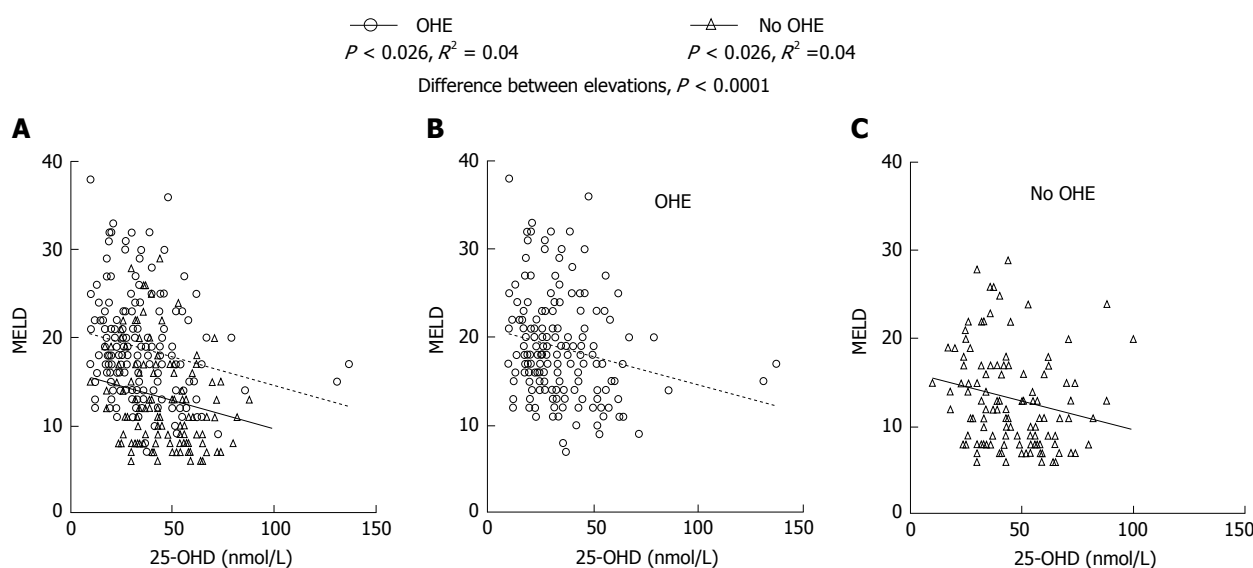
25-OHD deficiency and the development of all-cause dementia and a reduction in cognitive capacity<sup>[48]</sup> which is not confined to older populations. A linear relationship between 25-OHD deficiency and cognitive impairment has been identified in younger adults (30-60 years) as well as adults older than 60 years<sup>[49]</sup>. A systematic review of vitamin D and cognitive impairment concluded that "25-OHD insufficiency likely negatively affects specific cognitive functions, such as explicit episodic memory"<sup>[31]</sup> but there is a need for robust clinical investigation in this area.

Although it is plausible that 25-OHD deficiency could impact on cognitive function in CLD, neither a causative relationship nor a mechanism for this has been demonstrated. The level at which 25-OHD deficiency adversely affects brain function is unknown. 25-OHD is associated with verbal fluency, a marker of executive function and therefore a marker of cognitive function. Individuals with supratherapeutic 25-OHD levels of > 100 nmol/L scored significantly higher on verbal fluency tasks than those with inadequate 25-OHD levels<sup>[50]</sup> further supporting the role of vitamin D in the development of cognitive decline.

Neurobehavioral abnormalities are the major clinical component of HE and have shown to be associated with increased levels of inflammatory cytokines<sup>[51]</sup>. Systemic inflammation and changes in hepatic metabolism (*i.e.*, increased ammonia levels) are increasingly recognised as a precipitants of HE and worsen existing HE<sup>[52]</sup>. Vitamin D has been shown to have anti-inflammatory properties<sup>[52]</sup>. It can be postulated that vitamin D deficiency is associated with an increase in systemic inflammation thereby giving rise to the development of HE.

It is unclear whether there is a steady decline in brain function as 25-OHD levels drop or whether there is a threshold from which point there is a significant reduction in brain function in patients with cirrhosis. To show a causative relationship of the vitamin D with HE it is now necessary to examine the effects of vitamin D supplementation on encephalopathy symptoms in





**Figure 5** Overt hepatic encephalopathy, disease severity and 25-hydroxyvitamin D. Patients were stratified into those who had overt HE and those who did not. The combined data is presented in (A) and the sub groups of patients with OHE (B) and those without OHE (C). Vitamin D deficiency correlates with MELD score in both patients with and without OHE. Across the range of MELD scores from 10-30 patients with OHE (B) had significantly lower levels of 25-OHD than those who did not (C). 25-OHD: 25-hydroxyvitamin D; OHE: Overt hepatic encephalopathy; MELD: Model for end stage liver disease.

patients with CLD.

To date, the literature suggests association not causality. There is sufficient association between cognitive and behavioral changes associated with 25-OHD deficiency to suggest a role for vitamin D deficiency in the development of HE in patients with chronic liver disease. Further studies are required to investigate the relationship between 25-OHD levels and the development of HE in patients with CLD.

## COMMENTS

### Background

Hepatic encephalopathy (HE) describes a complex range of neuropsychiatric symptoms and is associated with the development and progression of hepatic cirrhosis. HE has been described as a form of dementia which is largely reversible. Low vitamin D levels [25-hydroxyvitamin D (25-OHD)] are associated with dementia and impaired cognition in the general population. The association between low 25-OHD levels and HE has not been previously investigated.

### Research frontiers

It is not known whether the association between low 25-OHD levels and HE is causative. The association described requires further investigation to determine the precise role of 25-OHD deficiency in the development of HE. Historically, the assays used to determine 25-OHD levels have varied significantly. Consistent assay methodology should be implemented to investigate this relationship further.

### Innovations and breakthroughs

It has been established that patients with chronic liver disease (CLD) have low levels of 25-OHD which are associated with overt HE. Monitoring of 25-OHD vitamin D levels and regular and appropriate vitamin D supplementation in patients with cirrhosis may help prevent the development of HE.

### Applications

Monitoring 25-OHD levels and replacement therapy is an important aspect of the overall management of patients with CLD.

### Terminology

Automated immunoassays are used for routine analysis of serum 25-OHD levels

and there is wide variation between the different assay techniques. At the time of this investigation, the Diasorin RIA assay was identified as the most reliable method of measuring serum 25-OHD levels.

### Peer-review

This is very interesting paper about the relationship between 25-OH deficiency and HE. Author concluded that there is a significant association between low-25-OHD levels and the development of HE.

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Prospective Study

# Hepatic encephalopathy before and neurological complications after liver transplantation have no impact on the employment status 1 year after transplantation

Henning Pflugrad, Anita B Tryc, Annemarie Goldbecker, Christian P Strassburg, Hannelore Barg-Hock, Jürgen Klempnauer, Karin Weissenborn

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## Abstract

### AIM

To investigate the impact of hepatic encephalopathy



before orthotopic liver transplantation (OLT) and neurological complications after OLT on employment after OLT.

## METHODS

One hundred and fourteen patients with chronic liver disease aged 18-60 years underwent neurological examination to identify neurological complications, neuropsychological tests comprising the PSE-Syndrome-Test yielding the psychometric hepatic encephalopathy score, the critical flicker frequency and the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS), completed a questionnaire concerning their occupation and filled in the short form 36 (SF-36) to assess health-related quality of life before OLT and 12 mo after OLT, if possible. Sixty-eight (59.6%) patients were recruited before OLT, while on the waiting list for OLT at Hannover Medical School [age:  $48.7 \pm 10.2$  years, 45 (66.2%) male], and 46 (40.4%) patients were included directly after OLT.

## RESULTS

Before OLT 43.0% of the patients were employed. The patients not employed before OLT were more often non-academics (employed: Academic/non-academic 16 (34.0%)/31 *vs* not employed 10 (17.6%)/52,  $P = 0.04$ ), had more frequently a history of hepatic encephalopathy (HE) (yes/no; employed 15 (30.6%)/34 *vs* not employed 32 (49.2%)/33,  $P = 0.05$ ) and achieved worse results in psychometric tests (RBANS sum score mean  $\pm$  SD employed  $472.1 \pm 44.5$  *vs* not employed  $443.1 \pm 56.7$ ,  $P = 0.04$ ) than those employed. Ten patients (18.2%), who were not employed before OLT, resumed work afterwards. The patients employed after OLT were younger [age median (range, min-max) employed 47 (42, 18-60) *vs* not employed 50 (31, 29-60),  $P = 0.01$ ], achieved better results in the psychometric tests (RBANS sum score mean  $\pm$  SD employed  $490.7 \pm 48.2$  *vs* not employed  $461.0 \pm 54.5$ ,  $P = 0.02$ ) and had a higher health-related quality of life (SF 36 sum score mean  $\pm$  SD employed  $627.0 \pm 138.1$  *vs* not employed  $433.7 \pm 160.8$ ;  $P < 0.001$ ) compared to patients not employed after OLT. Employment before OLT ( $P < 0.001$ ), age ( $P < 0.01$ ) and SF-36 sum score 12 mo after OLT ( $P < 0.01$ ) but not HE before OLT or neurological complications after OLT were independent predictors of the employment status after OLT.

## CONCLUSION

HE before and neurological complications after OLT have no impact on the employment status 12 mo after OLT. Instead younger age and employment before OLT predict employment one year after OLT.

**Key words:** Hepatic encephalopathy; Employment; Neurological complications; Cognitive function; Health-related quality of life; Liver transplantation

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**Core tip:** This prospective study is the first to consider

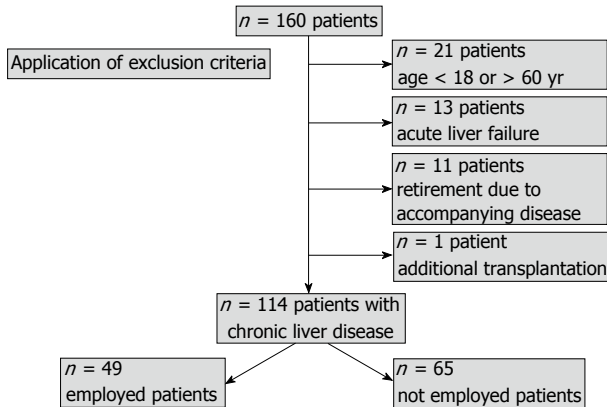
hepatic encephalopathy prior to liver transplantation, neurological complications after liver transplantation as well as socio-economic factors as risk factors for unemployment 1 year after transplantation. Our data confirm that employment status before liver transplantation is most important in predicting the employment status 12 mo after transplantation. However, neither prior-liver transplantation hepatic encephalopathy nor neurological complications after liver transplantation are independent risk factors for unemployment 1 year after transplantation.

Pflugrad H, Tryc AB, Goldbecker A, Strassburg CP, Barg-Hock H, Klempnauer J, Weissenborn K. Hepatic encephalopathy before and neurological complications after liver transplantation have no impact on the employment status 1 year after transplantation. *World J Hepatol* 2017; 9(10): 519-532 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i10/519.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i10.519>

## INTRODUCTION

During the last 35 years specialized transplantation centres with outpatient clinics for follow-up and improvement of immunosuppressive therapy have significantly increased survival rates after orthotopic liver transplantation (OLT)<sup>[1]</sup>. Thus, additional indicators of treatment quality besides mortality, such as employment after OLT, emerged. Employment after OLT indicates reintegration into society, regain of cognitive and physical capability and increased health-related quality of life (HRQoL)<sup>[2]</sup>. Although reintegration of patients into work is pursued, actually, only about 50% of the patients work after OLT, and there are significant differences between different countries with rates ranging between 17% and 55%<sup>[3-8]</sup>. Reasons why patients do not work after OLT are believed to be manifold. Local social security insurance system, age, sex, level of vocational training, level of school education, type of work, disability, unemployment before OLT, underlying liver disease, high morbidity due to liver disease and complications after OLT have been discussed<sup>[4,9]</sup>. Hereof, employment itself and the type of employment before OLT were considered to be the most important predictors of post OLT employment<sup>[3]</sup>. Interestingly, neither hepatic encephalopathy (HE) before OLT nor neurological complications after OLT have been considered in this respect so far even though both can significantly impact patients' physical and mental ability before and after OLT.

HE is a frequent complication of liver cirrhosis caused by liver insufficiency and porto-systemic shunts<sup>[10]</sup>. It is based on neurochemical and neurophysiological disorders of the brain and although the pathogenesis of HE is not completely understood, ammonia is believed to be of major importance<sup>[11]</sup>. HE is characterized by deficits in motor accuracy and motor speed as well as cognitive impairment especially concerning attention, whereas verbal abilities maintain unaffected<sup>[12]</sup>. HE is



**Figure 1 Patients' distribution.** Flow chart showing loss of patients due to exclusion criteria and distribution into the groups "employed" and "not employed" before liver transplantation.

present in about 10%-50% of patients at the time of transplantation and about 35%-45% of OLT patients have a history of HE<sup>[13]</sup>. Neurological complications like encephalopathy, seizures, tremor, psychotic disorders and posterior reversible encephalopathy syndrome occur in about 30% of the patients after OLT<sup>[14]</sup>. They result in a high morbidity and prolonged in-hospital stay<sup>[15]</sup>. HE prior OLT and neurological complications after OLT have not been distinctly considered as risk factors for unemployment after OLT so far, probably because HE was considered to be completely reversible<sup>[16]</sup>, and neurological complications after OLT - though frequent - are usually impairing the patients cognitive function only transiently<sup>[14,15,17]</sup>.

However, HE is known to have an impact on patients' working ability before OLT, especially of blue collar workers<sup>[18]</sup>, and neurological complications possibly impair recovery of working capability after OLT<sup>[15]</sup>. The main hypothesis of this prospective study was that hepatic encephalopathy before OLT and neurological complications after OLT are significantly associated with unemployment one year after OLT. Furthermore, we analysed whether employment status before OLT, occupation, underlying liver disease, health-related quality of life, quality adjusted life years (QALYs), age and sex are significantly associated with the employment status one year after OLT.

## MATERIALS AND METHODS

### Patients

All patients included into this study took part in a long-term prospective follow-up study of patients after liver transplantation ( $n = 160$ ). Patients with liver cirrhosis, admission to the waiting list for liver transplantation, acute liver failure and age between 18 and 80 years were included into the follow-up study. For the present study patients with acute liver failure, neurological or psychiatric diseases not related to hepatic encephalopathy, additional transplantation of another organ, regular intake of medication with an effect on the central nervous system (CNS), age older than 60 years at OLT because of the high

probability of age related retirement and the expected low probability of reintegration into employment after OLT, retirement due to conclusion of work life, accompanying disease or age were excluded. Finally, data of 114 patients with chronic liver disease were considered for the analysis (Figure 1). Sixty-eight (59.6%) patients were recruited before OLT, while on the waiting list for liver transplantation at Hannover Medical School [age:  $48.7 \pm 10.2$  years, 45 (66.2%) male], and 46 (40.4%) patients were included directly after OLT [age:  $44.9 \pm 11.4$  years, 27 (58.7%) male].

All subjects gave written informed consent. The study was approved by the local ethics committee and performed according to the World Medical Association Declaration of Helsinki (revised in 2008).

### Methods

Patients recruited before OLT regularly underwent neurological examinations by a neurologist of the group before OLT if possible and all patients included underwent a neurological examination on day 1, day 7, day 90 and approximately 12 mo after OLT. If the examination was not possible 12 mo after OLT, it was done at a later point of time within the follow-up study. Additional neurological examinations were performed when necessary. Encephalopathy, posterior reversible encephalopathy syndrome, alterations of consciousness, seizures, hallucinations, confusion, infections of the CNS, intracerebral bleeding or stroke were classified as neurological complications. Neurological complications were assessed as a categorical variable independent from the time of occurrence within the immediate hospital stay after OLT.

If possible, a psychometric test battery for the assessment of attention, concentration, memory, speed of information processing, visuo-constructive abilities, motor speed and accuracy and executive functions comprising the PSE-Syndrome-Test, a battery that provides the psychometric hepatic encephalopathy score (PHES)<sup>[19]</sup>, the critical flicker frequency (CFF)<sup>[20]</sup> and the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS)<sup>[21,22]</sup> were applied by a neurologist of the group trained in these tests. The median interval between the psychometric testing before OLT and the transplantation was 4 mo (interquartile range 5 mo, min 0, max 33) and the median interval between OLT and psychometric testing after OLT was 12 mo (interquartile range 5 mo, min 10, max 62). Furthermore, the patients were asked to complete questionnaires concerning occupation and HRQoL [short form 36 (SF-36)]<sup>[23]</sup>. The SF-36 evaluation was performed according to its scoring algorithm yielding 8 domain scores: Physical functioning (PF), physical role functioning (PRF), bodily pain (BP), general health perception (GHP), vitality (VIT), social role functioning (SRF), emotional role functioning (ERF) and mental health (MH) which were summated for the SF-36 sum score. The default summary measures physical health and mental health were not calculated because they are based on

**Table 1 Self-reporting questionnaire occupation *n* (%)**

<i>n</i> = 114	Before OLT	After OLT
Employed	36 (31.6)	33 (34.4) <sup>1</sup>
Retired	48 (42.1)	49 (51.0) <sup>1</sup>
Unemployed	7 (6.1)	2 (2.1) <sup>1</sup>
Certified unfit for work	10 (8.8)	2 (2.1) <sup>1</sup>
Homemaker	8 (7.0)	6 (6.3) <sup>1</sup>
Student	5 (4.4)	4 (4.2) <sup>1</sup>
Deceased	-	18 (15.8)

<sup>1</sup>Percent value is based on *n* = 96 survivors. OLT: Orthotopic liver transplantation.

American standard values.

The six-dimension health state short form (SF-6D)<sup>[24]</sup> was derived from the SF-36 by generating six multi-level dimensions that provide a health status which ranges from 1 (full health) to 0 (death). It is based on preference weights gained from the United Kingdom general population and estimates a preference-based single index measure for health to measure QALYs.

Individual test results were evaluated by comparison to norm values given in the test manuals. The scores of the psychometric batteries were adjusted for age and education. Reasons for missing data before OLT were language issues, inclusion of the patient after OLT or refusal by the patient to complete the tests or questionnaires. After OLT data is missing due to refusal by the patient to complete the tests or questionnaires, language issues or death after OLT.

Age, occupation, underlying liver disease, laboratory Model of End Stage Liver Disease (labMELD) score and medication were assessed and documented. The history of HE was taken from reliable case records in which HE was diagnosed and scored by physicians according to the West Haven criteria<sup>[25]</sup>.

**Self-reporting questionnaire occupation:** The patients selected which of the following specifications applied to their situation: Employed, retired (receiving pension due to illness), unemployed, certified unfit for work, homemaker or in training at school or university. Patients on a full time or part time job, students and homemakers were classified as employed. Although Patients with the status "student" or "homemaker" were not working for a wage, they were classified as employed because the authors are convinced that studying or keeping the house requires physical and mental capability which equals the requirements that are needed to work for a wage. The not employed patient group consisted of patients that were unemployed, temporarily certified unfit for work or retired due to liver disease (Table 1). For subgroup analysis the patients were allocated according to their employment status before and after OLT into the groups employed before and after OLT, employed before but not employed after OLT, not employed before and after OLT as well as not employed before but employed after OLT. Patients with a university degree were classified as academics and patients with a vocational training for qualification were classified as non-academics. These

data were surveyed retrospectively for patients included after OLT from case records or by anamneses.

**Statistical methods:** Normality of distribution was assessed by the Shapiro-Wilk test. Differences between the groups of employed and not employed patients concerning age, labMELD score, psychometric test results and SF-36 scores were evaluated with the Mann-Whitney test for not normal distributed values and Analysis of variance (ANOVA) for normal distributed values. The Wilcoxon test or the paired sample T-test was used to compare related values concerning psychometric test results and SF-36 scores surveyed before and after OLT. Categorical variables comprising sex, profession, history of HE and neurological complications were tested by Fisher's Exact Test and the underlying liver disease was tested by the  $\chi^2$  test. Binary logistic regression analysis (Method = Enter) was applied to identify independent prognostic factors for employment after OLT as the dependent variable considering employment before OLT, underlying liver disease, labMELD score, history of HE, neurological complications after OLT, age, sex, profession, SF-36 sum score before OLT and SF-36 sum score 12 mo after OLT as independent parameters. For the regression model the Omnibus Test of Model Coefficients, the -2 Log likelihood, the Nagelkerke R Square and the effects size Cohen's *d* values are shown. For the variables in the Equation significant at the 0.05 level, Wald statistic, *P* value, the Odds ratio [Exp(B)] and the confidence interval for the Odds ratio [Exp(B)] are given. Normally distributed values are shown as mean  $\pm$  SD and not normally distributed values are shown as median with range. A *P*-value  $\leq$  0.05 was considered significant for all tests applied. The statistical methods of this study were reviewed by Prof Hecker, former Head of the Biostatistics Department at Hannover Medical School.

## RESULTS

### Before OLT

Forty-nine (43.0%) of the 114 patients were employed at the time of OLT compared to 65 (57.0%) who were not employed (Figure 2). The two patient groups did not differ with regard to age, sex, the severity of liver disease according to the labMELD score, or with regard to aetiology of liver disease. Patients who were not employed before OLT had significantly more often a positive history of HE, were more frequently non-academic (82% vs 66%) and showed a significantly lower value in the SF-36 domain score physical functioning, whereas all other SF-36 domain scores and the QALYs did not differ. Furthermore, they achieved significantly worse results in the PHES and the RBANS sum score (Table 2).

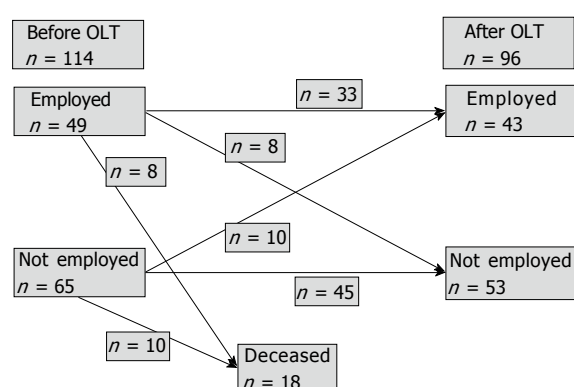
### Twelve month after OLT

Twelve month after OLT 43 (44.8% of those who survived) patients were employed (including students and homemakers) and 53 (55.2%) patients were not employed. Eighteen of the included 114 patients died after OLT

**Table 2** Characteristics of employed and not employed patients before orthotopic liver transplantation

<i>n</i> = 114	Employed ( <i>n</i> = 49)	Not employed ( <i>n</i> = 65)	<i>P</i> value
Age median (range, min-max)	49 (42,18-60)	50 (36, 24-60)	0.06
Sex (male/female)	30 (61.2%)/19	42 (64.6%)/26	0.85
Profession	16 (34.0%)/31	10 (17.6%)/52	0.04
Academic/ non-academic	(NS 2)	(NS 3)	
labMELD median (range, min-max)	18 (33, 7-40)	18 (33, 7-40)	0.16
Aetiology of liver disease			0.44
PSC	16	14	
PBC	0	1	
Alcohol	5	10	
HCV	7	11	
HBV	5	13	
AIH	3	1	
M. Wilson	3	1	
Others	10	14	
History of HE (+/-)	15 (30.6%)/34	32 (49.2%)/33	0.05
PHES, median (min/max)	0 (-8/+5) ( <i>n</i> = 27)	-2 (-18/+4) ( <i>n</i> = 37)	0.04
CFF, mean ± SD	42.7 ± 3.9 ( <i>n</i> = 26)	42.3 ± 5.0 ( <i>n</i> = 35)	0.77
RBANS	92.2 ± 17.0 ( <i>n</i> = 24)	89.6 ± 18.6 ( <i>n</i> = 32)	0.59
Immediate memory, mean ± SD			
RBANS Visuospatial/constructional, median (range, min-max)	84 (60, 66-126) ( <i>n</i> = 24)	84 (66, 60-126) ( <i>n</i> = 32)	0.26
RBANS	99.3 ± 11.6 ( <i>n</i> = 24)	96.2 ± 14.4 ( <i>n</i> = 32)	0.40
Language, mean ± SD			
RBANS	91.8 ± 17.3 ( <i>n</i> = 24)	84.2 ± 16.6 ( <i>n</i> = 32)	0.10
Attention, mean ± SD			
RBANS	97 (36, 86-122) ( <i>n</i> = 24)	94.5 (64, 44-108) ( <i>n</i> = 32)	0.24
Delayed memory, median (range, min-max)			
RBANS	472.1 ± 44.5 ( <i>n</i> = 24)	443.1 ± 56.7 ( <i>n</i> = 32)	0.04
Sum score, mean ± SD			
RBANS	92.1 ± 11.6 ( <i>n</i> = 24)	84.9 ± 14.4 ( <i>n</i> = 32)	0.05
Total scale, mean ± SD			

*P* value ≤ 0.05 is considered significant. NS: Not specified; OLT: Orthotopic liver transplantation; labMELD: Laboratory Model of End Stage Liver Disease; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis; HE: Hepatic encephalopathy; PHES: Psychometric hepatic encephalopathy score; CFF: Critical flicker frequency; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status.



**Figure 2** Employment status before and 12 mo after orthotopic liver transplantation. The distribution of patients into the groups “employed” and “not employed” before and 12 mo after OLT is displayed. *n* = 114 patients before OLT; *n* = 96 survivors 1 year after OLT; *n* = 18 patients deceased after OLT. OLT: Orthotopic liver transplantation.

(15.8%). The cause of death was multi-organ failure in 5 cases, sepsis, heart failure or abdominal bleeding in 3 cases each, subarachnoid haemorrhage in one case, meningitis/encephalitis in one case and unknown in 2 cases. Eight of these were employed (16.3%) and 10

(15.4%) were not employed before OLT. Eight of the patients who were employed before OLT did not return to employment after OLT (19.5% of those who survived, *n* = 41), while 33 (80.5% of those who survived) returned to work. Of those survivors who were not employed before OLT (*n* = 55), 10 (18.2%) returned to work within the year after transplantation, while 45 (81.8%) remained not employed (Figure 2).

Patients not employed 12 mo after OLT were significantly older and showed significantly worse results in the psychometric tests after OLT than the employed patients (Table 3).

HE (*P* = 0.10) before OLT and neurological complications (*P* = 0.11) after OLT were more frequent in not employed patients after OLT, but the difference did not reach statistical significance at the 0.05 level. Concerning the HRQoL, all SF-36 domain scores and the QALYs were significantly higher in the group of employed patients compared to the not employed patients after OLT (Table 4). There was no significant difference for all other parameters tested.

Patients employed before and after OLT (*n* = 33) other than patients employed before but not employed



**Table 3** Characteristics of employed and not employed patients after orthotopic liver transplantation

<i>n</i> = 96	Employed ( <i>n</i> = 43)	Not employed ( <i>n</i> = 53)	<i>P</i> value
Age	47 (42, 18-60)	50 (31, 29-60)	0.01
median (range, min-max)			
Sex (male/female)	23 (53.5%)/20	36 (67.9%)/17	0.21
Profession	13 (31.0%)/29	8 (15.1%)/45	0.08
Academic/non-academic	(NS 1)		
labMELD median (range, min-max)	18 (33, 7-40)	19 (33, 7-40)	0.16
Aetiology of liver disease			0.41
PSC	15	13	
PBC	1	0	
Alcohol	4	8	
HCV	7	6	
HBV	3	12	
AIH	2	1	
M. Wilson	2	1	
Others	9	12	
History of HE (+/-)	13 (30.2%)/30	25 (47.2%)/28	0.10
Neurological complications (+/-)	17 (39.5%)/26	30 (56.6%)/23	0.11
PHES, median (min/max)	0 (-5/+2)	-1 (-10/+4)	0.10
	( <i>n</i> = 30)	( <i>n</i> = 43)	
CFF, mean $\pm$ SD	45.8 $\pm$ 4.0	42.0 $\pm$ 4.1	< 0.001
	( <i>n</i> = 29)	( <i>n</i> = 40)	
RBANS	101.1 $\pm$ 15.1	92.1 $\pm$ 17.7	0.03
Immediate memory, mean $\pm$ SD	( <i>n</i> = 30)	( <i>n</i> = 41)	
RBANS Visuospatial/constructional, median (range, min-max)	84 (64, 62-126)	89 (57, 64-121)	0.17
	( <i>n</i> = 30)	( <i>n</i> = 41)	
RBANS	103.2 $\pm$ 13.9	93.0 $\pm$ 16.5	0.01
Language, mean $\pm$ SD	( <i>n</i> = 30)	( <i>n</i> = 41)	
RBANS	101.2 $\pm$ 13.7	89.3 $\pm$ 15.5	0.001
Attention, mean $\pm$ SD	( <i>n</i> = 30)	( <i>n</i> = 41)	
RBANS	98 (109, 10-119)	95 (44, 75-119)	0.13
Delayed memory, median (range, min-max)	( <i>n</i> = 30)	( <i>n</i> = 41)	
RBANS	490.7 $\pm$ 48.2	461.0 $\pm$ 54.5	0.02
Sum score, mean $\pm$ SD	( <i>n</i> = 30)	( <i>n</i> = 41)	
RBANS	97.4 $\pm$ 13.6	89.6 $\pm$ 14.2	0.02
Total scale, mean $\pm$ SD	( <i>n</i> = 30)	( <i>n</i> = 41)	

*P* value  $\leq$  0.05 is considered significant. NS: Not specified; OLT: Orthotopic liver transplantation; labMELD: Laboratory Model of End Stage Liver Disease; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis; HE: Hepatic encephalopathy; PHES: Psychometric hepatic encephalopathy score; CFF: Critical flicker frequency; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status.

after OLT (*n* = 8) showed significantly better values in the CFF (*P* = 0.04) and higher scores in the RBANS domain scores immediate memory (*P* = 0.04) and attention (*P* = 0.04) after OLT (Table 5). Furthermore, the health related quality of life scores after OLT were significantly higher in patients reintegrated into employment after OLT compared to the patients not reemployed after OLT (Table 6).

In the subgroup of 10 patients (18.2%) that were not employed before OLT but returned to work within 1 year after OLT, 5 were male (50%) and the median age was 41 (range 34, min 26, max 60) years. The qualification was a vocational education in 8 and a university degree in 2 cases. The reason for OLT was primary sclerosing cholangitis (PSC) in 3 patients, primary biliary cirrhosis (PBC), alcoholic liver disease, hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, kryptogenic cirrhosis, biliary atresia and Budd-Chiari syndrome in 1 patient, respectively. The median labMELD score was 20 (range 24, min 8, max 32). Four patients had a history of HE before OLT and 6 patients had a neurological

complication directly after OLT. All 10 patients returned to a job working for a wage after OLT. The comparison of the psychometric test results and the health related quality of life scores after OLT of this subgroup compared to patients that were not employed before and after OLT (*n* = 45) showed no significant group differences except that the patients reintegrated into employment after OLT were significantly younger (*P* = 0.03) and had significantly better CFF values (*P* < 0.01) after OLT than the patients that stayed not employed after OLT (Tables 7 and 8).

#### **Paired comparison of psychometric tests and questionnaires surveyed before and 12 mo after OLT**

Thirty-seven patients filled in the questionnaires before and 12 mo after OLT. Of these, 16 patients were employed and 21 patients were not employed one year after OLT.

The HRQoL and the QALYs significantly increased after OLT in the 16 patients that were employed after OLT (Table 9). In contrast, the patients not employed after OLT (*n*

**Table 4 Short form 36 domain scores and six-dimension health state short form score of employed and not employed patients**

SF-36 domain score	Before OLT		P value	After OLT		P value
	Employed (n = 27)	Not employed (n = 35)		Employed (n = 30)	Not employed (n = 41)	
PF, mean ± SD	70.7 ± 25.1	47.4 ± 27.4	0.001	82.3 ± 19.2	59.4 ± 28.2	< 0.001
PRF, median (range, min-max)	50 (100, 0-100)	25 (100, 0-100)	0.14	100 (100, 0-100)	25 (100, 0-100)	< 0.001
BP, median (range, min-max)	74 (100, 0-100)	51 (100, 0-100)	0.14	100 (69, 31-100)	74 (88, 12-100)	0.001
GHP, median (range, min-max)	40 (77, 10-87)	35 (82, 0-82)	0.71	69.5 (87, 10-97)	50 (87, 10-97)	0.01
VIT, median (range, min-max)	45 (80, 10-90)	40 (85, 0-85)	0.43	70 (75, 20-95)	45 (85, 5-90)	0.001
SRF, median (range, min-max)	87.5 (87.5, 12.5-100)	62.5 (87.5, 12.5-100)	0.52	100 (50, 50-100)	62.5 (87.5, 12.5-100)	< 0.001
ERF, median (range, min-max)	100 (100, 0-100)	100 (100, 0-100)	0.94	100 (100, 0-100)	33.3 (100, 0-100)	< 0.001
MHI, median (range, min-max)	76 (60, 32-92)	64 (88, 8-96)	0.31	80 (56, 44-100)	68 (72, 28-100)	0.01
Sum score, mean ± SD	479.2 ± 193.9	419.7 ± 169.7	0.20	627.0 ± 138.1	433.7 ± 160.8	< 0.001
SF-6D QALYs, mean ± SD	0.71 ± 0.14	0.64 ± 0.15	0.06	0.8 ± 0.1	0.64 ± 0.12	< 0.001

P value ≤ 0.05 is considered significant. SF-36: Short form 36; SF-6D: Six-dimension health state short form; OLT: Orthotopic liver transplantation; PF: Physical functioning; PRF: Physical role functioning; BP: Bodily pain; GHP: General health perception; VIT: Vitality; SRF: Social role functioning; ERF: Emotional role functioning; MHI: Mental health; QALYs: Quality adjusted life years.

**Table 5 Comparison of patients employed before and after orthotopic liver transplantation to patients employed before but not-employed after orthotopic liver transplantation**

n = 41	Employed before and after OLT (n = 33)	Employed before but not employed after OLT (n = 8)	P value
Age, median (range, min-max)	50 (42, 18-60)	54 (30, 29-59)	0.13
Sex (male/female)	18 (54.5%)/15	6 (75.0%)/2	0.43
Profession	11 (34.4%)/21	3 (37.5%)/5	1.00
Academic/non-academic	(NS 1)		
labMELD median (range, min-max)	18 (33, 7-40)	17 (31, 9-40)	0.91
Aetiology of liver disease			0.19
PSC	12	2	
PBC	0	0	
Alcohol	3	0	
HCV	6	0	
HBV	2	2	
AIH	2	1	
M. Wilson	2	0	
Others	6	3	
History of HE (+/-)	9 (27.3%)/24	2 (25.0%)/6	1.00
Neurological complications (+/-)	11 (33.3%)/22	5 (62.5%)/3	0.23
PHES after OLT, median (min/max)	1 (-4/+2)	0 (-7/+4)	0.93
	(n = 25)	(n = 7)	
CFF after OLT, mean ± SD	45.3 ± 3.7	41.7 ± 5.0	0.04
	(n = 24)	(n = 7)	
RBANS after OLT	101.6 ± 14.1	89.4 ± 10.0	0.04
Immediate memory, mean ± SD	(n = 26)	(n = 7)	
RBANS after OLT Visuospatial/	84 (64, 62-126)	89 (57, 64-121)	0.68
constructional median (range, min-max)	(n = 26)	(n = 7)	
RBANS after OLT	103.2 ± 14.6	95.1 ± 24.2	0.27
Language, mean ± SD	(n = 26)	(n = 7)	
RBANS after OLT	100.8 ± 14.1	87.1 ± 18.6	0.04
Attention, mean ± SD	(n = 26)	(n = 7)	
RBANS after OLT	98 (109, 10-119)	95 (17, 88-105)	0.16
Delayed memory, median (range, min-max)	(n = 26)	(n = 7)	
RBANS after OLT	492.0 ± 47.8	457.0 ± 56.8	0.11
Sum score, mean ± SD	(n = 26)	(n = 7)	
RBANS after OLT	97.7 ± 13.7	88.3 ± 14.6	0.12
Total scale, mean ± SD	(n = 26)	(n = 7)	

P value ≤ 0.05 is considered significant. NS: Not specified; OLT: Orthotopic liver transplantation; labMELD: Laboratory Model of End Stage Liver Disease; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis; HE: Hepatic encephalopathy; PHES: Psychometric hepatic encephalopathy score; CFF: Critical flicker frequency; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status.

= 21) did not show a significant change concerning their HRQoL with the exception of the SF-36 domain scores physical functioning and general health perception, which both increased significantly after OLT (Table 10).

Forty-two patients completed the PHES (n = 16 employed after OLT), 36 patients the RBANS (n = 13 employed after OLT) and 38 patients the CFF (n = 15 employed after OLT) before and after OLT.

**Table 6** Short form 36 domain scores and six-dimension health state short form score of patients employed before and after orthotopic liver transplantation compared to patients employed before but not-employed after orthotopic liver transplantation

SF-36 domain score	Before OLT		P value	After OLT		P value
	Employed before and after OLT (n = 17)	Employed before and not employed after OLT (n = 4)		Employed before and after OLT (n = 26)	Employed before and not employed after OLT (n = 7)	
PF, mean $\pm$ SD	75.9 $\pm$ 23.8	55.0 $\pm$ 31.6	0.15	84.6 $\pm$ 17.0	45.0 $\pm$ 23.5	< 0.001
PRF, median (range, min-max)	50 (100, 0-100)	62.5 (50, 25-75)	0.97	100 (100, 0-100)	25 (50, 0-50)	0.001
BP, median (range, min-max)	84 (100, 0-100)	81 (48, 52-100)	0.90	100 (49, 51-100)	52 (78, 22-100)	0.03
GHP, median (range, min-max)	50 (72, 10-82)	35 (42, 25-67)	0.64	72 (77, 20-97)	60 (72, 15-87)	0.31
VIT, median (range, min-max)	45 (80, 10-90)	42.5 (60, 20-80)	0.97	70 (75, 20-95)	50 (70, 10-80)	0.04
SRF, median (range, min-max)	87.5 (87.5, 12.5-100)	87.5 (50, 50-100)	0.70	100 (37.5, 62.5-100)	50 (62.5, 37.5-100)	< 0.01
ERF, median (range, min-max)	100 (100, 0-100)	66.7 (100, 0-100)	0.70	100 (100, 0-100)	33.3 (100, 0-100)	0.05
MH, median (range, min-max)	72 (60, 32-92)	74 (24, 56-80)	0.83	82 (48, 52-100)	76 (56, 44-100)	0.22
Sum score, mean $\pm$ SD	512.1 $\pm$ 193.4	487.1 $\pm$ 172.2	0.82	652.9 $\pm$ 100.7	418.5 $\pm$ 121.5	< 0.001
SF-6D QALYs, mean $\pm$ SD	0.72 $\pm$ 0.14	0.72 $\pm$ 0.15	0.94	0.83 $\pm$ 0.1	0.65 $\pm$ 0.10	< 0.001

P value  $\leq$  0.05 is considered significant. SF-36: Short form 36; SF-6D: Six-dimension health state short form; OLT: Orthotopic liver transplantation; PF: Physical functioning; PRF: Physical role functioning; BP: Bodily pain; GHP: General health perception; VIT: Vitality; SRF: Social role functioning; ERF: Emotional role functioning; MH: Mental health; QALYs: Quality adjusted life years.

**Table 7** Comparison of patients not employed before and after orthotopic liver transplantation to patients not employed before but employed after orthotopic liver transplantation

n = 55	Not Employed before and after OLT (n = 45)	Not employed before but employed after OLT (n = 10)	P value
Age, median (range, min-max)	50 (28, 32-60)	41 (34, 26-60)	0.03
Sex (male/female)	30 (66.7%)/15	5 (50%)/5	0.47
Profession	5 (11.1%)/40	2 (20%)/8	0.6
Academic/non-academic			
labMELD median (range, min-max)	19 (33, 7-40)	20 (24, 8-32)	0.74
Aetiology of liver disease			0.2
PSC	11	3	
PBC	0	1	
Alcohol	8	1	
HCV	6	1	
HBV	10	1	
AIH	0	0	
M. Wilson	1	0	
Others	9	3	
History of HE (+/-)	23 (51.1%)/22	4 (40%)/6	0.73
Neurological complications (+/-)	25 (55.6%)/20	6 (60%)/4	1.0
PHES after OLT, median (min/max)	-1 (-10/+4) (n = 36)	0 (-5/+2) (n = 5)	0.63
CFF after OLT, mean $\pm$ SD	42.0 $\pm$ 4.0 (n = 33)	47.9 $\pm$ 5.2 (n = 5)	< 0.01
RBANS after OLT	92.7 $\pm$ 19.0 (n = 34)	97.8 $\pm$ 22.9 (n = 4)	0.62
Immediate memory, mean $\pm$ SD			
RBANS after OLT Visuospatial/constructional, median (range, min-max)	90.5 (55, 66-121) (n = 34)	84 (11, 78-89) (n = 4)	0.32
RBANS after OLT	92.5 $\pm$ 14.8 (n = 34)	103.5 $\pm$ 10.3 (n = 4)	0.16
Language, mean $\pm$ SD			
RBANS after OLT	89.7 $\pm$ 15.1 (n = 34)	104.0 $\pm$ 12.6 (n = 4)	0.08
Attention, mean $\pm$ SD			
RBANS after OLT	96 (44, 75-119) (n = 34)	98.5 (34, 71-105) (n = 4)	1.0
Delayed memory, median (range, min-max)			
RBANS after OLT	461.9 $\pm$ 54.8 (n = 34)	482.3 $\pm$ 57.6 (n = 4)	0.49
Sum score, mean $\pm$ SD			
RBANS after OLT	89.9 $\pm$ 14.3 (n = 34)	95.0 $\pm$ 14.5 (n = 4)	0.51
Total scale, mean $\pm$ SD			

P value  $\leq$  0.05 is considered significant. OLT: Orthotopic liver transplantation; NS: Not specified; labMELD: Laboratory Model of End Stage Liver Disease; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus; AIH: Autoimmune hepatitis; HE: Hepatic encephalopathy; PHES: Psychometric hepatic encephalopathy score; CFF: Critical flicker frequency; RBANS: Repeatable Battery for the Assessment of Neuropsychological Status.

**Table 8** Short form 36 domain scores and six-dimension health state short form score of patients not employed before and after orthotopic liver transplantation compared to patients not employed before but employed after orthotopic liver transplantation

SF-36 domain score	Before OLT		P value	After OLT		P value
	Not employed before and after OLT (n = 25)	Not employed before but employed after OLT (n = 6)		Not employed before and after OLT (n = 34)	Not employed before but employed after OLT (n = 4)	
PF, mean ± SD	48.2 ± 26.8	57.5 ± 26.0	0.45	62.4 ± 28.5	67.5 ± 28.4	0.74
PRF, median (range, min-max)	25 (100, 0-100)	62.5 (100, 0-100)	0.45	25 (100, 0-100)	25 (100, 0-100)	1.0
BP, median (range, min-max)	51 (100, 0-100)	56.5 (78, 22-100)	0.79	74 (88, 12-100)	52.5 (69, 31-100)	0.70
GHP, median (range, min-max)	35 (67, 15-82)	43.5 (52, 0-52)	0.64	46 (87, 10-97)	41 (70, 10-80)	0.73
VIT, median (range, min-max)	40 (80, 5-85)	45 (65, 0-65)	0.79	45 (85, 5-90)	42.5 (35, 40-75)	0.70
SRF, median (range, min-max)	62.5 (87.5, 12.5-100)	81.3 (50, 50-100)	0.42	62.5 (87.5, 12.5-100)	68.8 (50, 50-100)	0.77
ERF, median (range, min-max)	100 (100, 0-100)	66.7 (67.7, 33.3-100)	0.21	33.3 (100, 0-100)	66.7 (66.7, 33.3-100)	0.48
MH, median (range, min-max)	68 (88, 8-96)	68 (80, 16-96)	0.79	68 (68, 28-96)	54 (56, 44-100)	0.57
Sum score, mean ± SD	419.4 ± 166.3	480.8 ± 180.4	0.43	436.9 ± 169.1	458.5 ± 237.3	0.82
SF-6D QALYs, mean ± SD	0.64 ± 0.15	0.71 ± 0.14	0.35	0.64 ± 0.12	0.65 ± 0.14	0.87

P value ≤ 0.05 is considered significant. SF-36: Short form 36; SF-6D: Six-dimension health state short form; OLT: Orthotopic liver transplantation; PF: Physical functioning; PRF: Physical role functioning; BP: Bodily pain; GHP: General health perception; VIT: Vitality; SRF: Social role functioning; ERF: Emotional role functioning; MH: Mental health; QALYs: Quality adjusted life years.

**Table 9** Paired analysis of the short form 36 domain scores and the six-dimension health state short form score of patients employed after orthotopic liver transplantation surveyed before and 12 mo after orthotopic liver transplantation

SF-36 domain score, n = 16	Before OLT	After OLT	P value
PF, mean ± SD	71.6 ± 26.8	84.1 ± 18.6	0.04
PRF, mean ± SD	50.0 ± 47.4	82.8 ± 35.0	0.02
BP, mean ± SD	70.7 ± 34.5	88.8 ± 18.8	0.04
GHP, mean ± SD	46.9 ± 24.0	66.8 ± 24.0	0.01
VIT, mean ± SD	45.9 ± 23.8	68.4 ± 15.2	0.01
SRF, mean ± SD	70.3 ± 33.2	90.6 ± 15.5	0.06
ERF, mean ± SD	70.8 ± 43.7	91.7 ± 19.3	0.10
MH, mean ± SD	70.8 ± 18.7	80.0 ± 13.6	0.17
Sum score, mean ± SD	497.0 ± 191.7	653.2 ± 128.6	0.01
SF-6D QALYs, mean ± SD	0.71 ± 0.12	0.81 ± 0.10	0.02

P value ≤ 0.05 is considered significant, no correlation between first and second measurement. SF-36: Short form 36; SF-6D: Six-dimension health state short form; OLT: Orthotopic liver transplantation; PF: Physical functioning; PRF: Physical role functioning; BP: Bodily pain; GHP: General health perception; VIT: Vitality; SRF: Social role functioning; ERF: Emotional role functioning; MH: Mental health; QALYs: Quality adjusted life years.

In the group of patients employed 12 mo after OLT, the PHES and the RBANS did not change significantly whereas the CFF increased significantly after OLT (PHES:  $n = 16$ ; median 1.0, range 19 (min -14, max 5) before OLT, median 1.0, range 7 (min -5, max 2) after OLT,  $P = 0.26$ ; CFF:  $n = 15$ ; before OLT mean  $43.3 \text{ Hz} \pm 3.8$ , after OLT mean  $45.6 \text{ Hz} \pm 4.6$ ,  $P = 0.04$ ; RBANS:  $n = 13$ ; immediate memory  $P = 0.08$ , visuospatial/constructional  $P = 0.17$ , language  $P = 0.21$ , attention  $P = 0.34$ , delayed memory  $P = 0.44$ , sum score  $P = 0.70$ , total scale  $P = 0.79$  (Figure 3).

The patients not employed 12 mo after OLT showed a significant increase in the PHES ( $n = 26$ ,  $P = 0.04$ ) whereas the CFF ( $n = 23$ ,  $P = 0.28$ ) did not change significantly [before OLT PHES median -1.0, range 22 (min -18, max 4), CFF mean  $41.0 \text{ Hz} \pm 4.4$ ; after OLT PHES median -1.0, range 13 (min -9, max 4), CFF mean

**Table 10** Paired analysis of the short form 36 domain scores and the six-dimension health state short form score of patients not employed after orthotopic liver transplantation surveyed before and 12 mo after orthotopic liver transplantation

SF-36 domain score, n = 21	Before OLT	After OLT	P value
PF, mean ± SD	48.1 ± 28.3	65.5 ± 29.0	0.03
PRF, mean ± SD	38.1 ± 40.0	41.6 ± 39.0	0.76
BP, mean ± SD	61.1 ± 31.3	70.0 ± 24.8	0.25
GHP, mean ± SD	41.8 ± 15.6	56.8 ± 22.2	0.01
VIT, mean ± SD	44.3 ± 20.0	51.2 ± 22.2	0.14
SRF, mean ± SD	65.5 ± 29.3	67.3 ± 21.1	0.80
ERF, mean ± SD	55.6 ± 45.1	57.1 ± 44.9	0.91
MH, mean ± SD	66.3 ± 16.3	69.1 ± 18.7	0.53
Sum score, mean ± SD	420.7 ± 163.8	478.6 ± 148.0	0.21
SF-6D QALYs, mean ± SD	0.64 ± 0.15	0.66 ± 0.10	0.46

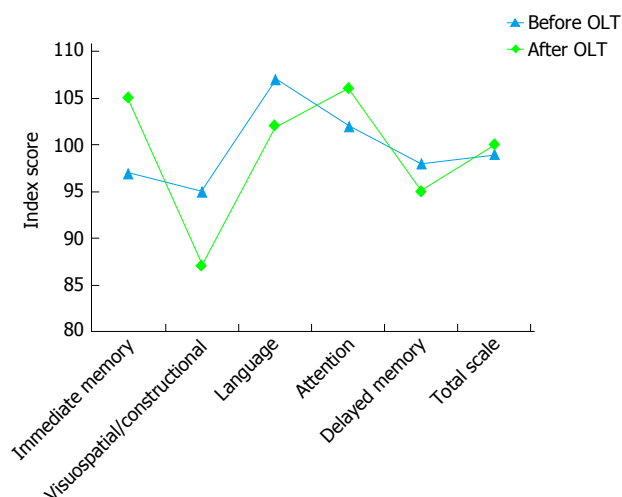
P value ≤ 0.05 is considered significant, no correlation between first and second measurement. SF-36: Short form 36; SF-6D: Six-dimension health state short form; OLT: Orthotopic liver transplantation; PF: Physical functioning; PRF: Physical role functioning; BP: Bodily pain; GHP: General health perception; VIT: Vitality; SRF: Social role functioning; ERF: Emotional role functioning; MH: Mental health; QALYs: Quality adjusted life years.

$41.9 \text{ Hz} \pm 4.1$ ]. The RBANS domain score Attention increased significantly 12 mo after OLT ( $n = 23$ ,  $P < 0.01$ , mean  $82.9 \pm 16.2$  before OLT,  $91.2 \pm 15.6$  after OLT) whereas all other RBANS domain scores did not change significantly (Figure 4).

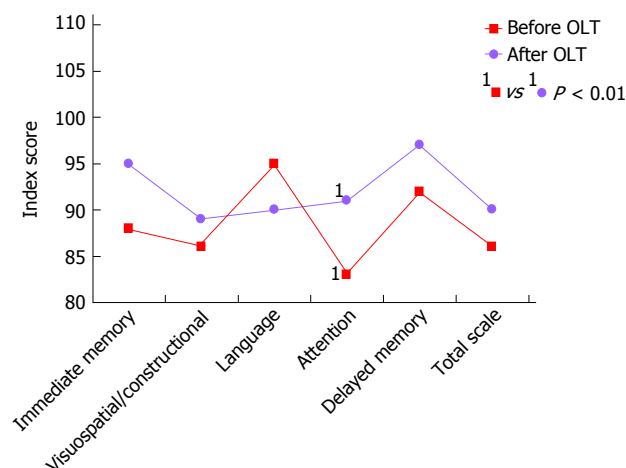
### Binary logistic regression

Using binary logistic regression analysis (Method enter, Omnibus Test of Model Coefficients  $\chi^2 = 52.840$ ,  $P < 0.001$ , -2 Log likelihood = 77.581, Nagelkerke R Square = 0.571, Cohen's  $d = 0.70$ ), employment status before OLT [Wald statistic = 21.5,  $P < 0.001$ , odds ratio (OR) = 19.64, confidence interval for OR 5.58 to 69.14] and age in years (Wald statistic = 8.17,  $P < 0.01$ , OR = 0.90, confidence interval for OR 0.84 to 0.97) were independent predictors of the employment status 12 mo after OLT ( $n = 95$ ,  $n = 1$  excluded due to missing value





**Figure 3** Paired comparison of repeatable battery for the Assessment of Neuropsychological Status domain scores of patients employed after liver transplantation surveyed before and 12 mo after orthotopic liver transplantation. Thirty-six patients completed the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) test before and 12 mo after orthotopic liver transplantation (OLT). Of these 13 patients (36.1%) were employed after liver transplantation. This figure shows the paired analysis of the RBANS results of the patients employed after OLT achieved before and 12 mo after OLT. The RBANS Total scale and the domain scores Immediate memory, Visuospatial/constructional ability, Language ability, Attention and Delayed memory are displayed.



**Figure 4** Paired comparison of repeatable battery for the Assessment of Neuropsychological Status domain scores of patients not employed after orthotopic liver transplantation surveyed before and 12 mo after orthotopic liver transplantation. Thirty-six patients completed the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) test before and 12 mo after orthotopic liver transplantation (OLT). Of these 23 patients (63.9%) were not employed after OLT. This figure shows the paired analysis of the RBANS results of the patients not employed after OLT achieved before and 12 mo after OLT. The RBANS Total scale and the domain scores Immediate memory, Visuospatial/Constructional ability, Language ability, Attention and Delayed memory are displayed. \*Indicates a statistical significant increase in the RBANS domain score Attention after OLT at the  $P < 0.01$  level.

concerning profession). No significant effects were found for the underlying liver disease, history of HE before OLT, labMELD score, profession, sex, SF-36 sum score before OLT and neurological complications after OLT. In a subgroup of patients who filled in the SF-36 after transplantation (Method enter, Omnibus Test of Model Coefficients  $\chi^2 = 50.579$ ,  $P < 0.001$ , -2 Log likelihood = 46.137, Nagelkerke  $R^2 = 0.685$  and Cohen's  $d = 0.94$ ,  $n = 71$ ) the employment status before OLT (Wald statistic = 11.84,  $P < 0.001$ , OR = 20.13, confidence interval for OR = 3.64-111.27) and the SF-36 sum score after OLT (Wald statistic = 7.18,  $P < 0.01$ , OR for increment of 10 points = 1.10, confidence interval for OR = 1.03 -1.17) were independent predictors of the employment status after OLT.

## DISCUSSION

This prospective study evaluated the impact of hepatic encephalopathy before OLT and neurological complications after OLT on the employment status 12 mo after liver transplantation. Moreover, health-related quality of life, age, sex, employment status before OLT and professional category were registered to identify factors which might be significantly associated with the employment status one year after OLT.

In contrast to our hypothesis, we did not find a significant impact of HE before or neurological complications after OLT on the employment status 12 mo after OLT, though the not employed patients after OLT showed a trend towards a higher frequency of HE before OLT and neurological complications after OLT in comparison to

patients employed after OLT. Instead, the employment status after OLT was independently predicted by the employment status before OLT, age and health-related quality of life after OLT.

Hepatic encephalopathy is associated with high morbidity and has a direct impact on health-related quality of life before liver transplantation<sup>[26]</sup>. Impairment of motor and cognitive function lead to premature retirement of patients with HE<sup>[18]</sup>. Blue collar workers with liver cirrhosis are more frequently considered unfit for work than white collar workers, probably due to the fact that HE significantly affects motor function while language ability is preserved<sup>[18]</sup>. In accordance herewith, our patients who were not employed before OLT had more frequently a history of HE and had predominantly a vocational education for qualification compared to employed patients.

The credo that HE is completely reversible has been put into question recently, since it was shown that patients who had suffered HE before OLT, had an incomplete recovery of their cognitive function about 1 year afterwards<sup>[13,27,28]</sup>. This could well interfere with the patients' working ability. However, we did not find a significant impact of a HE history upon the employment status after OLT in our patients. Instead, like others, we observed an improvement in cognitive function in our patients after OLT with only a few patients showing abnormal test results 12 mo after OLT, for example, in the PHES (9 of 73 examined; 12.3%)<sup>[13,27]</sup>. Of these, only one patient was employed whereas 8 patients were not employed. There was no relation to any specific underlying cause of liver disease, such as alcoholism.

Neurological complications affecting the CNS are frequent in the first weeks after OLT and are known to prolong the in-hospital stay<sup>[14,15]</sup>. Although the distinctive impairment of cognitive function by neurological complications in the first weeks after OLT might only be transient<sup>[17]</sup>, long term impairment might occur and influence the working capability. Nevertheless, our results did not indicate that neurological complications significantly impair the working capability 1 year after OLT and thus underline the good prognosis of neurological complications in the first weeks after OLT as long as they are promptly diagnosed and treated sufficiently. Our results still showed a trend indicating a higher frequency of neurological complications after OLT in the group of patients not employed 12 mo after OLT.

Eighty point five percent ( $n = 33$ ) of the surviving patients employed before OLT ( $n = 41$ ) returned to work afterwards, indicating the importance of the pre transplant working status upon a patient's occupational fate. This is in accordance with the findings of other studies<sup>[2-5,8,29]</sup> which came to similar results, irrespective of the country or continent where the study was performed<sup>[9]</sup>.

It is no surprise that age was a predictor for post OLT employment status as well, since it may be hypothesized that younger patients have a higher physical and cognitive health resource than older patients, facilitating the return to work. Additionally, social insurance companies might be more eager to reintegrate young patients into work because of the costs of early retirement. Also, employers might have a higher confidence in young patients to be capable of working compared to older patients<sup>[8]</sup>.

In our study, patients who were working 1 year after OLT had a significantly higher SF-36 and SF-6D score than those who did not, and the subgroup of patients that returned to their pre OLT job after transplantation had significantly better health related quality of life scores than patients who were employed before OLT but did not return to employment after OLT. Furthermore, the SF-36 score at 12 mo after OLT was an independent predictor for employment after OLT in the subgroup of patients who filled in this form. Aberg *et al.*<sup>[2]</sup> assessed HRQoL in 354 patients after OLT [age at OLT (mean) 48 years, 42% male] compared to 6050 age and gender matched controls. They showed that the employed OLT patients had significantly higher HRQoL scores than retired patients and concluded that employment is an indicator of HRQoL. Our data do not allow a decision, whether the scores are higher due to the fact that the patients were able to return to a normal life and therefore perceived themselves as physically and mentally fit, or if better physical and mental condition facilitated the return to employment after OLT. However, it is conceivable that patients who have reached independence and the economic status they had before OLT have more confidence in their physical and cognitive functions than those who are not. In consequence, reintegration of patients after OLT into employment should be considered an important tool to achieve patients' well-being. The

significant difference between patients who are working and those who are not employed after OLT and additionally between the subgroup of patients that were employed before and after OLT compared to patients that did not return to employment after OLT with regard to cognitive function (RBANS) in this study, however, indicates that besides socio-economic factors also medical factors must be considered (Tables 3 and 5).

In contrast to some other studies<sup>[8,30]</sup> and in accordance with Hunt *et al.*<sup>[31]</sup> we did not find a significant gender difference with regard to employment status after OLT. The differing results between the studies may be due to lacking comparability of the classification of "work" especially as not all studies classified "homemakers" as employed.

Education has also been reported to have an impact on employment after OLT<sup>[3,4]</sup>. Our results were not able to confirm this assumption probably due to the low number of patients with a university degree (21 of 96 survivors; 21.9%). Nevertheless, a trend ( $P = 0.06$ ) towards a higher frequency of vocational training in the group of patients not employed after OLT was observed. But the effect of education on post OLT employment was not observed by all authors<sup>[31]</sup>, and obviously it is not exclusively the level of education that affects the probability to return to work after OLT, but also the type of work done before OLT. Adams *et al.*<sup>[32]</sup> as well as Weng *et al.*<sup>[6]</sup> showed that patients working in non-office jobs were less likely to return after OLT than patients working in an office. This may be due to different physical demands<sup>[29]</sup>. However, considering the observation that blue collar workers with chronic liver disease are more often not employed than white collar workers might as well be just a sequel of the pre OLT health status.

Contradictory results have been achieved considering the effect of the underlying liver disease - especially alcoholism<sup>[7,8,33-35]</sup> and hepatitis C<sup>[3]</sup> - upon the proportion of subjects employed after OLT. Alcoholic liver disease was estimated to have no effect<sup>[33,34]</sup>, to increase<sup>[7]</sup> or to decrease<sup>[8,35]</sup> the probability of resuming work after OLT. In our study the underlying liver disease had no effect on the employment status after OLT.

Patients with chronic liver disease are not employed before OLT due to various reasons. Cirrhosis-associated morbidity might be the most frequent because being frequently certified unfit for work might lead to unemployment and employers as well as social insurance companies might aspire the patients' retirement. This assumption includes the hypothesis that patients staying employed before OLT might be less impaired and might have a shorter period of time of severe liver disease. It might alleviate returning to work after OLT and achieving the economic status as well as the financial independence they had before OLT. The employer might be more eager to reintegrate these patients after OLT because the circumstances signal that work capability exists. Still, our data do not support this assumption, if the labMELD score is considered representative for patients' health status.

Although patients who were not employed after OLT differed with regard to psychometric test results from those who were employed, the majority of the not employed patients achieved results within the normal range. The PHES, for example, was only abnormal in 8 of 43 patients (18.6%). Resuming work after OLT for patients who were not employed before OLT seems quite unlikely as only 10 (18.2%) of the not employed patients of our cohort returned to work after OLT. Similar results were described in other studies<sup>[9]</sup>. Probably the time off work is too long, determining low confidence in patients and employers that reintegration is possible. Furthermore, bureaucracy and fear of losing pension claims might play a role. Additionally, our data (Tables 3-6) and that of others<sup>[9]</sup> indicate that returning to the pre OLT job might be impaired by poor physical or impaired mental functioning. Achieving an occupational retraining, however, is extensive and support for patients might be low. To solve these problems, interventions based on the individual needs and obstacles of each single patient are needed to facilitate reemployment after OLT. Although so far data about the efficiency of interventions before and after OLT to facilitate reemployment after OLT are missing, the main aim seems to be to keep the patients with chronic liver disease employed before OLT<sup>[36]</sup>. To achieve this aim, liver related complications like hepatic encephalopathy and ascites need to be prevented or if applicable treated as soon as possible. The patients' mobility might be maintained by regular physiotherapy. Furthermore, education programs for employers about working capabilities of patients with chronic liver disease might prevent loss of employment before OLT. Such interventions might also increase the health related quality of life. After OLT, rehabilitation programs that focus on the individual physical and mental job requirements for each patient might be conducive to reintegrate the patient into the pre OLT job and to increase the health related quality of life. In addition, employers need to be educated about the working capabilities of patients after OLT. If the reintegration into the pre OLT job is not possible, collaboration with social workers and employment support agencies might be needed to match the patient to an appropriate alternative job. In this respect, the reduction of bureaucratic barriers seems to be particularly important concerning the encouragement of patients to resume work after OLT while at the same time, if needed, providing them with full medical coverage<sup>[36]</sup>.

Limitations of our study are that our results can only be compared to studies that also classified "homemakers" and "students" as employed, because some studies only classified subjects as employed if they were working for a wage. Furthermore, 46 patients (40.4%) were included after OLT. Data for the psychometric tests and quality of life scores before OLT were missing for these patients. However, all other variables were available because all patients included underwent neurological examination after OLT and detailed case records were available for all patients including the HE history, occupation, underlying liver disease, labMELD score and medication. Finally, our

results are only based on patients within the German health-care system, which might limit the transferability to other countries. Nevertheless, our findings are well in line with those of former studies, indicating the effect of the pre transplant employment status upon the post transplant working career, independent of the different health care systems.

As a result, our data confirm that employment status before OLT is most important in predicting the employment status 12 mo after OLT. Neither prior-OLT HE nor neurological complications after OLT are independent risk factors for unemployment 1 year after OLT. However, our results show a trend for both values to be more frequent in patients not employed after OLT, indicating the need to analyse a larger sample to finally answer the question if HE before OLT and neurological complications after OLT affect working ability after transplantation.

In conclusion, education of patients, employers and social insurance companies is needed to emphasise that it is worth analysing, on a single subject basis, if a patient is capable of being reintegrated into work after OLT. Obstacles should be identified in every single case because resuming work after OLT might improve the post OLT care and increase the health-related quality of life in patients after OLT.

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## COMMENTS

### Background

Specialized transplantation centres and improvement of immunosuppressive therapy have significantly increased survival rates after orthotopic liver transplantation (OLT). Thus, besides mortality other indicators of treatment quality emerged. Employment after OLT is considered to indicate treatment quality and socio-economic factors before OLT are esteemed crucial in this respect. However, currently only about 50% of patients are reintegrated into employment after OLT and the reasons for not returning to the pre OLT job are not well described. The relevance of hepatic encephalopathy (HE) before OLT and neurological complications after OLT has not been considered so far although both can significantly impact patients' physical and mental ability before and after OLT. This prospective study was designed to evaluate the impact of HE before and neurological complications after OLT in addition to socio-economic factors upon the employment status 1 year after OLT.

### Research frontiers

Outcome of patients after OLT improved during the last 35 years and thus the focus on the patients' mental and physical well-being after OLT increased. Especially reintegration into employment was identified as an important factor as it is important for the physical and mental health after OLT. However, only about 50% of the patients return to their jobs after OLT. This study contributed to this research field by evaluating whether hepatic encephalopathy before OLT or neurological complications after OLT have an impact on the employment status of the patients 1 year after OLT.

### Innovations and breakthroughs

The available studies identified employment before OLT, the type of employment and younger age as the main predicting factors for reintegration into employment

after OLT. This study contributed by showing that neither prior-OLT hepatic encephalopathy nor neurological complications after OLT are independent risk factors for unemployment 1 year after OLT. Furthermore, their study confirmed that employment status before OLT is most important in predicting the employment status 12 mo after OLT.

### Applications

This study showed that neither hepatic encephalopathy before OLT nor neurological complications after OLT increase the probability of unemployment one year after OLT. Especially employment before OLT predicts the reintegration into employment after OLT and thus interventions should focus on how to keep patients with liver cirrhosis employed before OLT. Furthermore interventions are needed during the rehabilitation after OLT that focus on the physical and mental needs required for the pre OLT job of each patient.

### Terminology

Hepatic encephalopathy: A frequent complication of liver cirrhosis caused by liver insufficiency and porto-systemic shunts. It is based on neurochemical and neurophysiological disorders of the brain and ammonia is believed to be of major importance. It is characterized by deficits in motor accuracy and motor speed as well as cognitive impairment especially concerning attention, whereas verbal abilities maintain unaffected. Neurological complications: encephalopathy, seizures, tremor, psychotic disorders and posterior reversible encephalopathy syndrome occur in about 30% of the patients after OLT.

### Peer-review

In this well-written article, Pflugrad *et al* explore factors associated with employment after OLT, which is essential for quality of life and meaningful transplant outcomes. They found that hepatic encephalopathy before or central nervous system complications after OLT were not independent predictors of employment, unlike pre-OLT employment, age and post-OLT functional status.

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## Hepatocellular carcinoma in non-alcoholic steatohepatitis: Current knowledge and implications for management

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### Abstract

With the prevalence of hepatitis C virus expected to decline, the proportion of hepatocellular carcinoma (HCC) related to non-alcoholic steatohepatitis (NASH) is anticipated to increase exponentially due to the growing epidemic of obesity and diabetes. The annual incidence rate of developing HCC in patients with NASH-related cirrhosis is not clearly understood with rates ranging from 2.6%-12.8%. While multiple new mechanisms have been implicated in the development of HCC in NASH; further prospective long-term studies are needed to validate these findings. Recent evidence has shown a significant proportion of patients with non-alcoholic fatty liver disease and NASH progress to HCC in the absence of cirrhosis. Liver resection and transplantation represent curative therapeutic options in select NASH-related HCC patients but have placed a significant burden to our healthcare resources and utilization. Currently NASH-related HCC is the fastest growing indication for liver transplant in HCC candidates. Increased efforts to implement effective screening and preventative strategies, particularly in non-cirrhotic NASH patients, are needed to reduce the future impact imposed by NASH-related HCC.

**Key words:** Non-alcoholic steatohepatitis; Cirrhosis; Non-alcoholic fatty liver disease; Obesity; Hepatocellular carcinoma

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**Core tip:** Non-alcoholic steatohepatitis (NASH) is antici-

pated to account for a greater proportion of hepatocellular carcinoma (HCC) incidence due to the growing epidemic of obesity and diabetes. Currently NASH-related HCC is the fastest growing indication for liver transplant in HCC candidates. Increased efforts to implement effective screening and preventative strategies particularly in non-cirrhotic NASH patients possibly based on genetic susceptibility are needed to reduce the future impact imposed by NASH-related HCC.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers Worldwide, HCC being the sixth most common cancer, and is the second leading cause of cancer-related death<sup>[1]</sup>. HCC largely occurs in the background of chronic liver disease and cirrhosis of the liver<sup>[2]</sup>. The leading liver disease etiologies for cirrhosis in patients with HCC include but are not limited to chronic hepatitis B, chronic hepatitis C virus (HCV), and alcoholic liver disease. With advent of curative treatments for HCV, the risk of progression to cirrhosis and development of HCC secondary to HCV is anticipated to decline. However, in recent years, non-alcoholic fatty liver disease (NAFLD) has quickly risen as one of leading etiologies for liver disease. NAFLD is a spectrum of chronic liver disease ranging from simple hepatic steatosis to liver cell injury and inflammation known as non-alcoholic steatohepatitis (NASH). The rising incidence of NAFLD/NASH has subsequently led to a dramatic rise in NASH-related HCC incidence<sup>[3]</sup>. Numerous studies have demonstrated that NASH can lead to advanced fibrosis and cirrhosis, thereby increasing the risk of developing HCC<sup>[4-6]</sup>. Among patients with NAFLD or NASH, liver disease is the third leading cause of death<sup>[4]</sup>, while HCC represents the main cause of death in this group<sup>[7]</sup>. The cumulative annual incidence rate for developing HCC in patients with NASH-related cirrhosis is approximately 2.4%-12.8%<sup>[8]</sup>. In the absence of NASH or cirrhosis, NAFLD can present with HCC. These patients usually present with less aggressive tumors and are less likely to be diagnosed by surveillance compared to HCC that develops in the setting of viral hepatitis<sup>[9-11]</sup>. A similar rising trend has been reported in NASH progressing to HCC in the absence of cirrhosis<sup>[12-14]</sup>. In NASH, several risk factors for HCC development have been identified including metabolic syndrome and insulin resistance causing changes in serum cytokines, persistent inflammation, and altered gut microflora and bile composition<sup>[15]</sup>.

## EPIDEMIOLOGY

Currently, NAFLD affects more than 80 million Americans, making it the most common etiology for liver disease in the United States. With the incidence of obesity, diabetes and metabolic syndrome continuing to increase in the United States and Europe, NAFLD/NASH may become the most common cause of HCC in developed countries in the near future<sup>[16]</sup>. In 2012, primary liver cancer was recognized overall as the second most common cause of cancer-related death in the world. In the United States, HCC is the most rapidly rising cause of cancer and cancer-related deaths with an incidence that has tripled over the last decade. This high likelihood for mortality reflects a poor prognosis without therapeutic intervention<sup>[17]</sup>. HCC is the most prevalent histological subtype accounting for 70%-85% of primary liver malignancies<sup>[18]</sup>. Compared to HCC in alcoholic liver disease and viral hepatitis, there is a lack of strong epidemiological data regarding the incidence and prevalence of HCC in NAFLD<sup>[19]</sup>. While the prevalence of NAFLD is thought to be highest among Hispanics and Caucasians, the ethnic distribution among NAFLD/NASH-related HCC patients has yet to be defined<sup>[20]</sup>. NASH-related HCC patients are predominantly male; however, gender has not been proven to be a statistical risk factor NASH progression to HCC<sup>[21]</sup>. Studies analyzing demographic and clinical characteristics of NASH-related HCC patients are outlined in Table 1. Reports indicate that NASH can be verified by histological evaluation in up to 47% of all NAFLD cases among obese individuals<sup>[22,23]</sup>. Amongst a growing population of diabetes which has surpassed 26 million in the United States, the prevalence of biopsy-proven NAFLD and NASH has been reported to be as high as 74% and 11%, respectively<sup>[24,25]</sup>.

This rise in the incidence of NASH-related HCC has impacted trends in liver transplantation as well. A retrospective cohort study amongst adult liver transplant recipients from 2002-2012 indicated that there was 4-fold increase in patients undergoing liver transplant for NASH-related HCC compared to 2-fold increase in number of patients undergoing transplantation for HCV-related HCC<sup>[26]</sup>. During this 10-year span, NASH also became the second leading cause of HCC-related liver transplantation in America, steadily increasing from 8.3% in 2002 to 10.3% in 2007 and to 13.5% in 2012<sup>[16]</sup>, and most likely will surpass 15% by 2017.

## PROGRESSION OF NASH/NAFLD TO HCC

NAFLD is the hepatic manifestation of metabolic syndrome, with insulin resistance driving the alteration in physiology. As mentioned earlier, it ranges from isolated hepatic steatosis, to NASH with or without cirrhosis, and progression to HCC. The diagnosis of NASH is based on histological evidence of hepatic steatosis or magnetic resonance spectroscopic evidence > 5% fat accumulation of liver weight without the presence of secondary causes

**Table 1** Reported studies of hepatocellular carcinoma in patients with cirrhotic and non-cirrhotic non-alcoholic fatty liver disease/non-alcoholic steatohepatitis, and their clinical characteristics

Ref.	All (n)	NASH/NAFLD (n)	Study type	Clinical characteristics	Cirrhotic NASH with HCC		Non-cirrhotic NASH with HCC	
					Histological diagnosis	Clinical diagnosis	Histological diagnosis	Clinical diagnosis
Cotrim <i>et al</i> <sup>[97]</sup>	110	110	Cohort	Age, 67 ± 11 yr; male, 72 (65.5%); non-Hispanic white, N/A	32 (29.1%)	58 (52.7%)	20 (18.2%)	0
Van Meer <i>et al</i> <sup>[98]</sup>	933	91 <sup>1</sup>	Cohort	Age, 64 yr; male, 60 (66%); non-Hispanic white, N/A	N/A	N/A	91 (100%)	N/A
Shrager <i>et al</i> <sup>[99]</sup>	9	9	Case series	Age, 58 yr; male, 8 (88.9%); non-Hispanic white, N/A	5 (55.5%)	N/A	4 (44.4%)	N/A
Kikuchi <i>et al</i> <sup>[93]</sup>	42	38	Case series	Age, 66.5 yr; male 26 (62%); non-Hispanic white, N/A	34	N/A	4	N/A
Chagas <i>et al</i> <sup>[100]</sup>	394	7	Prospective	Age, 63 ± 13 yr; Male 4 (57%); non-Hispanic white, N/A	6	N/A	1	N/A
Ertle <i>et al</i> <sup>[101]</sup>	150	36	Cohort	Age, 68.6 ± 8.4 yr; male 32 (88.9%); non-Hispanic white, N/A	5	14 <sup>2</sup>	10	7 <sup>2</sup>
Tokushige <i>et al</i> <sup>[102]</sup>	2299	292	Cohort	Age, 72 ± 8.4 yr; male, 181 (62%)	181 <sup>3</sup>	N/A	111 <sup>3</sup>	N/A
Hashizume <i>et al</i> <sup>[103]</sup>	1310	10	Case series	Age 71.5 yr; male 6 (66.7%)	5	N/A	4	N/A
Kawada <i>et al</i> <sup>[13]</sup>	807	8	Cohort	Age 73 yr; male 3/6 (50%); non-Hispanic white, N/A	2	N/A	6	N/A
Malik <i>et al</i> <sup>[104]</sup>	143	143	Case control	Age 59 ± 7.6 yr; male 44 (44.9%); 16 non-Hispanic White, 1 Asian	17	N/A	0	N/A
Takuma <i>et al</i> <sup>[105]</sup>	11	11	Case series/ Literature review	Age 73.8 ± 4.9 yr; male 5 (45%)	4	N/A	7	N/A
Perumpail <i>et al</i> <sup>[106]</sup>	44	6	Cohort	Age 72 ± 8 yr; male 5 (83.3%)	NA	NA	6	N/A
Ascha <i>et al</i> <sup>[107]</sup>	510	195	Cohort	Age 56.5 yr; male 86 (44.1%)	NA	NA	N/A	25 <sup>4</sup>
Mohamad <i>et al</i> <sup>[108]</sup>	83	83	Cohort retrospective	Age 64.8 ± 10.4 yr; male 54 (65.1%); non-Hispanic White, 77 (92.8%)	47	N/A	36	N/A

<sup>1</sup>Histological data available in 86 patients only; <sup>2</sup>AASLD Radiological criteria used for diagnosis; <sup>3</sup>Results based on both liver biopsy and abdominal imaging. Differentiating data not available in the study; <sup>4</sup>Histologic confirmation obtained in 59% of the patients diagnosed with HCC. HCC: Hepatocellular carcinoma; EMT: Epithelial to mesenchymal transition; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; N/A: Not available.

such as alcohol abuse, endocrine disorders, chronic HCV infection or familial hypobetalipoproteinemia<sup>[27]</sup>. Recent evidence has demonstrated an association between NASH and HCC to be exclusive to patients who had progressed to cirrhosis, suggesting causality<sup>[8]</sup>.

Compared to benign course of simple steatosis, patients with NASH are more likely to develop progressive advanced liver disease. Matteoni *et al*<sup>[28]</sup> demonstrated increased rates of cirrhosis in patients with NASH compared to those with fatty liver without NASH (25% vs 3%, respectively), and increased risk of liver disease-related death (11% vs 2%, respectively). In a much larger study across the entire spectrum of NAFLD which included 420 patients, Rafiq *et al*<sup>[29]</sup> demonstrated a higher mortality in those with NASH/NAFLD when compared to the general population; liver-related deaths occurred in 13% vs < 1% in general population, and 3%

of those with NAFLD developed cirrhosis. Another study further confirmed increased rate of liver-related deaths among patients with NASH when compared with those without NASH (17.5% vs 3%, respectively). In patients with compensated cirrhosis, NASH-related cirrhosis patients had better survival outcomes compared to HCV-related cirrhosis patients. However, in decompensated cirrhosis both cohorts had comparable poor outcomes<sup>[30,31]</sup>. Currently, both the American Association for the Study of Liver Diseases, and the European Association for the Study of Liver Disease recommend screening for HCC in patients with NASH related cirrhosis every 6-12 mo<sup>[32]</sup>.

## HCC IN NON-CIRRHOTIC NAFLD/NASH

Emerging evidence suggests that a significant proportion of patients with NAFLD-associated HCC, do not have



histologic evidence of cirrhosis. In a study conducted by Kawada *et al.*<sup>[13]</sup>, of 1168 patients who underwent hepatic resection for HCC, 6 of 8 patients with NASH-related HCC did not demonstrate cirrhosis. This study suggested that the presence of cirrhosis in NASH-related HCC was lower compared to HCV-related HCC. These data suggest that compared to patients HCV, HCC may develop at an earlier stage those with NASH. Paradis *et al.*<sup>[33]</sup> analyzed 128 HCC patients who were recruited over 12 years, and reported significant number of patients with NASH developed HCC in the absence of fibrosis when compared to HCC in the setting of other underlying chronic liver disease (65% with F0-F2 in NASH group vs 26% in chronic liver disease)<sup>[33,34]</sup>. To explain this phenomenon in non-cirrhotic NAFLD patients, one proposed hypothesis is the malignant transformation of hepatic adenoma. Few published reports have suggested that in the presence of metabolic syndrome, hepatocellular adenoma may incur a malignant transformation<sup>[19,35]</sup>.

## HCC IN CIRRHOTIC NAFLD/NASH

During the last two decades, various studies have tried to determine the relationship between NAFLD/NASH, cryptogenic cirrhosis and HCC. A recent meta-analysis by White *et al.*<sup>[8]</sup> showed that approximately 60% HCC cases attributed to NAFLD/NASH had cirrhosis either before or at the time of diagnosis. This meta-analysis also included review of cohort and longitudinal studies which showed that NASH-associated cirrhosis consistently carried an increased HCC risk ranging between 2.4% and 12.8%<sup>[8]</sup>. Additionally, this study reported the risk of developing HCC is lower in patients with cirrhosis due to NAFLD/NASH when compared to those with chronic HCV (NAFLD/NASH, 26.9% vs HCV, 19.7%).

The true prevalence of NASH and NASH-related HCC is likely underestimated. In up to 6.9%-29% of HCC, the underlying etiology of liver disease is unknown and is considered secondary to cryptogenic cirrhosis<sup>[19]</sup>. Features suggestive of NASH are more frequently observed in HCC arising in patients with cryptogenic cirrhosis than in age- and sex-matched HCC patients of well-defined viral or alcoholic etiology<sup>[36]</sup>. Although the prevalence of NAFLD/NASH-related HCC is not well defined, the increasing incidence of obesity and diabetes, suggests the impact of NAFLD/NASH-related HCC will continue to grow.

## MORTALITY IN NAFLD/NASH

Long term outcomes in NAFLD and NASH has been evaluated in several studies and distinctive differences between NASH and non-NASH subtypes of NAFLD have been shown<sup>[28,29,37-42]</sup>. Type 2 diabetes mellitus has been shown to increase the risk of both liver-related mortality and overall mortality in NAFLD patients<sup>[43,44]</sup>. In light of these findings NAFLD patients with type II diabetes should be prioritized in future treatment protocols<sup>[44]</sup>. A population-based study published in 1996 followed 153852 subjects and found that diabetic patients had a

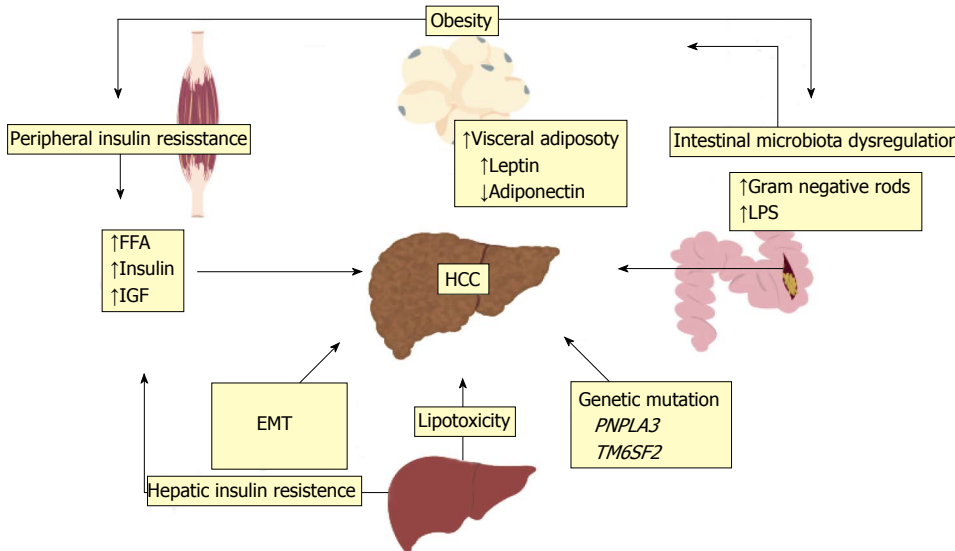
standardized incidence ratio of 4.1 for HCC<sup>[45]</sup>. However, another retrospective analysis from United States Veteran Registry noted increased the risk of primary liver cancer in patients with diabetes only in the presence of other risk factors such as hepatitis C or B or alcoholic cirrhosis<sup>[46]</sup>. These observations were not supported by further analysis that found an incremented HCC risk in diabetic patients independently from alcoholic liver disease and viral hepatitis<sup>[47,48]</sup>. In a recent meta-analysis, Younossi *et al.*<sup>[49]</sup> reported that in NAFLD patients, annual incidence of HCC was 0.44 per 1000 person-years (95%CI: 0.29-0.66), whereas for those with NASH, the annual HCC incident rate was 5.29 per 1000 person-years (95%CI: 0.75-37.56). Among NAFLD cohort, the pooled liver-specific and overall mortality incidence rates were 0.77 per 1000 person-years (95%CI: 0.33-1.77 events) and 15.44 per 1000 person-years (95%CI: 11.72-20.34 events), respectively. Among the NASH cohort, the pooled liver-specific and overall mortality incidence rates were 11.77 per 1000 person-years (95%CI: 7.10-19.53 events) and 25.56 per 1000 person-years (95%CI: 6.29-103.8 events), respectively.

Although cardio-vascular (CV) events remain the major cause of death in patients with NAFLD and NASH, the CV mortality rate amongst the NASH and non-NASH subtypes of NAFLD is similar<sup>[42,50-52]</sup>. Since patients with NASH have significantly higher liver-related mortality than those with non-NASH NAFLD, treatment strategies should be designed to ameliorate the risks for cardiovascular mortality<sup>[28,29,38,40-42,49,50]</sup>. Further, patients with NASH and type 2 diabetes mellitus, will need increased attention and linkage of care to reduce liver disease-related complications and to reduce their risk of HCC<sup>[53-55]</sup>.

## RISK FACTORS AND PROPOSED

### MECHANISMS FOR NASH-RELATED HCC

Development of HCC in the setting of chronic liver disease is a complex but gradual process that requires transition through a dysplasia-carcinoma sequence. Several putative oncogenic mechanisms has been incriminated that lead to genomic instability, including telomere erosion, chromosome segregation defects and alterations in the DNA-damage-response pathways<sup>[56,57]</sup>. Obesity and diabetes are involved in the mechanisms involved in the development of HCC in NAFLD. The development of HCC in NAFLD is likely multifactorial; involving low grade chronic systemic inflammatory response, increased lipid storage and lipotoxicity, gut disbiosis with elevated levels of lipopolysaccharide (LPS) and hyperinsulinemia with insulin resistance and increased IGF levels<sup>[19]</sup>. In addition patients with HCC from NAFLD in general has a distinctive phenotype with presentation in older age, being less aggressive and less likely to be diagnosed by surveillance compared with HCC caused by viral hepatitis<sup>[9-11]</sup>. Other factors such as genetic polymorphism and, increased iron absorption may also lead to development of HCC in NASH<sup>[14]</sup>. Proposed mechanisms for NASH-related HCC are depicted in Figure 1.



**Figure 1 Risk factors and proposed mechanisms for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis-related hepatocellular carcinoma.** The development of NAFLD and NASH-related HCC is multifactorial. Proposed pathogenic mechanisms include obesity, peripheral and hepatic insulin resistance from type 2 diabetes, increased hepatic lipid storage and lipotoxicity, EMT, genetic mutations and intestinal microbiota dysregulation. HCC: Hepatocellular carcinoma; EMT: Epithelial to mesenchymal transition; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; FFA: Free fatty acid; IGF: Insulin-like growth factor; LPS: Lipopolysaccharide; *PNPLA3*: Patatin-like phospholipase domain-containing 3; *TM6SF2*: Transmembrane 6 superfamily member 2.

Cytokines carry out the intercellular communication signals, cellular interactions along with growth and differentiation. Disease states cause imbalances in cytokine levels promoting aberrant signaling and modulating inflammatory responses seen in epithelial to mesenchymal transition pathologic process<sup>[15]</sup>. Imbalances in the levels of cytokines such as tumor necrosis factor (TNF)-alpha, leptin, adiponectin and interleukin-6 (IL-6) play a pivotal role in NASH<sup>[58-60]</sup>.

### Obesity

Obesity is a significant risk for the development of HCC particularly in patients with NASH, who have a higher predisposition for obesity. Obese (body mass index > 30 kg/m<sup>2</sup>) patients have a reported 1.93-fold higher risk of developing primary liver cancer. Obesity and excessive visceral adipose tissue has been associated with a chronic inflammatory state due to increased levels of leptin. Leptin, a profibrotic and proangiogenic cytokine, activates the Janus kinase (JAK) pathway, thereby initiating an intracellular signaling cascade of pro-inflammatory cytokines<sup>[61,62]</sup>. Obesity has also been associated reduced level of adiponectin, an anti-inflammatory cytokine. Additionally, obesity has been associated with other risk factors including insulin resistance, increased hepatic lipid storage and alteration of intestinal microflora.

### Insulin resistance

Diabetes has shown to be an independent risk factor for the development of HCC in NASH<sup>[61,63]</sup>. Excessive fat accumulation and obesity lead to hepatic and peripheral insulin resistance causing compensatory hyperinsulinemia. Evidence supports that insulin and insulin-like growth factor (IGF) may promote the development

of primary liver cancer by activating various oncogenic pathways<sup>[61]</sup>. Both IGF-1 and insulin receptor substrate stimulates growth by activating the mitogen-activate protein kinase (MAPK) pathway and increases the transcription of c-fos and c-jun, known proto-oncogenes. Activation of MAPK pathway subsequently activates the Wnt/ $\beta$ -catenin signaling cascade leading to fibrosis and hepatocarcinogenesis<sup>[61,62]</sup>.

### Lipotoxicity

Increased lipid accumulation in the liver arises from lipolysis within peripheral adipose tissue, dietary sources and de novo hepatic lipogenesis<sup>[19,64]</sup>. This increased lipid accumulation causes hepatic lipotoxicity resulting in the excessive production of saturated and monounsaturated free fatty acids (FFAs)<sup>[65]</sup>. These FFAs undergo  $\beta$ -oxidation leading to formation of reactive oxygen species. Reactive oxygen species induce endothelial reticulum stress, mitochondrial damage and gene transcription promoting inflammatory cell signaling pathways.

### Intestinal microflora dysregulation

Other novel pathogenic pathway between the gut and liver has been demonstrated, which is driven by dietary changes leading to gut dysbiosis that has the potential to generate hepatic inflammation can ultimately influence HCC. In NASH patients, small intestinal bacterial overgrowth<sup>[66,67]</sup> and increased TNF- $\alpha$  levels, elevated expression of Toll-like receptor (TLR) 4 and increased levels of serum IL-8<sup>[67]</sup> has been demonstrated.

LPS, a major component of outer membrane of gram-negative bacteria, is an endotoxin that causes inflammation upon entering the systemic circulation. The involvement of LPS in the development of HCC is sus-

pected by the observation that LPS removal by gut sterilization results in diminished tumor growth in patients with chronic liver injury<sup>[68,69]</sup>. In two recent studies, the investigators observed in NASH patients, increased levels of TNF-alpha, interleukin-8 and elevated expression of TLR 4 and small intestinal bacterial overgrowth<sup>[66,67]</sup>. NASH patients also have less gut gram-negative *Bacteroidetes* and an increase in alcohol producing bacteria when compared to patients with simple steatosis, which raises a question as to whether these strains are involved in the pathogenesis of NASH<sup>[70,71]</sup>.

Several recent studies have identified potential link between gut dysbiosis and NAFLD in both in animal models and human<sup>[66-72]</sup>. There is incremental evidence for gut microbiome in the pathogenesis of NASH based on these findings, suggesting potential therapeutic role of correcting of gut dysbiosis to a more healthy phenotype in limiting progression of NASH. Evidence linking gut microbiota, NASH, and HCC development is reported from Dapito *et al.*<sup>[69]</sup>. They treated mice with diethylnitrosamine (DEN) followed by carbon tetrachloride (CCL4) to promote fibrosis-driven HCC<sup>[69]</sup>. They found that TLR4-deficient mice had limited HCC growth; DEN/CCL4-treated wild-type mice that received antibiotics also had reduced tumor growth, suggesting that the microbiota played a role in HCC progression possibly *via* LPS-TLR4 axis.

Gut microbiota can catalyse generation of secondary bile acids such as sDCA, which is known to induce DNA damage<sup>[72]</sup>. Yoshimoto *et al.*<sup>[68]</sup> found that DCA can promote the activation of a senescence-associated secretory phenotype in HSCs, reflected by the secretion of IL-1 $\beta$ . Further they observed limited obesity-induced HCC development in the absence of IL-1 $\beta$ , and alleviation of HCC development with antibiotic treatment. In addition, lowering of DCA or feeding of DCA, limited or enhanced HCC growth respectively. Although the role for bile acids in NASH HCC progression need further exploration, these studies certainly lay the foundation for future exploratory studies in both animal models and human.

### Genetic polymorphisms

Genetic polymorphism is also one of the factors that may account for development of HCC in NAFLD. Genetic predisposition plays an important role in susceptibility to the metabolic syndrome and NASH. Recent genome-wide association studies have identified a single nucleotide polymorphism in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene. Specifically, a C-to-G genotype in the *rs738409* gene, encoding the I148M protein variant, determines differences in hepatic fat accumulation<sup>[73]</sup>. Although the physiological and biological functions of *PNPLA3* within the liver, which effect fat accumulation and NASH, remain unclear, the association of *rs738409* polymorphisms with HCC is evident<sup>[74]</sup>.

It has also been suggested a polymorphism in the transmembrane 6 superfamily member 2 gene (*TM6SF2*) may increase the risk of NASH progression to HCC<sup>[75]</sup>. *TM6SF2* mutation encodes for a loss of function sub-

stitution of lysine to glutamic acid. This *TM6SF2* variant was associated with liver injury in NAFLD and NASH patients. While there is an increased prevalence of the *TM6SF2* variant in NAFLD and NASH patients, conflicting preliminary data exists regarding its role in the progression to HCC.

### Other risk factors

Increased intrahepatic iron accumulation has been associated with NASH progression to HCC. Although clinical data is limited, Sorrentino *et al.*<sup>[76]</sup> demonstrated higher hepatic iron storage levels among NASH-related HCC patients compared to NASH patients. The underlying mechanism of increased iron absorption in NASH patients may be related to oxidative DNA damage but further studies are required to understand the role of iron accumulation in NAFLD and HCC<sup>[19,76]</sup>.

Other significant risk factors for NASH progression to HCC include advanced age and concomitant chronic alcohol consumption<sup>[77]</sup>. Alcohol consumption among NASH patients has an associated 3.6-fold increased risk for development of HCC. Also emerging evidence has suggested a possible correlation between obstructive sleep apnea and NAFLD and NASH but its association to development of HCC has not been investigated<sup>[78]</sup>.

## SURVEILLANCE

With the increasing prevalence of NAFLD/NASH and associated HCC, chemopreventive and perhaps reconsideration of current surveillance guidelines are needed<sup>[16]</sup>. The current AASLD guidelines recommend screening for HCC every 6 mo in patients with cirrhosis. However, the current guidelines lack recommendations for surveillance of NASH patients without cirrhosis who are at risk for developing HCC. This is further supported by a study performed by Mittal *et al.*<sup>[79]</sup> in which the data collected on about 1500 HCC patients where HCC related to NASH received less surveillance and treatment compared with HCC arising in underlying etiologies related to HCV and alcohol.

The lack of longitudinal data in the non-cirrhotic NASH population makes it difficult to develop good evidence - based screening guideline. There is a need for studies addressing the screening guidelines for surveillance of HCC in NASH particularly for non-cirrhotic individuals. We suspect that earlier screening may be needed in patients with NASH who have multiple risk factors for HCC<sup>[19]</sup>.

## CURRENT THERAPEUTIC OPTIONS

The biological heterogeneity of HCC makes it difficult to clarify the key mechanisms of cancer development and thus to develop and implement effective therapies<sup>[80]</sup>. A few chemopreventive agents have shown promise in the prevention and treatment of steatohepatitis and fibrosis; however these are small individual studies and thus there is a lack of a general consensus due to paucity

of data. There is currently no effective chemoprevention to decrease the incidence of HCC. Exceptions include nucleoside analogues used to reduce viral replication in those with hepatitis B, and DAAs for HCV which have very high cure rates<sup>[81]</sup>.

### Medical therapy

Regular exercise and controlled caloric intake is the mainstay of therapy for NAFLD, however the extent to which these are effective to prevent the development of HCC is unclear. Physical activity has been reported to have a preventive effect on development of HCC. A large prospective cohort study, which included over 400000 participants suggested that increased physical activity might have a role in HCC prevention that is independent of weight reduction<sup>[82]</sup>. Preliminary data suggests that statins, metformin and S-Adenosylmethionine are potential chemopreventive agents<sup>[16]</sup>.

Patients with NASH have been found to be deficient in vitamin E and D; vitamin D deficiency is thought to play a role in hepatic carcinogenesis<sup>[83,84]</sup>. Other dietary antioxidants such as vitamin C, selenium, coenzyme Q12 and certain phytochemicals have also been touted have chemopreventive potential<sup>[85]</sup>. NASH patients have been shown to have low levels of serum lycopene<sup>[83]</sup>. There is a strong inverse relationship between serum lycopene levels and the risk of GI cancers<sup>[86]</sup>.

Metformin has an antitumor effect in HCC *via* suppression of mTOR pathway<sup>[87]</sup>. Although it may not have a role in the treatment of NASH, metformin may have a role in decreasing the incidence of HCC in NASH<sup>[16]</sup>. A review of two recent meta-analyses included 22650 cases of HCC in approximately 334000 patients with type 2 diabetes revealed that metformin reduced incidence of HCC by 50% whereas sulfonylurea and insulin increased incidence of HCC by 62% and 161% respectively<sup>[88]</sup>. The use of metformin has also been shown to increase survival of HCC patients who have cirrhosis<sup>[89]</sup>.

Statins have shown a protective effect in individuals who are at risk for development of steatohepatitis and F2-F4 fibrosis<sup>[90]</sup>. The protective effect of statins in diabetics is thought to be due to anti-inflammatory properties of statins mediated through the inhibition of JAK<sup>[91]</sup>. A recent Swedish case control study which evaluated almost 4000 HCC patients treated with statin that were matched with 19970 controls showed that the odds ratio for HCC amongst statin users was 0.88, suggesting a modest but beneficial effect of statins in reducing the risk of HCC<sup>[92]</sup>.

The heterogeneity of HCC makes it difficult to clarify the mechanism of cancer development and to develop effective therapeutics. However, an integrative functional genomics approach will contribute to the discovery of potential molecular features critical for HCC development. These studies will provide us with better treatment strategies that may be effective to treat all HCC patients including those with NASH.

### Surgical therapy

Curative treatment options including liver resection and

liver transplantation in select early-stage HCC candidates. The Barcelona Clinic Liver Cancer staging system and therapeutic algorithm has been applied to HCC candidates including those with NASH-related HCC<sup>[93]</sup>. Non-cirrhotic NASH-related HCC patients who underwent curative surgical resection have shown to have superior survival than those with HCV and alcohol-related HCC<sup>[11]</sup>.

Since the implementation of the Model for End-Stage Liver Disease (MELD) system for liver allocation in 2002 the number of HCC liver transplantations has dramatically increased. In 2012, they accounted for 23.2% of all liver transplantations in the United States<sup>[26]</sup>. HCC liver candidates are eligible to receive a MELD exception which upgrades their priority and thus, increases their likelihood of receiving liver transplant and survival. Subsequently, a higher number of HCC candidates have sought listing for liver transplant. A recent study using United Network for Organ Sharing data from 2004-2013<sup>[94]</sup> demonstrated that NASH-related HCC candidates have lower rates for receiving MELD exception and have longer time to transplant compared to HCV-related HCC. Despite this, NASH-related HCC was the fastest growing indication for liver transplantation from 2002-2012<sup>[26]</sup>. NASH-related HCC liver transplant recipients have better outcomes compared HCV-related HCC with a 5-year post-transplant survival approaching 68%<sup>[95]</sup>. NASH-related HCC liver transplant recipients with morbid obesity and CV risk factors tend to have poorer outcomes<sup>[96]</sup>. Further research is needed to evaluate NASH-related HCC post liver transplant survival risk factors and exploring why this growing cohort is less likely to receive a MELD exception.

## CONCLUSION

With the prevalence of HCV expected to decline, NASH is anticipated to account for a greater proportion of HCC incidence in the near future due to the growing epidemic of obesity and diabetes. The annual incidence rate of developing HCC in patients with NASH-related cirrhosis is not clearly understood with rates ranging from 2.6%-12.8%. Recent evidence has shown a significant proportion of patients with NAFLD and NASH progress to HCC in the absence of cirrhosis. While liver resection and transplantation represent curative therapeutic options in select NASH-related HCC candidates, they also have placed a significant burden to our healthcare resources and utilization. Currently NASH-related HCC is the fastest growing indication for liver transplant in HCC candidates. Increased efforts to implement effective screening and preventative strategies, particularly in non-cirrhotic NASH cohort, are needed to reduce the future impact imposed by NASH and NASH-related HCC.

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Retrospective Study

## Efficacy and safety of dual therapy with daclatasvir and asunaprevir in elderly patients

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### Abstract

#### AIM

To survey the efficacy and safety of dual therapy with daclatasvir and asunaprevir in the elderly hepatitis C virus (HCV) patients multicentricity.

#### METHODS

Interferon-ineligible/intolerant patients and non-responders to previous pegylated-interferon/ribavirin therapy with chronic HCV genotype 1b infection were enrolled. Child B, C cirrhotic patients were excluded.

Patients received oral direct acting antiviral treatment consisting of 60 mg daclatasvir once daily plus 200 mg asunaprevir twice daily for 24 wk. We divided the patients into two groups of 56 elderly patients ( $\geq 75$  years-old) and 141 non-elderly patients ( $< 75$  years old) and compared the efficacy and safety.

## RESULTS

Ninety-one point one percent of elderly patients and 90.1% of non-elderly patients achieved sustained virological response at 24 wk (SVR<sub>24</sub>). In the former, 1.8% experienced viral breakthrough, as compared with 3.5% in the latter (not significant). Adverse events occurred in 55.4% of the former and 56.0% of the latter. In the former, 7 cases (12.5%) were discontinued due to adverse events, and in the latter 9 cases were discontinued (6.4%, not significant).

## CONCLUSION

Dual therapy with daclatasvir and asunaprevir achieved the same high rates of SVR<sub>24</sub> in HCV elderly patients without more adverse events than in the non-elderly patients.

**Key words:** Asunaprevir; Chronic hepatitis; Daclatasvir; Dual oral therapy; Elderly patients; Hepatitis C virus infection; Hepatitis C virus; Liver cirrhosis

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**Core tip:** Recently, it was demonstrated that dual oral therapy with daclatasvir and asunaprevir without pegylated-interferon/ribavirin was well tolerated and achieved high sustained virological response rates in Japanese patients with chronic hepatitis C virus genotype 1b infection, including patients with liver cirrhosis (Child A stage). However, the efficacy and side effects of these drugs was previously studied in non-elderly patients (less than 70 years of age). Those in elderly patients, who are supposed to have higher incidence of hepatocellular carcinoma, have not been studied. We demonstrated that efficacy and side effects in elderly patients were nearly the same as in non-elderly patients.

Tarao K, Tanaka K, Nozaki A, Sato A, Ishii T, Komatsu H, Ikeda T, Komatsu T, Matsushima S, Oshige K. Efficacy and safety of dual therapy with daclatasvir and asunaprevir in elderly patients. *World J Hepatol* 2017; 9(11): 544-550 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i11/544.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i11.544>

## INTRODUCTION

Recently, it was demonstrated that dual oral therapy with daclatasvir and asunaprevir without pegylated-interferon/ribavirin (PegIFN $\alpha$ /RBV) was well tolerated and achieved high sustained virological response (SVR)

rates in difficult-to-treat Japanese patients with chronic hepatitis C virus (HCV) genotype 1b infection, including patients with liver cirrhosis (Child A stage)<sup>[1-4]</sup>.

It is generally accepted that the average age of the patients with HCV-associated liver disease in Japan is increasing, and indeed patients more than 60 years of age represent more than 70% of all patients<sup>[5]</sup>. Moreover, Kumada *et al*<sup>[6]</sup> recently analyzed the age distribution of 3388 persistent HCV-infected patients and found that the median age was 70 years, and 2249 (66.4%) were elderly patients of more than 65 years.

Also, recently, Asahina *et al*<sup>[7]</sup> demonstrated that the risk for hepatocellular carcinoma (HCC) after interferon treatment was age-dependent and increased predominantly when the age at primary liver biopsy was  $> 65$  years. They also demonstrated that progression of fibrosis over time was significantly accelerated in older patients. In addition, elderly patients with HCV-associated chronic hepatitis are thought to develop liver cirrhosis more rapidly, and HCC might develop more frequently as a result. An increase in the aged population is an impending problem, and we must eradicate the HCV infection as soon as possible in elderly patients.

We therefore retrospectively examined the efficacy and safety of dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protein inhibitor, asunaprevir, in elderly patients ( $\geq 75$  years of age) with hepatitis C chronic hepatitis or liver cirrhosis (Child A stage), who may have suffered from longer periods of HCV infection, in many large hospitals in Kanagawa Prefecture in Japan.

## MATERIALS AND METHODS

### Study design

This study included two populations of patients with HCV genotype 1b infection: Null responders ( $< 2 \log_{10}$  decline in serum HCV RNA levels after 12 wk of prior PegIFN $\alpha$ /RBV), and PegIFN $\alpha$ /RBV ineligible/intolerant patients. The latter group was either patients who discontinued prior therapy with PegIFN $\alpha$ /RBV due to intolerance after  $< 12$  wk, or patients who were treatment-naïve but poor candidates for PegIFN $\alpha$ /RBV for medical reasons such as advanced age or complications of depression, anemia, myelosuppression, diabetes or cardiovascular or renal dysfunction. Of the cirrhotic patients, only those with Child-Pugh stage A were enrolled<sup>[8]</sup>, and patients with Child-Pugh stages B and C were omitted.

The patients were out-patients and visited the following hospitals in Kanagawa Prefecture of Japan between 1 September 2014 and 30 March 2015: Tarao's Gastroenterological Clinic, Yokohama City University Medical Center, Yokohama Seibu Hospital of St. Marianna University, Yokohama Municipal Citizens Hospital, Yokosuka General Hospital Uwamachi, and National Hospital Organization Yokohama Medical Center.

Elderly patients were defined as those equal to or over 75 years old. Enrolled patients were divided into

**Table 1** Background of elderly ( $\geq 75$  years old) and non-elderly ( $< 75$  years old) patients

Parameter	Elderly patients, <i>n</i> = 56	Non-elderly patients, <i>n</i> = 141	<i>P</i>
Cirrhosis/chronic hepatitis	30/26	44/97	0.003
Age in years	77.8 (75-83)	65.3 (34-74)	
Male/female	23/33 (41.1%/58.9%)	54/87 (38.3%/61.7%)	
HCV genotype 1b, %	100	100	
HCV RNA, mean log <sub>10</sub> IU/mL	5.85 $\pm$ 0.77	6.12 $\pm$ 0.70	
Pegylated-interferon/ribavirin non-responder, %	17.9	25.5	0.251

HCV: Hepatitis C virus.

two groups, elderly patients ( $\geq 75$  years old) and non-elderly patients ( $< 75$  years old), and efficacy and safety assessments were compared. The primary efficacy end-point was the proportion of patients with undetectable HCV RNA at 24 wk post-treatment (SVR<sub>24</sub>).

Written informed consent was obtained from all patients. The study was approved by institutional review boards in each hospital and conducted in compliance with the Declaration of Helsinki.

### Patients

Eligible patients were men and women, aged 34-83 years, with HCV genotype 1 infection with chronic hepatitis or compensated liver cirrhosis. Patients with cirrhosis were confined to Child-Pugh stage A<sup>[8]</sup>, and patients with stage B and C cirrhosis were excluded. Exclusionary laboratory findings included alanine aminotransferase (ALT)  $> 5 \times$  upper limit of normal (ULN), total bilirubin  $> 2$  mg/dL, albumin  $< 3.5$  g/dL, hemoglobin  $< 9.0$  g/dL, white blood cells  $< 1500$  mm<sup>3</sup>, platelets  $< 50000$ /mm<sup>3</sup>, and creatinine  $> 1.8 \times$  ULN. No patients had prior exposure to HCV direct-acting antivirals.

### Analysis of resistant-associated variants

At pre-treatment points and the resistant-associated variants (RAVs) in NS5A (Y93H) were investigated by PCR-invader assay. PCR-invader assays were conducted by BML Inc. (Saitama, Japan), and weakly positive and negative samples were defined as substitution-negative. In 132 out of 197 cases, the RAVs was examined and the results were as follows: Y93H  $\leq 1\%$  in 116 cases (87.9%), 2%-5% in 14 cases (10.6%), 23% in 1 case (7.6%), 64% in 1 case (7.6%).

### Study drug dosing

Patients received 24 wk of treatment with 60 mg daclatasvir once daily combined with 200 mg asunaprevir twice daily, and participated in 24 wk of post-treatment follow-up. HCV RNA, physical examinations, adverse events, laboratory parameters and concomitant medications were assessed at day 1 (baseline), treatment weeks 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24, and post-treatment weeks 4, 8, 12 and 24.

### Statistical analysis

Pearson's  $\chi^2$  test and the Student's *t*-test were used for statistical analyses. Statistical significance was considered

to exist at  $P < 0.05$ .

## RESULTS

A total of 197 patients were enrolled in this retrospective study, and included 56 elderly patients and 141 non-elderly patients. Backgrounds of the patients are shown in Table 1. In the elderly patients, the number of cirrhotic patients (53.6%) was significantly larger, as compared with 31.2% in the non-elderly patients ( $P = 0.003$ ). The average age was 77.8 years in the elderly patients and 65.3 years in the non-elderly patients. The male/female ratio was nearly the same in the two groups ( $P = 0.719$ ). HCV genotype was 1b in all patients. HCV RNA (mean log<sub>10</sub> IU/mL) was  $5.85 \pm 0.77$  in the former and  $6.12 \pm 0.70$  in the latter ( $P = 0.016$ ). Percentages of the non-responder patients in the prior PegIFN $\alpha$ /RBV therapy group were 17.9% in the former and 25.5% in the latter ( $P = 0.251$ ).

### Virologic outcomes

Of the elderly patients, 51 (91.1%) achieved SVR<sub>24</sub>, while in the non-elderly patients, 127 (90.1%) achieved SVR<sub>24</sub>. The ratio of patients achieving SVR<sub>24</sub> was nearly the same in the two groups (Table 2). The ratio of patients who achieved SVR<sub>24</sub> in the group that discontinued due to side effects was 71.4% (5/7) for the elderly and 77.8% (7/9) for the non-elderly patients (Table 2).

### Viral breakthrough

Only one patient out of 56 (1.8%) experienced viral breakthrough in the elderly group, as compared with 5 out of 141 (3.5%) in the non-elderly group (not significant,  $P = 0.519$ ). Post-treatment relapse was seen in 2 (3.6%) of the elderly patients, as compared with 7 (5.0%) of the non-elderly patients (not significant,  $P = 0.675$ ) (Table 2).

### Safety

Adverse events and laboratory abnormalities in each group are shown in Table 3. There were no significant differences in each event between elderly and non-elderly groups. The total number of patients who showed adverse events in the elderly group was 31 out of 56 (55.4%), which was nearly the same in the non-elderly group (79 out of 141, 56.0%) (Table 4).

Table 5 shows the causes of discontinuation and

**Table 2** Virologic outcomes

Parameter	Elderly patients, <i>n</i> = 56	Non-elderly patients, <i>n</i> = 141	<i>P</i>
SVR <sub>24</sub>	51 (91.1)	127 (90.1)	
Viral breakthrough	1 (1.8)	5 (3.5)	0.519
Post-treatment relapse	2 (3.6)	7 (5.0)	0.675
Ratio of patients who achieved SVR <sub>24</sub> in the discontinued cases due to side effects	5/7 (71.4)	7/9 (77.8)	

Data are presented as *n* (%). SVR<sub>24</sub>: Sustained virological response at 24-wk.

**Table 3** Adverse events and laboratory abnormalities

Event	Elderly patients, <i>n</i> = 56	Non-elderly patients, <i>n</i> = 141
Nasopharyngitis	5 (8.9)	6 (4.3)
Headache	4 (7.1)	9 (6.4)
Diarrhea	3 (5.4)	5 (3.5)
Pyrexia	2 (3.6)	12 (8.5)
Malaise	7 (12.5)	8 (5.7)
Anorexia	6 (10.7)	8 (5.7)
AST elevation	15 (26.8)	55 (39.0)
ALT elevation	14 (25.0)	54 (38.3)
Hb decrease	8 (14.3)	11 (7.8)
Total bilirubin increase	2 (3.6)	11 (7.8)
Creatinine increase	8 (14.3)	13 (9.2)

Data are presented as *n* (%). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Hb: Hemoglobin.

**Table 4** Total number of adverse events in the elderly and non-elderly patients

	Elderly patients	Non-elderly patients
No. of total patients enrolled	56	141
No. of patients who experienced adverse events	31	79
Percentage of patients who experienced adverse events	55.4	56.0

the numbers of patients in whom the drugs were discontinued due to adverse effects in each group. The levels of elevation of ALT and total bilirubin at which the drug was discontinued were 200 INU (5-folds of normal) for ALT and 3.0 mg/dL for total bilirubin. In the elderly group, 7 out of 56 cases (12.5%) were discontinued, and in the non-elderly group, 9 out of 141 (6.4%). The ratio of discontinuation was greater for the elderly patients but the difference was not significant ( $P = 0.336$ ). The ratio of patients who achieved SVR<sub>24</sub> in the discontinued due to side effects subgroup was 71.4% (5/7) in the elderly and 77.8% (7/9) in the non-elderly patients (Table 2).

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Informed consent was obtained from all patients for being included in the study. The protocol was approved by the ethics committees/institutional review boards

**Table 5** Causes of discontinuation and numbers of patients in whom the study drugs were discontinued due to adverse events in the elderly and non-elderly groups

Elderly patients	Non-elderly patients
16 w Malaise, Anorexia <sup>1</sup>	18 w Elevation of ALT <sup>1</sup>
6 w Malaise, Anorexia <sup>1</sup>	6 w Elevation of ALT <sup>1</sup>
6 w Elevation of ALT <sup>1</sup>	2 w Pyrexia <sup>1</sup>
8 w Elevation of ALT <sup>1</sup>	3 w Pyrexia
3 w Pyrexia	13 w Sepsis due to hemolytic streptococcus <sup>1</sup>
4 w Cough	18 w Abdominal fullness <sup>1</sup>
18 w Development of HCC <sup>1</sup>	2 w Elongation of PT
	3 w Elevation of total bilirubin <sup>1</sup>
	18 w Development of HCC <sup>1</sup>
Total 7/56 (12.5%)	9/141 (6.4%)

<sup>1</sup>Patients achieved sustained virological response at 24-wk. Level of elevation of ALT and total bilirubin at which the drug was discontinued: 200 INU (5-folds of normal) for ALT and 3.0 mg/dL for total bilirubin. HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase.

of participating centers and conformed to Good Clinical Practice guidelines and Declaration of Helsinki principles. This article does not contain any studies with animal subjects.

## DISCUSSION

In recent years, patients presenting with HCV-associated chronic hepatitis or liver cirrhosis have been older, especially those patients with liver cirrhosis<sup>[5-7]</sup>. Among these patients, the occurrence of HCC is also increasing. It is generally well accepted that aging is a potent risk factor for HCC development in patients with HCV-associated liver disease<sup>[7-11]</sup>. Peg-IFN $\alpha$ /RBV therapy was shown to be effective in preventing the development of HCC in younger patients<sup>[12]</sup>; however, older patients are poor candidates for Peg-IFN $\alpha$ /RBV therapy.

More recently, oral dual therapy with daclatasvir/asunaprevir was demonstrated to be very effective in eradicating HCV infection. Overall, 76.7% of patients achieved SVR<sub>12</sub> and SVR<sub>24</sub> in an initial trial<sup>[2]</sup>.

In this study, we demonstrated that the proportion of patients with SVR at 24 wk post-treatment was almost the same among elderly patients ( $\geq 75$  years old), who were thought to have longer durations of HCV infection, as among younger patients ( $< 75$  years old, 91.1% vs 90.1%).

We also demonstrated that the degree of side effects



was nearly the same in the elderly and younger patients. Hitherto, no report has dealt with age differences in the efficacy and side effects of oral dual administration of daclatasvir/asunaprevir. The question remains: Is there any essential benefit in eradicating HCV in elderly patients ( $\geq 75$  years old) with HCV-associated liver disease by administering daclatasvir/asunaprevir?

There is much evidence that the eradication of HCV virus (*i.e.*, SVR) by IFN or Peg-IFN $\alpha$ /RBV treatment brings about a low incidence of HCC development<sup>[12-18]</sup>. Yet, there is no definite evidence that the eradication of HCV virus by dual therapy with daclatasvir/asunaprevir can bring about a low incidence of HCC in SVR patients. There is some potential, however, for these drugs to lower the risk of HCC development. First, there is some evidence of lowering serum alpha-fetoprotein (AFP) levels after eradicating HCV virus by the dual therapy, which is one of the potential risk factors for HCC development in HCV-associated liver diseases<sup>[19-24]</sup>. Oka *et al.*<sup>[25,26]</sup> actually demonstrated that the serum AFP level was a potential risk factor for HCC development in cirrhotic patients.

Karino *et al.*<sup>[20]</sup> compared the changes of serum AFP levels after treatment by Peg-IFN $\alpha$ /RBV and by daclatasvir/asunaprevir treatment; they were 13.7–4.9 ng/mL on average for Peg-IFN $\alpha$ /RBV and 15.2–4.8 ng/mL for daclatasvir/asunaprevir. Moreover, the proportions of patients who showed below 5 ng/mL after SVR were 56% in Peg-IFN $\alpha$ /RBV and 65% in daclatasvir/asunaprevir treatment, suggesting nearly the same effect on AFP levels with both treatments. More recently, Miyaki *et al.*<sup>[24]</sup> examined the changes in AFP levels before and after the dual therapy (median 27 mo after the completion of the therapy), and found a significant decrease in SVR patients (AFP levels decreased to within the normal limit in all patients by 18 mo after treatment).

Second is the potential of dual therapy with daclatasvir/asunaprevir to reduce liver fibrosis. Miyaki *et al.*<sup>[24]</sup> also observed the changes of liver fibrosis markers before and after the administration of the drugs and found a significant increase in platelet counts and a significant decrease in liver fibrosis markers such as hyaluronic acid, type IV collagen and M2BPGi (a liver fibrosis glycomarker) at 27 mo (median) after completion of the treatment. van der Meer *et al.*<sup>[27]</sup> also demonstrated a significant increase in the platelet counts associated with a decrease in spleen volume after the completion of IFN therapy.

There is some evidence that eradication of HCV-virus by IFN might bring about a decrease in the fibrosis staging score of HCV-associated chronic hepatitis. Shiratori *et al.*<sup>[28]</sup> demonstrated that the fibrosis score after IFN therapy had regressed in patients with a sustained response at a rate of -0.28 U/year in the histological examination of the biopsied specimens, suggesting that the staging of fibrosis might be reduced by one step in every 4 years by IFN in SVR patients. And, it is well known that the staging of fibrosis has a close association with the incidence of HCC development<sup>[29]</sup>. Omata<sup>[29]</sup> surveyed

the relationship between the degree of fibrosis and the incidence of HCC in HCV-associated chronic hepatitis and found that the incidence of HCC was 0.46%/year in patients with slight fibrosis (F1 stage), while in patients with moderate fibrosis (F3 stage) it was 3%/year, and in patients with severe fibrosis (F4 stage) it was 7%/year.

In support of this concept, van der Meer *et al.*<sup>[27]</sup> demonstrated an increase in platelet counts associated with a decrease in spleen volume in the IFN-treated SVR patients among HCV-associated patients with Ishak 4-6 fibrosis, and concluded that the portal hypertension was decreased in those patients.

Considering the above evidence, it is possible that the eradication of HCV virus by dual therapy with daclatasvir/asunaprevir might bring about reduction in fibrosis and a lower incidence of HCC development even in patients over 75 years old, who have an impending risk of HCC development.

However, long-term observations of SVR patients after therapy with daclatasvir/asunaprevir will be necessary to make any final conclusions about the effect on prevention of HCC development.

## COMMENTS

### Background

Recently, direct acting antivirals (DAAs), including dual therapy with daclatasvir/asunaprevir, have been widely used in the therapy of chronic hepatitis C virus (HCV) genotype 1b infection. Dual therapy with daclatasvir/asunaprevir was demonstrated to be highly effective without serious side-effects. However, those results were studied in the non-elderly (patients under 70 years old). The effectiveness and safety in the elderly patients (> 70 years old) should be studied.

### Research frontiers

Although, DAAs were widely used in the treatment of HCV-associated liver diseases, few prior reports surveyed the difference in the efficacy and side effects of dual oral therapy with daclatasvir/asunaprevir between elderly patients and non-elderly patients.

### Innovations and breakthroughs

In this study, the effectiveness and safety of the therapy with daclatasvir/asunaprevir were demonstrated in elderly patients as well as in non-elderly patients.

### Applications

In Japanese, recently the aged population is increasing rapidly among cases of HCV-associated liver disease, and these individuals are at high risk for developing hepatocellular carcinoma. Eradicating HCV in this population is important and many approaches have been proposed. Now, the authors can eradicate HCV by oral therapy with daclatasvir/asunaprevir effectively and safely.

### Peer-review

The manuscript is an interesting one, discussing a very important issue as regarding new DAAs with HCV treatment.

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Observational Study

## Factors associated with success of telaprevir- and boceprevir-based triple therapy for hepatitis C virus infection

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**Data sharing statement:** Technical appendix, statistical code, and dataset available from the corresponding author at [kian.bichoupan@mssm.edu](mailto:kian.bichoupan@mssm.edu). Consent was not obtained but the presented data are anonymized and risk of identification is low.

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## Abstract

### AIM

To evaluate new therapies for hepatitis C virus (HCV), data about real-world outcomes are needed.

### METHODS

Outcomes of 223 patients with genotype 1 HCV who started telaprevir- or boceprevir-based triple therapy (May 2011-March 2012) at the Mount Sinai Medical Center were analyzed. Human immunodeficiency virus-positive patients and patients who received a liver transplant were excluded. Factors associated with sustained virological response (SVR24) and relapse were analyzed by univariable and multivariable logistic regression as well as classification and regression trees. Fast virological response (FVR) was defined as undetectable HCV RNA at week-4 (telaprevir) or week-8 (boceprevir).

### RESULTS

The median age was 57 years, 18% were black, 44% had advanced fibrosis/cirrhosis (FIB-4  $\geq 3.25$ ). Only 42% (94/223) of patients achieved SVR24 on an intention-to-treat basis. In a model that included platelets, SVR24 was associated with white race [odds ratio (OR) = 5.92, 95% confidence interval (CI): 2.34-14.96], HCV sub-genotype 1b (OR = 2.81, 95%CI: 1.45-5.44), platelet count (OR = 1.10, per  $\times 10^3$  cells/ $\mu$ L, 95%CI: 1.05-1.16), and *IL28B* CC genotype (OR = 3.54, 95%CI: 1.19-10.53). Platelet counts  $> 135 \times 10^3/\mu$ L were the strongest predictor of SVR by classification and regression tree. Relapse occurred in 25% (27/104) of patients with an end-of-treatment response and was associated with non-FVR (OR = 4.77, 95%CI: 1.68-13.56), HCV sub-genotype 1a (OR = 5.20; 95%CI: 1.40-18.97), and FIB-4  $\geq 3.25$  (OR = 2.77; 95%CI: 1.07-7.22).

### CONCLUSION

The SVR rate was 42% with telaprevir- or boceprevir-based triple therapy in real-world practice. Low platelets and advanced fibrosis were associated with treatment failure and relapse.

**Key words:** Sustained virologic response; Hepatitis C virus; Relapse; Telaprevir; Boceprevir; Triple-therapy;

Classification and regression; Adverse event; Real-world

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**Core tip:** A cohort of 223 hepatitis C virus (HCV)-infected patients at a tertiary referral center was analyzed. All patients were treated with telaprevir and boceprevir. Using both logistic regression and a machine learning techniques we identified baseline and on-treatment factors associated with sustained virologic response and relapse. We found that both low platelet count and advanced fibrosis or cirrhosis were associated with treatment failure. Information of the effectiveness of these protease inhibitors could be used to inform clinical trials of future HCV direct-acting antivirals.

Bichoupan K, Tandon N, Martel-Laferriere V, Patel NM, Sachs D, Ng M, Schonfeld EA, Pappas A, Crismale J, Stivala A, Khaitova V, Gardenier D, Linderman M, Olson W, Perumalswami PV, Schiano TD, Odin JA, Liu LU, Dieterich DT, Branch AD. Factors associated with success of telaprevir- and boceprevir-based triple therapy for hepatitis C virus infection. *World J Hepatol* 2017; 9(11): 551-561 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i11/551.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i11.551>

## INTRODUCTION

The hepatitis C virus (HCV) infects about 3% of the world's population. In the United States, HCV chronically infects an estimated 2.7 to 3.9 million people and is a leading cause of liver disease, liver cancer, and liver-related death<sup>[1,2]</sup>. In 2012, the Centers for Disease Control and Prevention announced a public health initiative to identify HCV-positive individuals and transition them into care<sup>[1]</sup>. The recommendation for increased screening was affirmed by the United States Preventive Services Task Force<sup>[3]</sup>. The goal of treatment is to induce a sustained virological response (SVR) and thereby interrupt, and potentially reverse the progression of liver disease, improve the quality of life, and reduce the risk of transmission.

In the era of direct-acting antiviral (DAAs) drugs, treatment for HCV is evolving rapidly. Data about outcomes in real-world clinical practice are needed to evaluate new medications. The first generation protease inhibitors, telaprevir (TVR) and boceprevir (BOC), were approved for treatment of genotype 1 HCV in combination with pegylated-interferon (PEG) and ribavirin (RBV) in 2011. In clinical trials, overall SVR rates ranged from 64% to 75% for TVR-based triple therapy and from 59% to 66% for BOC-based triple therapy<sup>[4,5]</sup>. These trials enrolled relatively few patients  $\geq 65$  years of age and few with liver cirrhosis<sup>[6-8]</sup>, although these patients are often in great need of treatment. Triple therapy with TVR or BOC is no longer the standard of care in the

United States and is no longer recommended by the American Association for the Study of Liver Disease and the Infectious Disease Society of America<sup>[9]</sup>; however, the effectiveness of these regimens in real-world practice may offer important information about currently available NS3/4A protease inhibitors. Information about their real-world effectiveness may benefit providers and patients in these regions of the globe.

Past investigations of factors associated with treatment outcome have yielded varied results<sup>[4,10]</sup>. Among patients receiving TVR-based therapy, the absence of fibrosis or cirrhosis has been associated with SVR<sup>[4,10-12]</sup>. Younger age was associated with SVR in one study<sup>[4]</sup>, but not in others<sup>[12,13]</sup>. Among patients receiving BOC-based triple therapy, younger age and lower fibrosis stage were associated with SVR among treatment-naïve patients, but not among treatment-experienced patients<sup>[4,7]</sup>. A study of veterans receiving either TVR- or BOC-based triple therapy found that failure to achieve SVR was associated with cirrhosis, prior null or partial response, *IL-28B* CT or TT genotype, high baseline viral load, black race, diabetes, high aspartate transaminase (AST) to platelet ratio index (APRI) and FIB-4 scores, low platelet counts, and low LDL cholesterol<sup>[14]</sup>.

TVR- and BOC-based triple therapies cause side effects and adverse events, including anemia, neutropenia, thrombocytopenia, rash, and liver decompensation<sup>[4,13-16]</sup>, which can lead to dose reductions and treatment discontinuations<sup>[4,7,14]</sup>. Several deaths have been reported<sup>[17]</sup>. A key clinical question is which patients should be treated now, and which should be advised to wait for less toxic and more effective therapies. A major concern is that the rate of liver damage may accelerate with age<sup>[18]</sup>. Modeling studies predict that HCV-related morbidity and mortality will increase sharply in the years ahead<sup>[19,20]</sup>. The aging of the HCV-positive population and the age-dependent increase in the risk of HCV-related morbidity and mortality create a need to develop and deploy HCV therapies as safely and effectively as possible.

In real-world clinical practice little is known about the factors associated with SVR rates and relapse with DAA's. This project investigates these factors and provides a benchmark for comparing newer therapeutic agents to the first generation protease inhibitors. We were especially interested in factors that may worsen over time, such liver fibrosis stage and platelet counts.

## MATERIALS AND METHODS

### *Study subjects and methods of data collection*

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the IRB of Mount Sinai (GCO #: 10-0032). The need for informed consent was waived as the work did not alter standard clinical practice. The study group was composed of 223 adults who initiated triple therapy with PEG-IFN/RBV and TVR or BOC (HCV NS3/4A protease inhibitors) at the Mount Sinai Medical Center between May 2011 and March 2012. Providers submitted names of patients and

Mount Sinai's electronic database was queried to capture the complete cohort of patients. The electronic database query identified patients with an ICD-9 code for HCV and a prescription for either TVR or BOC. Medical records were reviewed to verify patients identified through electronic phenotyping. All patients included in the analysis received at least one dose of TVR or BOC. Human immunodeficiency virus-positive patients and patients who previously received a liver transplant were excluded.

Data about the HCV treatment regimen, age, gender, race, body mass index (BMI), *IL28B* genotype, baseline clinical laboratory values [albumin, hemoglobin, AST, alanine transaminase (ALT), platelets, creatinine, ferritin, information to calculate the estimated glomerular filtration rate (eGFR), and alpha-fetoprotein (AFP)], indicators of liver fibrosis, past medical history, including diabetes (indicated by at least one of the following—prescription for metformin, pioglitazone, insulin, a recorded diagnosis of diabetes, fasting glucose above 125 mg/dL, or hemoglobin A1c above 6.5%), depression (indicated by use of at least one of the following medications—wellbutrin, aripiprazole, bupropion, citalopram, venlafaxine, escitalopram, paroxetine, or sertraline), outcome of prior HCV treatment, HCV sub-genotype and viral load were extracted from medical records. On-treatment changes in HCV viral load, ferritin, estimated GFR using the Chronic Kidney Disease Epidemiology Collaboration formula<sup>[21]</sup> were calculated and incident anemia (hemoglobin below 9 g/dL) was recorded.

If available, biopsy or transient elastography data were used to classify liver disease as cirrhosis (yes/no). Cirrhosis was defined as a liver biopsy score of 4 using the Batts-Ludwig system or a transient elastography value > 13.5 kPa. In addition, liver fibrosis stage was estimated from the APRI and the FIB-4 score. APRI scores > 1.5 and FIB-4 scores > 3.25 were coded as advanced fibrosis/cirrhosis. APRI and FIB-4 scores were calculated as follows:  $APRI = \frac{AST (U/L)}{(\text{upper limit of normal})} / [\text{platelet count } (10^9/L) \times 100]^{[22-24]}$ ,  $FIB-4 = \frac{\text{age (years)} \times AST (U/L)}{[\text{platelet count } (10^9/L) \times ALT (U/L)]^{1/2}}^{[25,26]}$ . The FIB-4 score is reliable and has been tested and validated in large HCV mono-infected cohorts<sup>[27]</sup>.

The standard treatment regimens are outlined in supporting Figures 1 (TVR) and 2 (BOC). Patients on TVR received 12 wk of TVR in combination with PEG/RBV. After week 12, TVR was discontinued. PEG/RBV dual therapy was continued for an additional 12 or 36 wk depending on prior treatment response, the presence of cirrhosis, and viral kinetics. With the exceptions noted below, patients on BOC-containing regimens began treatment with 4 wk of PEG-IFN/RBV dual therapy. Then BOC was added and patients received triple therapy for 24 to 44 wk depending on prior treatment response, the presence of cirrhosis and viral kinetics. Eight patients did not receive the standard PEG-IFN/RBV lead-in prior to starting BOC: Six did not have a lead-in phase, one received 19 wk of lead-in, and one received 24 wk of lead-in. Seventy-two patients, 63 on TVR and 9 on BOC, met the eligibility criteria for consideration of response

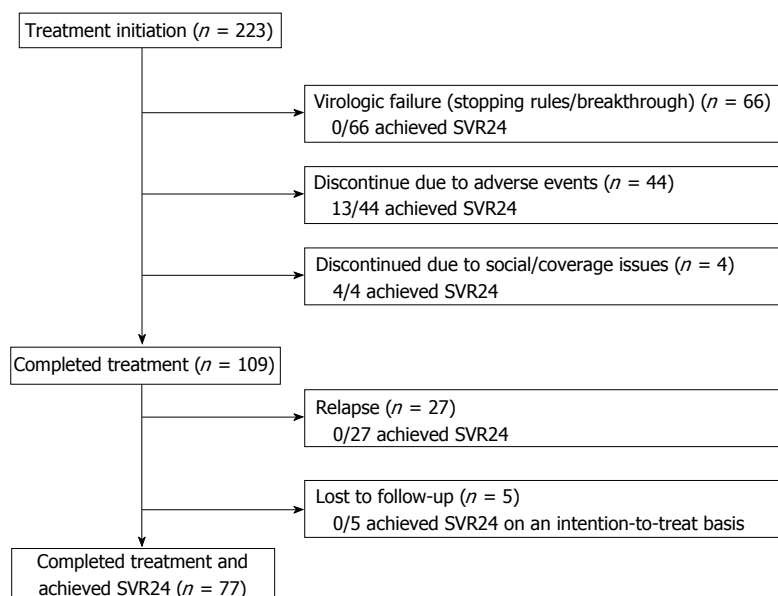


Figure 1 Outcomes of 223 patients initiating telaprevir- or boceprevir-based triple therapy. SVR: Sustained virological response.

guided therapy. Virologic stopping rules were followed.

During treatment, follow-up visits were scheduled for weeks 4, 8, 12, 24, 48, 60 and 72. Anemia was managed at the discretion of the provider. Generally, RBV dose was reduced at hemoglobin levels  $\leq 10$  g/dL. If hemoglobin levels remained low, erythropoietin was administered. Blood transfusions were used to treat intractable anemia<sup>[9]</sup>. Eltrombopag was not used.

SVR was defined as the absence of HCV RNA six months after the end-of-treatment (EOT), as measured by a real-time-polymerase chain reaction assay (Roche Cobas/Ampliprep Cobas Taqman version 2.0, Roche Molecular Systems Inc., Branchburg, NJ, United States). An HCV viral load below the lower limit of detection (18 IU/mL) was recorded as undetectable. Relapse was defined as the detection of HCV RNA after the absence of HCV RNA at EOT. A fast virologic response (FVR) was defined as undetectable HCV RNA 4 wk after the initiation of TVR or 8 wk after the initiation of BOC-based treatment.

### Statistical analysis

Subgroups were compared using *t*-tests or Mann-Whitney tests for continuous variables and  $\chi^2$  or Fisher-exact tests for categorical variables. Pearson correlation tests were used to assess associations between continuous variables. Univariable and multivariable logistic regression were used to analyze the association between the baseline factors listed above and SVR calculated on an intention-to-treat basis. Models for relapse were built using data from patients who completed the planned treatment regimen, had undetectable HCV RNA at the EOT, and who were not lost to follow-up. Factors with a *P*-value below 0.15 in univariable models were included in multivariable analyses; variables with a *P*-value below 0.05 were retained in final models and were considered to be

independently associated with the outcome.

When variables were highly correlated with each other, a series of models was built, each with only one member of the pair. In the case of variables that contained common components (e.g., APRI scores and FIB-4 scores) and variables that represent similar disease characteristics (FIB-4  $\geq 3.25$  and histologically/elastography-defined cirrhosis) only one was entered into a model at a time. Missing data for HCV sub-genotype and *IL28B* polymorphism were imputed as 1a or CT/TT, respectively. A sensitivity analysis was conducted to determine whether variables associated with SVR were specific to one of the two protease inhibitors. Regression analyses were conducted on a dataset containing patients on TVR alone.

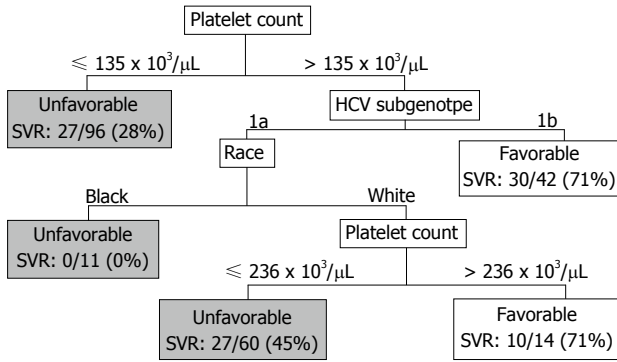
Classification and regression trees (CARTs) were built to identify baseline factors associated with SVR. Factors in the tree included race, cirrhosis (FIB-4 score), diabetes, HCV sub-genotype, *IL28B* genotype, history and previous response to HCV treatment (naïve/relapse vs null/intolerant), and platelet count.

The significance level was set to 0.05. Statistical analyses were conducted with SPSS and SAS.

## RESULTS

### Characteristics of the study group and treatment outcomes

Two complementary approaches were used to identify cases and ensure that the entire cohort of patients meeting the entry criteria were included. The traditional approach (patients identified by their providers) yielded 209 patients. A query of the Mount Sinai data warehouse identified an additional 14 patients, yielding a total of 223 patients whose baseline characteristics are presented in Table 1. One hundred and seventy-two (77%) were treated with TVR and 51 (23%) were



**Figure 2** Classification and regression trees analysis of baseline factors associated with sustained virologic response. SVR: Sustained virologic response; HCV: Hepatitis C virus.

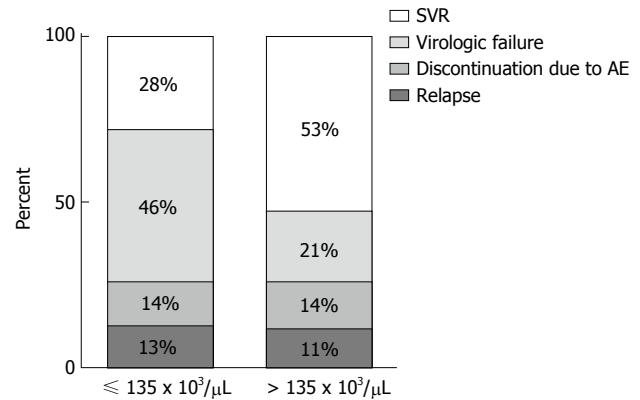
treated with BOC. The patients were predominantly male (65%) with a median age of 57 years (range: 22-74) and a BMI of 27.1 kg/m<sup>3</sup> (range: 16.3-49.3). Eighteen percent were black. Liver biopsy and/or transient elastography data were available for 202/223 (91%) patients of whom 96/202 (48%) had cirrhosis.

Figure 1 diagrams treatment outcomes. Of the 223 patients who initiate treatment, 66 patients had virologic failure during treatment; 44 discontinued treatment early due to adverse events (13 achieved SVR); four discontinued due to social/coverage issues (four achieved SVR); 109 (49%) completed the intended treatment regimen and were viral load negative at the EOT, but 27 relapsed and five did not return for testing at week-24 post EOT and could not be reached after repeated attempts by providers to contact them. On an intention-to-treat basis, the overall SVR rate was 42%. The overall SVR rate was 45% (77/172) for patients on TVR and 33% (17/51) for patients on BOC ( $P = 0.15$ ) in an unadjusted analysis that did not correct for baseline differences. The SVR rates for treatment-naïve, prior non-responders, prior-interferon intolerant patients, and prior relapsers were 41%, 35%, 33% and 62%, respectively ( $P = 0.04$ ).

### Baseline factors associated with SVR

The baseline characteristics of the SVR and non-SVR groups are presented in Supporting Table 1. The SVR group had a significantly higher proportion of patients with the favorable *IL28B* CC genotype, with HCV sub-genotype 1b and higher levels of albumin and platelets, and this group had a significantly lower proportion of blacks, patients with diabetes, liver cirrhosis, a history of non-response to PEG/RBV treatment, lower levels of AFP and AST, and lower APRI and FIB-4 scores. Univariable logistic regression analysis of baseline variables showed *IL28B* CC genotype, higher platelets, higher albumin, HCV sub-genotype 1b, an HCV treatment history that included no prior treatment or relapse were positively associated with SVR and that black race, diabetes, high AST, high AFP, diagnosis of cirrhosis, or and advanced fibrosis/cirrhosis with APRI >1.5 or FIB-4 ≥ 3.25 were negatively associated with SVR (Table 2).

Before building multivariable models, the Pearson's



**Figure 3** Treatment outcome stratified by low and high baseline platelet count. Treatment outcomes of patients with platelets ≤ 13.5 × 10<sup>3</sup>/μL and > 13.5 × 10<sup>3</sup>/μL are shown in a stacked column graph. The SVR rate in the low platelet group was 28% vs 53% in the high platelet group. SVR: Sustained virologic response.

correlation coefficient for pairs of variables was determined to ensure that two highly correlated variables were not included in the same model. The multivariable logistic regression model shown in Table 2 includes the platelet count and excludes variables highly correlated with the platelet count. SVR was positively associated with *IL28B* CC genotype [odds ratio (OR) = 3.54, 95% confidence interval (CI): 1.19-10.53], higher platelet counts (OR = 1.10, per × 10<sup>4</sup> cells/μL, 95%CI: 1.05-1.16), white race (OR = 5.92, 95%CI: 2.34-14.96) and sub-genotype 1b HCV (OR = 2.81, 95%CI: 1.45-5.44). Four additional logistic regression models were built in which the platelet count was excluded and variables that are highly correlated with platelet count were examined individually (Table 2). In all four models white race, sub-genotype 1b, and CC *IL28B* genotype were positively associated with SVR. Variables indicative of more advanced liver disease - lower albumin, higher FIB-4 score, cirrhosis on biopsy/fibroscan - were significantly associated with treatment failure in individual models. A forest plot showing the interaction between treatment history and various baseline characteristics on outcome is presented in Supporting Figure 3.

An additional multivariable logistic regression analysis was conducted on the subgroup of patients receiving TVR-based triple therapy. Results were similar to those for the entire study group. SVR was positively associated with platelets (OR = 1.07 per × 10<sup>4</sup> cells/μL; 95%CI: 1.01-1.14), white race (OR = 5.22; 95%CI: 1.76-15.50), and HCV sub-genotype 1b (OR = 3.27; 95%CI: 1.51-7.07).

### On-treatment factors associated with SVR

Table 3 shows the association between SVR and changes that occurred during treatment. As expected, SVR was strongly associated with viral kinetics. Patients whose HCV viral load decreased rapidly after starting the protease inhibitor and who thus had a FVR were significantly more likely to achieve an SVR ( $P < 0.01$ ). SVR was also significantly associated with a greater decrease in



**Table 1** Baseline characteristics of the study group

	<i>n</i>	Continuous variables: Median (IQR)/categorical variables: <i>n</i> (%)	Range
Telaprevir	223	172 (77)	-
Demographics and anthropometrics			
Age, yr	223	57 (51-61)	22-74
Gender, male	223	144 (65)	-
Race, white	223	182 (82)	-
BMI, kg/m <sup>3</sup> , 17-24: Normal; 25-30: Overweight; > 30: Obese <sup>a</sup>	186	27.1 (24.5-30.7)	16.3-49.3
Past medical history			
Diabetes	223	48 (22)	-
Depression	223	47 (21)	-
<i>IL28B</i> genotype	223		
CC		20 (9)	-
CT		50 (22)	-
TT		21 (10)	-
Unknown		132 (59)	-
Treatment history	223		
Naïve		68 (31)	-
Non-responder		95 (43)	-
Relapser		45 (20)	-
Intolerance		12 (5)	-
Unknown		3 (1)	-
HCV treatment related characteristics			
HCV viral load, log IU/mL	221	6.31 (5.89-6.66)	3.07-7.64
Sub-genotype	223		
1a		118 (53.5)	-
1b		72 (32)	-
1a/1b		1 (0.5)	-
Unknown		32 (14)	-
Laboratory tests			
Hemoglobin, g/dL, female:12-15.5 g/dL; male: 13.5-17.5 g/dL <sup>a</sup>	220	14.3 (13.2-15.3)	9.2-18.2
AFP, ng/mL, 1.6-4.5 ng/mL <sup>a</sup>	158	6.85 (3.58-13.83)	0.80-208.60
Albumin g/dL, 3.5-4.9 g/dL <sup>a</sup>	219	4.20 (3.90-4.50)	2.60-5.30
AST, U/L, 1-50 U/L <sup>a</sup>	221	62 (39-106)	19-324
ALT, U/L, 1-53 U/L <sup>a</sup>	221	67 (44-107)	15-403
Platelets, × 10 <sup>3</sup> /μL, 150-450 × 10 <sup>3</sup> /μL <sup>a</sup>	223	152 (106-195)	14-365
Creatinine, mg/dL, 0.60-1.40 mg/dL <sup>a</sup>	205	0.91 (0.81-1.04)	0.58-10.2
Ferritin, ng/mL, 15-150 ng/mL <sup>a</sup>	54	184 (113-373)	22-893
eGFR, mL/min/1.73 m <sup>2</sup> < 60 mL/min per 1.73 m <sup>2ab</sup>	204	88 (75-99)	6-125
Indication of liver fibrosis			
APRI score	221	0.82 (0.43-1.82)	0.10-12.48
FIB-4 score	221	2.65 (1.77-5.66)	0.35-35.30
FIB-4, advanced fibrosis/cirrhosis <sup>c</sup>	221	98/221 (44)	-
Cirrhosis, biopsy	189	85/189 (45)	-
Transient elastography score, kPa	80	11.8 (7.2-20.3)	3.9-55.1
Cirrhosis, transient elastography <sup>d</sup>	80	36/80 (45)	-
Cirrhosis, transient elastography/biopsy	202	96/202 (48)	-

<sup>a</sup>Normal range; <sup>b</sup>Estimated glomerular filtration rate calculated with epidemiology formula; <sup>c</sup>FIB-4 score ≥ 3.25 kPa; <sup>d</sup>Transient elastography > 13.5 kPa. BMI: Body mass index; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; eGFR: Estimated glomerular filtration rate; APRI: Aspartate transaminase to platelet ratio index.

eGFR in the first 4 wk. Treatment discontinuation due to adverse events was less common in the group that achieved SVR than the group that failed therapy (14% vs 24%), however, this difference was not statistically significant,  $P = 0.06$ . There was a trend of patients achieving SVR to having higher median changes in ferritin levels than patients who failed to achieve SVR (146 ng/mL vs 62 ng/mL,  $P = 0.08$ ).

#### Baseline and on-treatment factors associated with relapse

Overall, 109 patients had an undetectable HCV viral load

at the time when they completed the intended therapy. Five were lost to follow-up. Relapse was confirmed in 27/104 (25%) of the patients who had follow-up data. Among the patients who were viral load negative at the time treatment ended and who later became viral load positive, the median time to confirmed relapse was 12 wk after the EOT (IQR = 5-12 wk, range = 2-34 wk); however, in some cases, relapse may have occurred before the time it was detected because of missed visits. Multivariable logistic regression analysis revealed an association between relapse and HCV sub-genotype 1a (OR = 5.15; 95%CI: 1.40-18.97) and advanced

**Table 2** Univariable and multivariable logistic regression for baseline factors associated with sustained virological response

	Univariable			Multivariable <sup>a</sup>		
	OR	95%CI	P	OR	95%CI	P
Protease inhibitor, telaprevir	1.62	0.84-3.12	0.15	-	-	-
Age, yr	0.98	0.96-1.01	0.23	-	-	-
Gender, male	1.11	0.64-1.94	0.71	-	-	-
Race, white	3.12	1.41-6.90	< 0.01	5.92	2.34-14.96	< 0.01
Diabetes	0.43	0.21-0.87	0.02	-	-	-
Depression	1.58	0.83-3.02	0.17	-	-	-
BMI, kg/m <sup>2</sup>	0.98	0.93-1.04	0.52	-	-	-
IL28B, CC vs CT/TT	3.56	1.31-9.64	0.01	3.54	1.19-10.53	0.02
Treatment history, naïve/relapser	1.86	1.08-3.20	0.03	-	-	-
HCV viral load, log IU/mL	0.79	0.54-1.14	0.21	-	-	-
Sub-genotype, 1b (vs all other)	2.06	1.17-3.65	0.01	2.81	1.45-5.44	0.02
Hemoglobin, g/dL	1.03	0.86-1.23	0.78	-	-	-
AFP, ng/mL	0.95	0.92-0.98	< 0.01	-	-	-
Albumin, g/dL	2.56	1.33-4.92	< 0.01	-	-	-
AST, U/L	0.99	0.99-0.99	0.02	-	-	-
ALT, per U/L	1.00	0.99-1.01	0.61	-	-	-
Platelets, per × 10 <sup>3</sup> /μL	1.08	1.03-1.13	< 0.01	1.10	1.05-1.16	< 0.01
Creatinine, per mg/dL	0.8	0.42-1.53	0.50	-	-	-
Ferritin, per ng/mL	0.99	0.99-1.00	0.63	-	-	-
eGFR, per mL/min per 1.73 m <sup>2</sup>	1.00	0.99-1.02	0.65	-	-	-
APRI	0.84	0.70-1.02	0.08	-	-	-
FIB-4	0.91	0.83-0.99	0.02	-	-	-
APRI > 1.5	0.44	0.24-0.82	0.01	-	-	-
FIB-4 ≥ 3.25	0.39	0.22-0.68	< 0.01	-	-	-
Cirrhosis transient elastography/biopsy	0.5	0.29-0.87	0.01	-	-	-

<sup>a</sup>Model includes platelets and no variable significantly correlated with platelets. BMI: Body mass index; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; eGFR: Estimated glomerular filtration rate; APRI: Aspartate transaminase to platelet ratio index.

**Table 3** Comparison of on-treatment variables in the sustained virologic response and non-sustained virologic response group

	Total cohort Categorical: <i>n</i> (%) Continuous: Median (IQR)	SVR ( <i>n</i> = 94)	Fail to achieve SVR ( <i>n</i> = 129)	P
Discontinuation due to adverse events	44/223 (20%)	13/94 (14%)	31/94 (24%)	0.06 <sup>a</sup>
Change in ferritin from baseline to week 4 of treatment, ng/mL	91 (45-212)	146 (81-331)	62 (-20-175)	0.08 <sup>b</sup>
Development of severe anemia	94/223 (42%)	54/94 (57%)	66/129 (51%)	0.35 <sup>a</sup>
Change in eGFR from baseline to week 4 <sup>c</sup> , mL/min per 1.73 m <sup>2</sup>	-4.41 (-14.87-3.23)	-6.59 (-16.98-0.52)	-1.68 (-13.99-5.00)	0.04 <sup>b</sup>
Fast viral kinetics	139/223 (62%)	83/94 (88%)	56/129 (43%)	< 0.01 <sup>a</sup>

<sup>a</sup>χ<sup>2</sup> test; <sup>b</sup>Mann-Whitney test; <sup>c</sup>Estimated glomerular filtration rate calculated with epidemiology formula. SVR: Sustained virological response; eGFR: Estimated glomerular filtration rate.

fibrosis/cirrhosis (FIB-4 score ≥ 3.25; OR = 2.77; 1.07-7.22) (Table 4). The absence of an FVR (OR = 4.77, 95%CI: 1.68-13.56) was the only on-treatment variable independently associated with relapse.

### Classification and regression tree analysis of factors associated with SVR

A CART analysis underscored the predictive value of the platelet count and was largely consistent with the results of the logistic regression analysis (Figure 2). The strongest baseline predictor was a platelet count > 135 × 10<sup>3</sup> cells/μL. Among patients with platelets > 135 × 10<sup>3</sup> cells/μL, HCV sub-genotype 1b and white race were predictive of SVR. Among patients with platelets > 135 × 10<sup>3</sup> cells/μL and sub-genotype 1a HCV, platelet counts > 236 × 10<sup>3</sup>/μL were predictive of SVR among whites; however, all of the black patients in this subgroup failed

therapy. As illustrated in Figure 3, among patients with platelets > 135 × 10<sup>3</sup>/μL the virologic failure rate was 21% vs 46% in the lower platelet group (*P* < 0.01), and the SVR rate was 53% vs 28% (*P* < 0.01).

## DISCUSSION

This study analyzed treatment outcomes in a real-world cohort of patients treated with TVR- and BOC-based triple therapy by experienced hepatologists. The results are important because TVR-based triple therapy is still used in many parts of the world<sup>[28]</sup>. Our cohort closely resembles the population of HCV-infected individuals in the United States. Twenty-two percent of the United States population is black, 38% have advanced fibrosis/cirrhosis, and the average age is approximately 50 years with 5% above the age of 65 years<sup>[28-31]</sup>. In our cohort,

**Table 4** Univariable and multivariable logistic regression of factors associated with relapse

	Univariable			Multivariable		
	OR	95%CI	P	OR	95%CI	P
Protease inhibitor, Telaprevir	0.41	0.15-1.10	0.08	-	-	-
Age, yr	1.00	0.96-1.05	0.95	-	-	-
Gender, female	1.36	0.53-3.48	0.52	-	-	-
Race, black	1.16	0.28-4.71	0.84	-	-	-
Diabetes	1.86	0.65-5.28	0.25	-	-	-
Depression	0.54	0.17-1.75	0.30	-	-	-
BMI, per kg/m <sup>2</sup>	0.98	0.88-1.10	0.76	-	-	-
<i>IL28B</i> , CC vs CT/TT <sup>a</sup>	-	-	-	-	-	-
Treatment history, naïve/relapser	1.10	0.45-2.72	0.84	-	-	-
HCV viral load, log IU/mL	1.74	0.80-3.78	0.16	-	-	-
Sub-genotype, 1a (vs all other)	6.26	1.75-22.45	0.01	5.15	1.40-18.97	0.01
Hemoglobin, g/dL	1.24	0.88-1.73	0.22	-	-	-
AFP, ng/mL	1.02	0.99-1.05	0.15	-	-	-
Albumin, g/dL	1.02	0.34-3.08	0.97	-	-	-
AST, U/L	1.01	1.00-1.02	0.06	-	-	-
ALT, U/L	1.00	0.99-1.01	0.23	-	-	-
Platelets, × 10 <sup>3</sup> /μL	0.95	0.89-1.02	0.19	-	-	-
Creatinine, mg/dL	1.41	0.72-2.74	0.31	-	-	-
Ferritin, ng/mL	1.00	0.99-1.01	0.26	-	-	-
eGFR, mL/min per 1.73 m <sup>2b</sup>	0.99	0.97-1.02	0.66	-	-	-
APRI score	1.16	0.94-1.43	0.17	-	-	-
FIB-4 score	1.03	0.94-1.13	0.49	-	-	-
APRI > 1.5	2.33	0.87-6.23	0.09	-	-	-
FIB-4 ≥ 3.25	2.77	1.12-6.86	0.03	2.77	1.07-7.22	0.04
Cirrhosis, transient elastography/biopsy	2.55	1.05-6.19	0.04	-	-	-

<sup>a</sup>Zero patients with *IL28B* CC genotype relapsed after completing treatment; <sup>b</sup>Estimated glomerular filtration rate calculated with epidemiology formula. BMI: Body mass index; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein; AST: Aspartate transaminase; ALT: Alanine transaminase; eGFR: Estimated glomerular filtration rate; APRI: Aspartate transaminase to platelet ratio index.

18% were black, approximately half had cirrhosis, and 10% were above the age of 65 years. Our most significant findings were the low 42% SVR rate and the association between treatment failure and factors indicative of more advanced liver disease such as low platelets, cirrhosis, high FIB-4 score, high AFP, and low albumin. CART analysis identified platelet counts > 135 × 10<sup>3</sup>/μL as the strongest baseline predictor of SVR. Seventy-two percent of patients with platelet counts < 135 × 10<sup>3</sup>/μL failed therapy.

Our clinical outcomes are consistent with those of the multicenter CUPIC trial, which examined TVR and BOC in 511 patients with compensated cirrhosis<sup>[32]</sup>. In both studies, non-responders to dual therapy had low SVR rates, whereas prior relapsers had an SVR rate of 62% in our study and 74% in the CUPIC cohort, supporting the use of triple therapy in prior relapsers. Discontinuations due to adverse events and virologic failure were common in both studies. Only 49% of our cohort and 52% of the CUPIC study group completed the planned treatment. The SVR rate in CUPIC was 48%, similar to the 42% in our study ( $P = 0.15$ ). These results highlight the need for more effective therapies.

The association we observed between high platelets and SVR is consistent with published data. The relationship between thrombocytopenia and failure to achieve SVR was demonstrated in ENABLE-1 and ENABLE-2<sup>[33]</sup>, two phase III multicenter randomized controlled trials in which patients on PEG/RBV were either treated with

eltrombopag (a drug that stimulates platelet production) or placebo. In both trials, the SVR rate was significantly higher in the patients receiving eltrombopag: ENABLE-1 (23% vs 14%,  $P < 0.01$ ) and ENABLE-2 (19% vs 13%,  $P = 0.02$ ).

We did not find an association between younger age and SVR. This association was previously reported in a study by Frei and colleagues in which patients < 60 years of age or ≥ 60 years of age were matched on gender, cirrhosis, HCV genotype, and prior treatment response<sup>[34]</sup>. Further studies are needed to clarify the relationship between age and SVR among patients receiving triple therapy.

In our study, relapse occurred in about one-quarter of the patients who completed treatment and had an EOT. Relapse is emerging as the most common cause of non-SVR with newer DAAs. Sofosbuvir is an NS5B inhibitor that recently received FDA approval. In clinical trials, 9% of patients treated with sofosbuvir and PEG/RBV relapsed and 22% of patients treated with sofosbuvir and RBV relapsed<sup>[35,36]</sup>. Simeprevir is an NS3/4A protease inhibitor that recently received FDA approval. In clinical trials of simeprevir, relapse occurred in 11% of treatment-naïve and 18.5% of treatment-experienced patients<sup>[37]</sup>. In our study, relapse was related to both viral and host factors. HCV sub-genotype 1a and advanced fibrosis/cirrhosis (FIB-4 ≥ 3.25) were independently associated with relapse. Additionally, the absence of FVR was also associated with relapse, consistent with

results of dual therapy<sup>[37]</sup>. Recent data suggest that viral double-stranded RNA may play a role in relapse<sup>[38]</sup>. Understanding the molecular basis of relapse may allow the development of drugs that specifically target the processes underlying this type of treatment failure.

The strengths of our study include the complementary methods used to ensure that the entire cohort of patients was included, use of a study group with a high percentage of blacks, older patients and patients with advanced liver disease who were treated in real-world clinical practice, and use of two methods - multivariable logistic regression and CART analysis - to identify factors associated with treatment outcome. The limitations include the relatively small number of patients on BOC, and the observational study design, which limits the ability to compare the two protease inhibitors to each other.

Unless and until treatments are available that greatly reduce the risk of hepatocellular carcinoma in patients who have advanced liver disease, it will be essential to treat patients before extensive liver damage has occurred, as noted by others<sup>[36]</sup>. Several newly approved DAAs are available for HCV-infected patients including: Simeprevir, sofosbuvir, and the combination of ombitasvir, paritaprevir with ritonavir, and dasabuvir. In clinical trials, these compounds achieved high SVR rates; however, their effectiveness in the real-world is unknown and needs to be determined. This study provides a standard against which new therapies can be objectively evaluated.

## COMMENTS

### Background

Hepatitis C virus (HCV) infects 3% of the world's population. In the United States, HCV chronically infects an estimated 2.7 to 3.9 million people and is a leading cause of liver disease. Prior to 2011, treatments for HCV involved pegylated-interferon and ribavirin and had low rates of success. New drugs for HCV-infection, called direct-acting antivirals (DAAs), were first approved in 2011 and provided higher rates of success with lower adverse event rates. Understanding the effectiveness of these DAAs bears relevance for future clinical trials and simulation studies. This paper successfully analyzed a real-world cohort of patients treated with telaprevir- and boceprevir-based therapies to provide better information on cohorts using this class of therapies in the future.

### Research frontiers

NS3/4A protease inhibitors are a relatively new class of therapies available to treat HCV-infection. This paper investigates the effectiveness of the first two FDA-approved protease inhibitors for HCV. Understanding the characteristics associated with successful treatment in HCV-infected patients is critical for the ongoing treatment of the HCV-infected population.

### Innovations and breakthroughs

The authors used traditional and machine learning methods to identify factors associated with achieving a sustained virologic response in HCV-treatment. The authors found that factors indicative of more advanced fibrosis or cirrhosis were associated with lower rates of sustained virologic response. They also found that specific cut-off points in platelet count that could be used to risk-stratify patients. The results imply that treating patients before they develop more advanced disease is an important message that should be relayed to care-providers.

### Applications

The results could help improve design of future clinical trials for NS3/4A protease

inhibitors for HCV. The population contained individuals with characteristics that were lacking in clinical trials, offering unique information on the effectiveness of NS3/4A protease inhibitors in this population. Currently, numerous protease inhibitors are on the market for HCV. Understanding the risk profile of this class of therapies and the factors associated with their risks and benefits could lead to improvements. Additionally, this real-world study could provide some additional information for future simulation studies to use.

### Terminology

Sustained virologic response - the absence of HCV RNA 12-24 wk after the end-of-treatment. Considered a cure in HCV-treatment. Direct-acting antivirals (DAAs) - a class of therapies that directly target HCV and prevent replication. DAAs are composed of NS3/4A protease inhibitors, nucleos(t)ide and non-nucleos(t)ide NS5B Polymerase Inhibitors, and NS5A inhibitors.

### Peer-review

This study will give some useful information to clinicians and provided a successful ways to evaluate the new medications. The manuscript read and organized well, and the data treated were also reasonable.

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## 18-Fluoro-deoxyglucose uptake in inflammatory hepatic adenoma: A case report

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### Abstract

Positron emission tomography computed tomography (PET-CT) using 18-Fluoro-deoxyglucose ( $^{18}\text{F}$ FDG) is an imaging modality that reflects cellular glucose metabolism. Most cancers show an uptake of  $^{18}\text{F}$ FDG and benign tumors do not usually behave in such a way. The authors report herein the case of a 38-year-old female patient with a past medical history of cervical intraepithelial neoplasia and pheochromocytoma, in whom a liver lesion had been detected with PET-CT. The tumor was laparoscopically resected and the diagnosis of inflammatory hepatic adenoma was confirmed. This is the first description of an inflammatory hepatic adenoma with an  $^{18}\text{F}$ FDG up-take.

**Key words:** Liver surgery; Liver tumor; Liver cancer; Benign tumor; Laparoscopy; Prognosis

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**Core tip:** In cancer therapy, the use of 18-Fluoro-deoxyglucose ( $^{18}\text{F}$ FDG) positron emission tomography computed tomography as a staging or prognostic tool, is increasing. This is also the case for primary or secondary

liver cancer. In this paper, the authors report the first description of an inflammatory hepatic adenoma with <sup>18</sup>FDG uptake.

Liu W, Delwaide J, Bletard N, Delvenne P, Meunier P, Hustinx R, Detry O. 18-Fluoro-deoxyglucose uptake in inflammatory hepatic adenoma: A case report. *World J Hepatol* 2017; 9(11): 562-566 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i11/562.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i11.562>

## INTRODUCTION

Hepatocellular adenomas (HCAs) are rare benign hepatic tumors that are more frequent in women and have been associated with oral contraceptive use<sup>[1]</sup>. The risk of malignant transformation of HCAs is small but non-negligible<sup>[2]</sup>. The commonest complication of HCAs is bleeding, an occurrence which has been linked to multiple factors such as the size of the adenoma, pregnancy, visualization of lesional arteries, left lateral lobe location and exophytic growth. Due to these risks, recent guidelines have recommended the resection of adenomas that present: A diameter larger than 50 mm, signs of hepatocarcinoma or focal dysplasia, activated  $\beta$ -catenin mutation, high level of serum alfafoetoprotein, hepatocellular adenomas developing in male gender or hepatocellular adenomas developing in a glycogen storage disease<sup>[3]</sup>. The resection is regularly performed as laparoscopic hepatectomy<sup>[4]</sup>. Positron emission tomography computed tomography (PET-CT) using 18-Fluoro-deoxyglucose (<sup>18</sup>FDG) is an imaging modality that is based on an enhancement of glucose consumption, a distinguishing feature of most cancers that is in part related to the over-expression of GLUT-1 glucose transporters and increased hexokinase activity. The use of PET-CT in primary or secondary liver cancer is increasing<sup>[5,6]</sup>. As HCAs are benign lesions, they are not assumed to be <sup>18</sup>FDG-avid, except in some rare cases. To the best of their knowledge, the authors described herein the first report of <sup>18</sup>FDG uptake by an inflammatory HCA (I-HCA), and reviewed the literature for other reports of <sup>18</sup>FDG uptake in other types of liver adenoma.

## CASE REPORT

A 38-year-old female patient had a past medical history of cervical intraepithelial neoplasia treated with cervical conisation, and a pheochromocytoma that was laparoscopically resected in 2011. She was followed up with yearly magnetic resonance imaging (MRI) that demonstrated a segment 1 liver tumor whose size increased of 20 mm in two years. This 50-mm lesion bore the MRI features of HCA, showing a heterogeneous signal intensity on T-2 weighted images and low-signal intensity on T-1 weighted images. The lesion was slowly and gradually enhanced after injection of gadolinium without significant

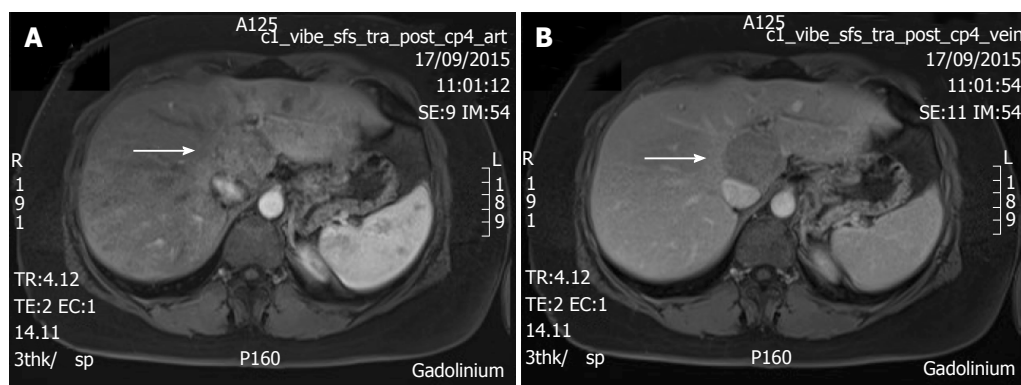
wash-out on portal phase (Figure 1). In addition, a left renal cyst was noticed, described as type 3 according to the Bosniak classification. An <sup>18</sup>FDG PET-CT (Figure 2) was performed to further confirm the nature of the hepatic lesion and exclude extrahepatic metastases. The liver lesion appeared hypermetabolic with a standardized uptake value (SUVmax) of 9.3. A percutaneous biopsy was performed and immunohistology allowed the diagnosis of I-HCA. Blood carcinoembryonic antigen, carbohydrate antigen 19.9 and alfafoetoprotein were negative. A discussion in a multi-disciplinary oncological team meeting led to the decision of the resection of the hepatic lesion. A laparoscopic resection of hepatic segment 1 was performed, extended to segments 2 and 3 due to the location of the tumor at the junction between the inferior vena cava, the left and middle hepatic veins and the left branch of the portal vein. During the same anesthesia, the left kidney mass was resected through a lombotomy, following the preferences of the urologist. The surgical specimen was analyzed and showed slightly clarified hepatocytes scattered throughout the lesion, fibrous tracts with vascular structures within, probably arteries with thick walls (Figure 3). Some inflammatory components surrounded these arteries and there was no significant sinusoidal dilatation. At immunohistochemistry, serum amyloid A was negative and anti-C reactive protein antibodies showed a significant expression of the inflammatory protein around blood vessels, confirming I-HCA (Figure 4). Inflammatory cells were CD3 positive (Figure 5). The immediate post-operative state was excellent, without significant pain and fast oral feeding. The length of hospital stay was 5 d. The patient was seen again one month later for an evaluation visit and no particular problems were observed.

## DISCUSSION

This report describes the occurrence of a 50-mm I-HCA that was highly avid for <sup>18</sup>FDG at PET-CT. The exact nature of this I-HCA was confirmed by surgical resection. To the best of the authors' knowledge, this is the first report of <sup>18</sup>FDG uptake by an I-HCA. HCAs are classified into four types, according to their genetic and histologic features (Table 1): HNF1 $\alpha$  inactivated HCA (H-HCA),  $\beta$ -catenin mutated HCA ( $\beta$ -HCA), I-HCA and unclassified HCA<sup>[7,8]</sup>. The actual risk of malignancy of all HCAs is evaluated at 4.2%<sup>[2,3]</sup>. The  $\beta$ -HCA subtype is associated with the highest risk of malignant transformation and must be resected (Table 1). After literature review, the authors found 22 other HCA cases with <sup>18</sup>FDG uptake in PET-CT<sup>[9-19]</sup> (Table 2), and none of them was the inflammatory type. Eighteen of them have a description of the histological findings with steatosis. Twelve reported a final diagnosis, which was either HNF1 $\alpha$  or hepatic adenomatosis.

The uptake of <sup>18</sup>FDG results from the increased metabolism of the cell. The intracellular FDG accumulation is proportional to the amount of glucose utilization<sup>[20]</sup> and most cancers do have increased cellular activity.

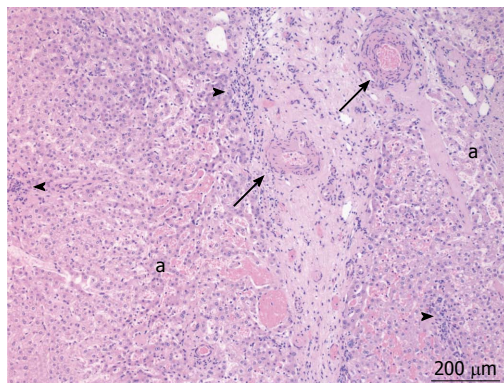




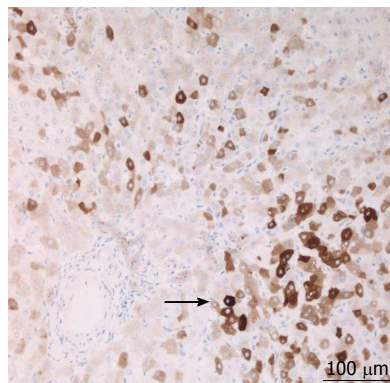
**Figure 1** T1 weighted magnetic resonance imaging with gadolinium injection, showing a 50-mm tumor in segment 1 (arrow). A: Arterial phase; B: Portal venous phase.



**Figure 2** Positron emission tomography computed tomography using 18-fluoro-deoxyglucose showing the 18-fluoro-deoxyglucose avidity of the segment 1 tumor. A: PET; B: CT; C: PET-CT fusion. PET: Positron emission tomography;  $^{18}\text{F}$ FDG: 18-fluoro-deoxyglucose; CT: Computed tomography.



**Figure 3** Pathology of the tumor that contains thickened arteries (arrows), inflammatory infiltrate (arrowheads), sinusoidal dilatation (a) (hematoxylin-eosin stain).



**Figure 4** Immunohistochemistry with anti-C reactive protein antibodies, positive in the adenomatous hepatocytes (arrow), confirming inflammatory hepatocellular adenoma.

The differential diagnosis of benign  $^{18}\text{F}$ FDG avid hepatic lesions might include focal steatosis, infectious, parasitic or inflammatory processes (e.g., hepatic abscess, cryptococcal infection, hepatic tuberculoma) and hepatic adenoma<sup>[21,22]</sup>. Focal fatty infiltration has been reported to be PET-avid<sup>[23]</sup>. In fact, as a response to fat accumulation, a subacute inflammatory hepatic reaction with infiltration of activated Kupffer cells may occur, resulting in a higher SUVmax than adjacent normal liver parenchyma. As

said above, five cases of hepatic adenoma showed fatty changes but none of them were of the inflammatory type. Only one had a few inflammatory infiltrates. Maybe the fatty change itself was sufficient enough to induce a PET-avid response, without obvious inflammatory infiltrate in histological examination. It is also possible, as suggested by Nakashima *et al.*<sup>[14]</sup>, that the high expression of glucose transporters might be responsible for the increased uptake. Indeed, one study demonstrated that in H-HCA the

**Table 1** Classification of hepatocellular adenomas

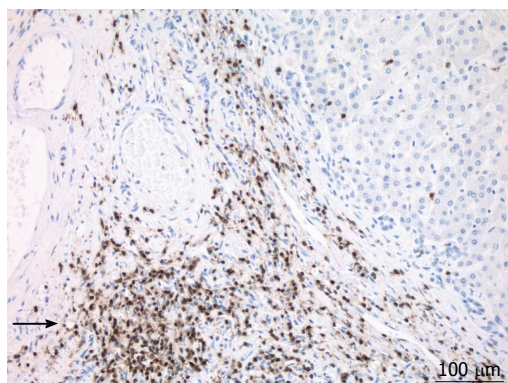
HCA subtype	Abbreviation	Proportion	Markers	Malignant transformation
HNF1 $\alpha$ inactivated	H-HCA	35%-40%	LFABP	Rare
$\beta$ -catenin activated	$\beta$ -HCA	10%	$\beta$ -catenin <sup>+</sup> /GS <sup>+</sup> activated	Yes
Inflammatory	I-HCA	50%	CRP <sup>+</sup>	No
Unclassified	U-HCA	5%	None	No

HCA: Hepatocellular adenoma.

**Table 2** Cases of 18-fluoro-deoxyglucose-avid hepatocellular adenomas reported in literature

Ref.	Gender	Age (yr)	Size (mm)	SUVmax	Diagnosis
[7]	Female	41	10	NA	HCA
[8]	Female	37	33	5	H-HCA
[9]	NA	44	30	6.2	HCA
[10]	Female	52	NA	4.09-9.8	Hepatic adenomatosis
[11]	Female	65	30	NA	Necrotic HCA
[12]	Male	69	40	10.4	H-HCA
[13]	4 cases	NA	73 $\pm$ 15	6 $\pm$ 0.5	HCA
[14]	Female	34	20-30	3.9	HCA
[15]	Male	73	25	11.9	Fatty liver
[16]	Female	44	23	7.9	H-HCA
[17]	9 cases	49 $\pm$ 16	27 $\pm$ 15	8.2 $\pm$ 4.3	H-HCA
This case	Female	38	50	9.3	I-HCA

HCA: Hepatocellular adenoma; <sup>18</sup>FDG: 18-fluoro-deoxyglucose; H-HCA: HNF1 $\alpha$  inactivated HCA; I-HCA: Inflammatory HCA; NA: Not available.



**Figure 5** Immunohistochemistry with anti-CD3 antibodies, positive in the inflammatory cells (arrow).

LFABP gene ablation significantly increased the *in-vitro* expression of GLUT-2 but not that of GLUT-1<sup>[24]</sup>. Another study demonstrated that HNF1 $\alpha$ -inactivated HCAs activate glycolysis due to a strong up-regulation of glucokinase<sup>[25]</sup>. These two components are features of most cancers (rise of GLUT-1 and hexokinase activity) with features of H-HCA (rise of GLUT-2 and glucokinase). However, due to the few reports published in literature, no conclusion can be made on the risk of cancer development in HCA with uptake of <sup>18</sup>FDG. Prospective and large series are needed to confirm the role of PET-CT in HCA evaluation and prognosis.

## COMMENTS

### Case characteristics

A 5-cm liver tumor was diagnosed in a 38-year-old woman.

### Clinical diagnosis

This tumor was asymptomatic and described at follow-up imaging after surgical resection of a pheochromocytoma.

### Differential diagnosis

Adenoma, hepatocellular carcinoma, other primary or metastatic hepatic tumors.

### Laboratory diagnosis

Blood tumor markers, and particularly alphafoetoprotein, were negative.

### Imaging diagnosis

Magnetic resonance imaging was compatible with hepatocellular adenoma, but the lesion was 18-fluoro-deoxyglucose (<sup>18</sup>FDG) avid at positron emission tomography computed tomography (PET-CT).

### Pathological diagnosis

Percutaneous biopsy and surgical specimen conformed inflammatory hepatocellular adenoma (I-HCA).

### Treatment

Laparoscopic liver R0 resection.

### Related reports

To the authors' knowledge, this case is the first report of a PET-CT FDG-avid I-HCA.

### Term explanation

Hepatocellular adenomas are benign liver lesions whose imaging diagnosis could be uncertain.

### Experiences and lessons

PET-CT positivity is not necessary linked to cancerous degeneration in liver adenomas.

## Peer-review

This paper reported a case of PET-avid hepatocellular adenomas and reviews related literature to show variety cause of PET-avid HCA.

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## Emerging concepts in alcoholic hepatitis

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### Abstract

Severe alcoholic hepatitis is implicated as a costly,

worldwide public health issue with high morbidity and mortality. The one-month survival for severe alcoholic hepatitis is low with mortality rates high as 30%-50%. Abstinence from alcohol is the recommended first-line treatment. Although corticosteroids remain as the current evidence based option for selected patients with discriminant function > 32, improvement of short-term survival rate may be the only benefit. Identification of individuals with risk factors for the development of severe alcoholic hepatitis may provide insight to the diverse clinical spectrum and prognosis of the disease. The understanding of the complex pathophysiologic processes of alcoholic hepatitis is the key to elucidating new therapeutic treatments. Newer research describes the use of gut microbiota modification, immune modulation, stimulation of liver regeneration, caspase inhibitors, farnesoid X receptors, and the extracorporeal liver assist device to aid in hepatocellular recovery. Liver transplantation can be considered as the last medical option for patients failing conventional medical interventions. Although the preliminary data is promising in patients with low risk of recidivism, controversy remains due to organ scarcity. This review article comprehensively summarizes the epidemiology, pathophysiology, risk factors, and prognostic indicators of severe alcoholic hepatitis with a focus on the current and emerging therapeutics.

**Key words:** Immune modulation; Alcoholic hepatitis; Gut microbiota modification; Extracorporeal liver assist device; Apoptosis inhibitors

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**Core tip:** Current research of alcoholic hepatitis pathophysiology *via* translational research has provided insight to novel therapeutic options. Recovery from severe alcoholic hepatitis with assistance of gut microbiota modification, immune modulators, stimulation of liver regeneration, caspase inhibitors, farnesoid X receptors, and extracorporeal liver assist device may be promising.

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## INTRODUCTION

Alcoholic hepatitis (AH), is one of the most severe manifestations of alcoholic liver disease. It is a public health issue and worldwide disease associated with high morbidity and mortality. Complications related to alcoholic liver disease result in costly hospitalizations. Current treatment strategies are limited. Abstinence is the first line treatment, however may not improve outcomes in patients with severe AH, defined as discriminant function  $> 32$ . The mainstay of therapy is corticosteroids, which have limited efficacy in specific populations. Pursuit of new treatment options for alcoholic hepatitis is the holy grail for patients ineligible or refractory to corticosteroids. The judicious use of early liver transplantation for severe alcoholic hepatitis has been explored although medical and ethical controversy remains. Exploration of maximal medical management with microbiota modification, immune modulation, liver regenerative factors, farnesoid X receptors (FXRs), caspase inhibitors, and extracorporeal liver assist device (ELAD) may be promising for patients with severe alcoholic hepatitis who do not have other options.

Sixty percent of the United States' population reports alcohol consumption<sup>[1]</sup>. Approximately 8%-10% of the United States population reports heavy alcohol use, which is defined as  $\geq 2$  drinks daily in men and  $\geq 1$  drink daily in women<sup>[2]</sup>. One standard drink contains approximately 14 g of alcohol, which is equivalent to 12 ounces (350 mL) of beer (4%-5% wt/vol), 6 ounces (177 mL) of wine (8%-10% wt/vol), and 2 ounces (59 mL) of hard liquor or whiskey (45% wt/vol)<sup>[1]</sup>. There are progressive and co-existing stages of disease in chronic alcoholism including steatosis, steatohepatitis, fibrosis, and development of compensated to decompensated cirrhosis. In a study examining hospitalized heavy alcohol drinkers with and without alcohol withdrawal, liver biopsies reveal steatosis in 44.9%, alcoholic hepatitis in 34.4%, liver cirrhosis with superimposed alcoholic hepatitis in 10.2%, and cirrhosis only in 10.5%<sup>[3]</sup>. In other studies, approximately 20% of individuals with chronic alcohol abuse are found to have AH when biopsied<sup>[4]</sup>.

Alcoholic hepatitis is an acute-on-chronic presentation of liver disease with a wide ranging spectrum of mild to florid, life-threatening injury<sup>[5]</sup>. It is a clinical syndrome associated with recent onset jaundice and coagulopathy in a person who has been a heavy drinker usually for more than a decade<sup>[6]</sup>. Although long standing alcohol abuse appears to be associated with the development of AH, the exact trigger for development is unclear. Other factors, such as environmental and genetic variables may play a pivotal role. The amount and duration of alcohol abuse needed to produce alcoholic hepatitis is variable depending on the individual patient. Alcohol consumption

of approximately 40 g daily for women and 50-60 g daily for men is recognized as a minimal threshold amount for patients at high risk of developing AH. Alcohol consumption is usually within less than 60 d prior to onset of jaundice with heavy alcohol use for more than 6 mo for severe alcoholic hepatitis clinical trial inclusion criterias<sup>[7]</sup>.

It has been reported that chronic alcohol abuse and binge drinking are associated with development of liver disease<sup>[8,9]</sup>. Binge drinking is defined as five or more drinks in men and four or more drinks in women within a period of approximately 2 h at least once a week<sup>[10]</sup>. Earlier studies implied that weekly binge drinking may be more deleterious than daily consumption of alcohol<sup>[2]</sup>. More recent studies suggest daily heavy drinkers had increased mortality from liver disease compared to binge drinkers<sup>[11]</sup>. It has been reported that the combination of chronic alcohol use with a binge drinking pattern may be more detrimental as animal studies showed mice with chronic ethanol fed diet with an addition of single high dose ethanol administration expressed more severe forms of liver injury and steatosis compared to animals with chronic ethanol feeding alone or single high dose of ethanol only<sup>[12]</sup>. Further studies are needed to delineate the pathophysiology of binge drinking and its' effects on alcoholic hepatitis.

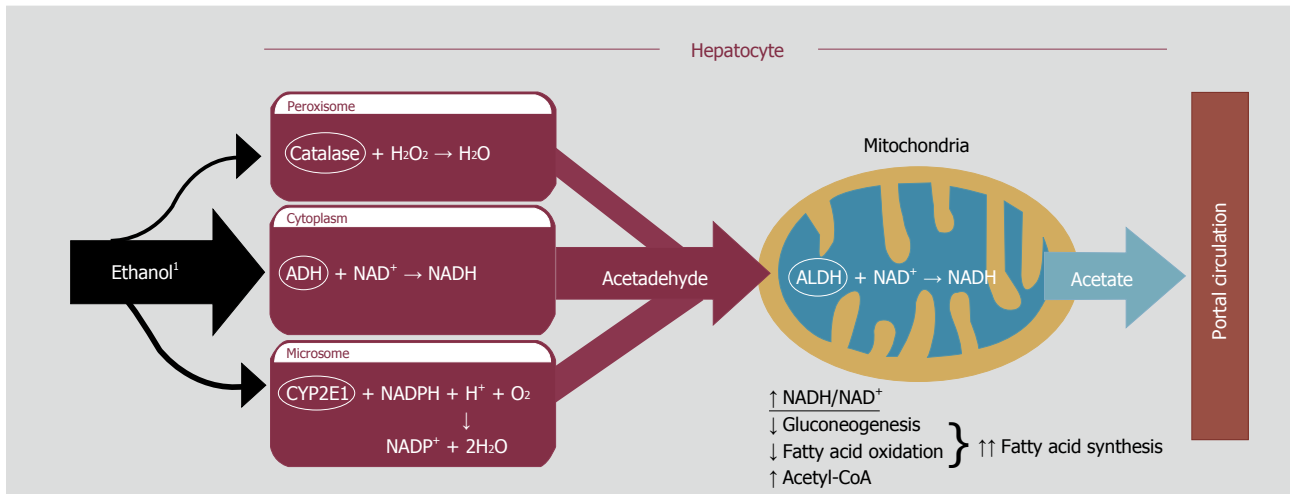
The true incidence of alcoholic hepatitis is unknown. Based on Denmark studies from 1999-2008, the annual incidence rate of alcoholic hepatitis was 46 per 1000000 in men and 34 per 1000000 in women<sup>[13]</sup>. In the United States, alcoholic hepatitis accounted for 325000 admissions annually in 2010 with average hospitalization cost of \$46264. The most common admitting diagnosis for patient hospitalized with AH was hepatic encephalopathy<sup>[14]</sup>.

## PATHOPHYSIOLOGY

The pathogenesis of liver disease related to alcohol consumption is not completely elucidated. Most studies simulating alcoholic hepatitis are recreated in animal models using an alcohol and fat infusion method<sup>[15]</sup>. The etiology of alcoholic hepatitis is complex and multifactorial. Principal factors include steatosis, oxidative stress, altered gut permeability, toxic metabolites, and formation of cytokines result in the initiation of an inflammatory cascade.

Ethanol is oxidized by three metabolic pathways: (1) alcohol dehydrogenase mainly; (2) cytochrome P450 2E1; and (3) catalase (Figure 1) Ten percent of ethanol oxidation occurs in the microsomal cytochrome P450 CYP2E1. Ethanol catalase driven reaction in the liver peroxisome is negligible<sup>[16]</sup>.

Ethanol is metabolized into acetaldehyde *via* the cytosolic alcohol dehydrogenase enzyme within hepatocytes. Acetaldehyde is converted into acetate and reduced nicotinamide adenine dinucleotide (NADH) *via* mitochondrial and cytosolic aldehyde dehydrogenase<sup>[17]</sup>. NADH is increased as a byproduct of ethanol metabolism.



**Figure 1 Ethanol metabolism in the hepatocyte.** <sup>1</sup>Ethanol inhibits the peroxisome-proliferator-activated receptor  $\alpha$  and adenosine monophosphate activated protein kinase with stimulation of sterol regulatory element binding protein 1, a membrane bound transcript factor to promote lipogenesis. ADH: Alcohol dehydrogenase; ALDH: Aldehyde dehydrogenase; NADH: Nicotinamide adenine dinucleotide.

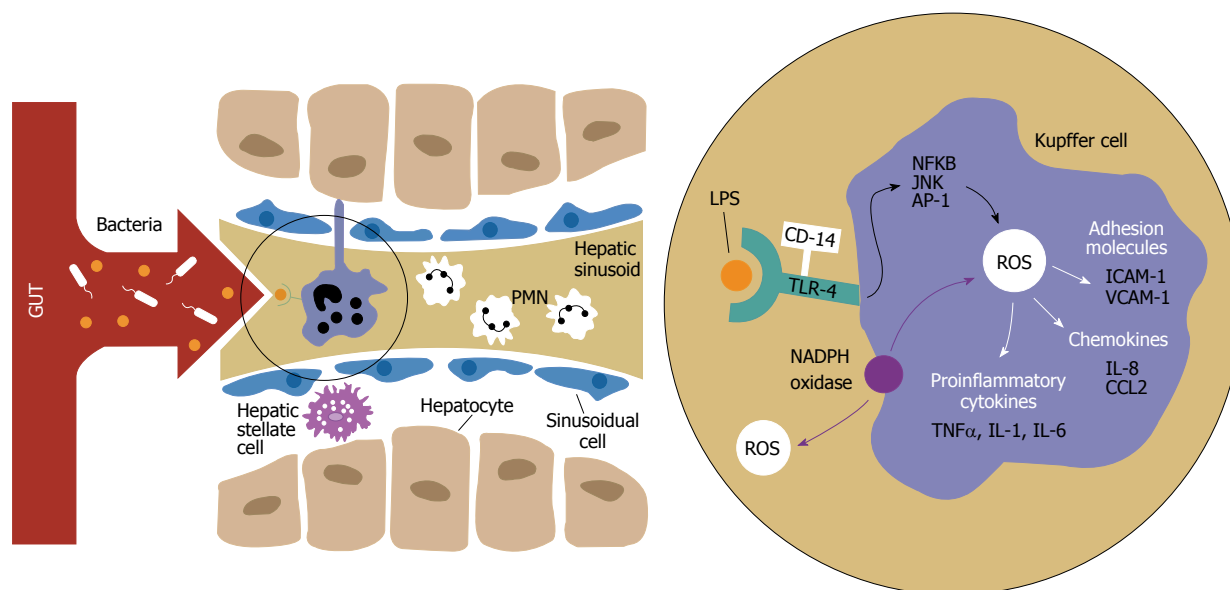
Elevated NADH/NAD<sup>+</sup> levels inhibit gluconeogenesis and fatty acid oxidation and is responsible for the high amounts of acetyl-coA found in heavy alcohol users<sup>[18]</sup>. Acetyl-coA induces fatty acid synthesis by serving as a precursor for fatty acid and cholesterol biosynthesis<sup>[19]</sup>. In addition, ethanol inhibits the peroxisome-proliferator-activated receptor  $\alpha$  and adenosine monophosphate activated protein kinase with stimulation of sterol regulatory element binding protein 1, a membrane bound transcription factor to promote lipogenesis<sup>[20-22]</sup>.

Acetaldehyde is direct hepatotoxin and a known carcinogen<sup>[23]</sup>. Acetaldehyde form adducts that are potent immunogens to activate inflammatory cytokines<sup>[24,25]</sup>. The production of reactive oxygen species inducing lipid peroxidation with additional cytotoxic effects of ethanol metabolism induce hepatocyte necrosis<sup>[26]</sup>. Damage-associated molecular patterns are produced after cell necrosis, which trigger inflammation, fibrosis, and abnormal hepatocyte regeneration<sup>[27]</sup>. After chronic ethanol consumption, the activity of the microsomal ethanol-oxidizing system increases by 5-10 fold, with an associated rise in cytochrome P-450, CYP2E1. CYP2E1 metabolism increases reactive oxygen species and acetaldehyde production, which diminishes hepatoprotective reduced glutathione and other defense systems leaving hepatocytes to be more vulnerable to oxidative stress<sup>[28,29]</sup>.

The endoplasmic reticulum (ER) regulates protein folding, maturation, misfolded protein degradation, and regulation of new protein entry<sup>[30]</sup>. When proteins are misfolded in the ER, the unfolded protein response is sensed by the binding immunoglobulin protein/glucose regulated protein 78 (GRP 78). This reaction produces oxidative stress and disassociation of the endoreticulum transmembrane transducers. The transducers are responsible for the activation and recruitment of c-Jun N-terminal (JNK), a stress kinase<sup>[31]</sup>. Multiple mechanisms, including downstream inflammation and increased oxida-

tive ER stress from hyperhomocysteinemia activates nuclear factor kappa beta (NFkB) and JNK to induce hepatocyte apoptosis *via* caspase activation<sup>[32,33]</sup>. Deficiencies of B vitamins or homocysteine metabolism mutations seen in chronic ethanol use cause accumulation of homocysteine, which induces the ER stress of the hepatocytes and vascular endothelial cells. In addition, ER stress is associated with fatty acid synthesis *via* the activation of SREBPs (sterol regulatory element-binding proteins), which enhance cholesterol and triglyceride biosynthesis and fibrosis *via* stellate cell activation<sup>[34,35]</sup>.

Ethanol induces gut dysbiosis and alters the permeability<sup>[36]</sup>. Increased gut permeability allows the endotoxins to infiltrate the liver through the portal vein<sup>[37]</sup> (Figure 2). Endotoxin levels are measured to be high in patients suffering from alcoholic hepatitis<sup>[38]</sup>. Bacterial lipopolysaccharide, an endotoxin, binds to the lipopolysaccharide binding protein to form a complex. The complex latches to the CD-14 molecule to activate Kupffer cells and macrophages *via* the toll-like receptor type 4 (TLR-4)<sup>[39]</sup>. This reaction stimulates mitogen-activated protein kinases [such as extracellular signal-regulated kinase (ERK-1/ERK-2), JNK and p38], NFkB, and activator protein 1 (AP-1). Reactive oxygen species produced by Kupffer cells cause the recruitment of adhesion molecules [intracellular adhesion molecule 1 and vascular adhesion protein 1, chemokines (IL-8 and C-C motif chemokine ligand 2), and inflammatory cytokines (tumor necrosis factor- $\alpha$ , IL-1 and IL-6)<sup>[40]</sup>. The enhanced inflammatory T-helper-type 1 (TH1) response to alcohol dehydrogenase in alcoholic hepatitis induces additional neutrophil recruitment<sup>[41,42]</sup>. Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase is an additional contributor to ROS<sup>[6]</sup>. Pro-inflammatory cytokine, IL-17 induces the migration of neutrophils into the hepatocytes and stimulates the hepatic stellate cells to produce IL-8 and chemokine CXC motif ligand 1 (CXCL1), which recruit other chemokines to attract other



**Figure 2** Acetaldehyde induced gut permeability with endotoxemia and inflammatory cascade. LPS: Lipopolysaccharide; TLR-4: Toll-like receptor type 4; ROS: Reactive oxygen species; NADPH: Nicotinamide adenine dinucleotide phosphate-oxidase; NFKB: Nuclear factor kappa beta; JNK: c-Jun N-terminal kinase; AP-1: Activator protein 1; ICAM-1: Intracellular adhesion molecule 1; VCAM-1: Vascular adhesion protein 1; IL-8: Interleukin 8; CCL2: C-C motif ligand 2; TNF $\alpha$ : Tumor necrosis factor alpha; IL-1: Interleukin 1; IL-6: Interleukin 6.

neutrophils<sup>[43]</sup>. IL-22 is stimulated by increased levels of IL-6 and TNF- $\alpha$ . Although IL-22 is produced by TH17, TH22 and natural killer cells, its receptor is mainly found in hepatocytes. It has a hepatoprotective effect against liver injury and secreted in parallel, to counteract the effects of IL-17<sup>[12]</sup>.

Peripheral neutrophilia is a characteristic finding in alcoholic hepatitis<sup>[44]</sup>. Normally, neutrophils are recruited to aid in tissue repair and recovery<sup>[45]</sup>. The innate immunity is impaired in patients with progressive liver dysfunction, contributing to multi-organ failure seen in patients with severe alcoholic hepatitis. Serum analysis of acute alcoholic hepatitis patients compared to patients with alcoholic cirrhosis and healthy controls show a significant reduction in antibacterial innate and adaptive immune responses. An impaired T cell response from AH patients produces fewer interferon gamma when exposed to lipopolysaccharide with impaired neutrophil phagocytosis and defective monocyte oxidative burst when stimulated by bacterial challenge. Defective monocyte oxidative burst reduces the expression of NADPH oxidase, which is responsible for generation of superoxide radicals required for bacterial killing. Higher rates of infection in AH may be explained by this impairment<sup>[46]</sup>. The T cells of AH patients exhibit increased numbers of PD ligand 1 (PD1), T-cell immunoglobulin and mucin domain 3 (TIM3), and galectin-9, which are ligands responsible for programmed cell death functioning. The blockade of the PD1 and TIM3 can restore the innate and adaptive immunity by increasing T cell and neutrophil antimicrobial activity<sup>[47]</sup>.

Other aldehydes produced along with acetaldehyde contribute to progressive hepatic fibrosis by inducing collagen synthesis. Collagen production activates

transforming growth factor  $\beta$  dependent, platelet-derived growth factor, and independent profibrotic pathways to active hepatic stellate cells, which contribute to portal hypertension<sup>[48]</sup>.

## RISK FACTORS

Studies have identified risk factors towards the development and progression of liver disease. Patterns of drinking, gender, genetic predisposition, and concomitant liver disease may increase the risk of susceptibility. Simultaneous alcohol consumption with food intake has been published to lower risk of alcoholic liver disease compared to those consuming alcohol alone<sup>[9]</sup>. Variant genes encoding for alcohol metabolism, such as alcohol dehydrogenase, aldehyde dehydrogenase, and cytochrome CYP2E1 might facilitate hepatotoxicity by increasing alcohol tolerance *via* delay of acetaldehyde formation or the metabolism of alcohol through other non-oxidative toxic pathways<sup>[49,50]</sup>. Acetaldehyde dehydrogenase gene polymorphisms may cause varying levels of alcohol sensitivity in Asians and women, who can develop alcoholic liver disease even if they do not consume alcohol as heavily as others. Women are twice as likely to develop hepatotoxicity with lower amounts and shorter duration of alcohol use compared to men, which may be attributable to gastric alcohol differences and higher proportion of body fat in women in addition to differences in dehydrogenase levels<sup>[51-53]</sup>. CYP2E1 gene polymorphisms can affect the metabolism of alcohol amongst those with different ethnic backgrounds and alcoholics, however the exact pathogenesis is yet to be elucidated<sup>[54]</sup>.

Variations in patatin-like phospholipase protein 3 (PNPLA3) has a strong association with cirrhosis develop-

ment in Caucasian and Mexican patients with alcoholism<sup>[55]</sup>. Patients with G allele of PNPLA3 have a higher risk of steatosis and fibrosis, as well as a significantly higher prevalence of alcoholic cirrhosis compared to those with C allele<sup>[56]</sup>. Recent data published from a genome wide association study found that severe alcoholic hepatitis risk is associated with PNPLA3 rs738409 variant, which until recently has been associated with cirrhosis development. Identification of SLC38A4 variant gene is another novel independent risk locus for severe AH<sup>[57]</sup>.

Caffeine consumption may have a protective effect against development of AH. Recent studies by Chalasani *et al.*<sup>[58]</sup> found the risk of AH was 27% with heavy alcohol users with PNPLA3 genotype CC with regular coffee consumption compared to 86% in heavy drinkers with PNPLA3 genotype GG, who did not consume coffee. PNPLA3 CC genotype subjects who were not regular coffee consumers had a 48% risk of AH. The risk of AH with PNPLA3 GC with and without regular coffee drinking was 37% and 62%, respectively. The risk of AH was 57% in patients with PNPLA3 GG gene who were regular coffee drinkers<sup>[58]</sup>.

Underlying obesity with body mass index (BMI)  $\geq 30$  likely potentiates the severity of alcoholic hepatitis. A common pathway is postulated for the generation of steatohepatitis through synergetic or additive effects of heavy alcohol use combined with obesity, although the exact mechanism is not well defined<sup>[59]</sup>. Diehl *et al.*<sup>[59]</sup> published a paper documenting a supra-additive interaction between obesity and heavy alcohol consumption. One unit of alcohol was equivalent to 8 g. Overweight or obese male subjects who consumed 15 or more alcohol units per week had an increased risk of liver related morbidity and mortality compared to controls. Another United Kingdom study examining 107, 742 women found that subjects with high BMI ( $\geq 25$  kg/m<sup>2</sup>) who drank  $\leq 15$  units of alcohol have an equivalent risk of chronic liver disease development compared to women with low BMI ( $< 25$ ) who drank  $\geq 15$  units per week. Women with BMI  $\geq 25$  who drank  $\geq 15$  units of alcohol weekly had the poorest outcomes. Even in overweight women who did not drink alcohol, the risk of negative outcomes were present<sup>[60]</sup>.

Alcoholics with other liver co-morbidities, such as hepatitis B, hepatitis C, and hemochromatosis have greater disease severity and likelihood to develop cirrhosis<sup>[61,62]</sup>. Underlying chronic liver disease may contribute to the development of acute-on-chronic presentation in AH.

## HEPATITIS B AND C WITH ALCOHOLIC LIVER DISEASE

The prevalence of hepatitis C patients with alcoholism is approximately 16% compared to the 1.5%-2% prevalence in the general population<sup>[63,64]</sup>. Patients with concomitant hepatitis C and alcoholism have 2- to 8-fold increase risk of all-cause mortality compared to patients without hepatitis C<sup>[65]</sup>. Alcohol abuse reduces survival

in patients with hepatitis C, especially in women<sup>[66]</sup>. Hepatitis C viral load was significantly increased within 4 mo when patients had higher amounts of alcohol consumption of 39-100 g/d compared to 0-50 g/d<sup>[67]</sup>. Alcohol induced liver fibrosis in patients with hepatitis C is dose-dependent and exhibited patients who ingest 30-40 g daily<sup>[68]</sup>. Mechanisms of the synergistic hepatotoxic effects of chronic alcohol abuse in patients with hepatitis C include altered cell-mediated immunity, increased oxidative stress, increase viral replication, hepatic steatosis, and inflammatory response from iron accumulation<sup>[62]</sup>.

Studies on viral hepatitis and chronic heavy alcohol use are mostly in patients with hepatitis C. Mechanisms of pathogenesis can also be applied to hepatitis B patients. Hepatitis B or C drinkers have an increase risk of hepatocellular carcinoma compared to non-drinkers<sup>[69,70]</sup>. Alcohol use did not effect viral efficacy in hepatitis B patients treated with entecavir or hepatitis C patients treated with interferon, however alcoholics may be less compliant with medication adherence<sup>[71,72]</sup>. Elevation of liver enzymes induced by alcohol can cause overtreatment of patients with chronic hepatitis B. It has been published that only 50% of patients with aminotransferase elevation was caused by immune active chronic hepatitis B among other etiologies<sup>[73]</sup>. Iron deposition is found in  $> 50\%$  patients with chronic hepatitis C or heavy alcohol consumption, which is not typically seen in hepatitis B<sup>[74]</sup>.

## HEMOCHROMATOSIS WITH ALCOHOLIC LIVER DISEASE

Hepcidin is a peptide produced in the liver for delivery of iron through the ferroportin transporter. When hepcidin levels are decreased in patients with progressive liver disease, iron is accumulated in the hepatocytes<sup>[75]</sup>. Concomitant iron accumulation and ethanol toxicity may be associated with increased production of oxidative stress. Patients with hemochromatosis who consumed more than 60 g of alcohol per day were 9 times more to develop cirrhosis than who consumed less<sup>[76]</sup>. Elevated hepatic iron concentration is associated with higher mortality in alcoholic cirrhosis patients<sup>[77]</sup>. Iron accumulation seen in alcoholic liver disease and hepatitis C is independent risk factor for hepatocellular carcinoma development<sup>[76]</sup>. Fifty percent of patients with hereditary hemochromatosis develop fibrosis with a 200-fold risk of hepatocellular carcinoma development<sup>[78]</sup>.

## NASH AND ALCOHOLIC LIVER DISEASE

Patients with risk factors for non-alcoholic steatohepatitis (NASH) are identified with insulin resistance, obesity, hyperlipidemia, and metabolic syndrome in the setting of minimal alcohol use compared to alcoholic liver disease patients<sup>[79]</sup>. Differentiating between alcoholic and NASH can be challenging as imaging, laboratory studies, and histologic findings can be non-diagnostic. Attaining a careful alcohol consumption history is cardinal, but can



be unreliable. Histologically, patients with NASH tend to have more advanced fatty degenerative hepatocytes, while there is generally a greater neutrophilic predominance and frequency of Mallory Denk bodies in hepatocytes with alcoholic liver disease. Mallory-Denk bodies are misfolded protein aggregates induced from ER stress, which are deposited into ubiquitin-rich cytoplasmic inclusions within ballooned hepatocytes<sup>[80,81]</sup>. Mallory-Denk bodies can be present in chronic cholestasis, Wilson's disease, NASH, and amiodarone toxicity. They are not exclusively seen in alcoholic hepatitis<sup>[82]</sup>. Patients with alcoholic liver disease tend to higher rates of perivenular fibrosis, phlebosclerosis, cholestasis, and ductal proliferation compared to NASH patients<sup>[83]</sup>. Using logistic regression, Dunn *et al.*<sup>[84]</sup> identified mean corpuscular volume, AST/ALT ratio, body mass index, and gender as the key variables to differentiating alcoholic liver disease from NASH patients of Caucasian ancestry. The alcoholic liver disease/nonalcoholic fatty liver disease index (ANI) created was found to have good diagnostic capacity compared other previous proposed biomarkers. ANI > 0 was consistent with an alcoholic liver disease diagnosis, while an ANI < 0 was likely due to nonalcoholic fatty liver disease. ANI is not as reliable in cirrhotic patients with Model of End-stage Liver Disease (MELD) score > 20, as well in patients with concomitant alcoholic and NASH disease<sup>[84]</sup>. A 20-year observational study of patients with uncomplicated hepatic steatosis concluded that 1.2% of non-alcoholic fatty liver disease patients developed cirrhosis compared to 22% of alcoholic fatty liver disease patients<sup>[85]</sup>.

## CLINICAL PRESENTATION

Symptoms of alcoholic hepatitis are nonspecific. Patients can experience fatigue, right upper quadrant abdominal pain, anorexia, fever, and weight loss. Development of jaundice may occur in a rapid fashion. Patients with alcoholic hepatitis can develop tender hepatomegaly, ascites, hepatic encephalopathy, upper gastrointestinal bleed, and sarcopenia. Signs of chronic alcohol abuse such as spider angiomas, splenomegaly, palmar erythema, gynecomastia, parotid gland enlargement, testicular atrophy, and Dupuytren's contractures may be present. Characteristic laboratory studies demonstrate a 2:1 aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio with typical values less than 300-400 mg/dL. Serum ALT levels are typically lower than AST in alcoholic hepatitis due to a reduced ALT activity in vitamin B6 depleted hepatocytes and mitochondrial injury causing release of mitochondrial AST<sup>[86]</sup>. Higher levels of aminotransferases may point towards an additional factor inducing hepatotoxicity (e.g., superimposed ischemic hepatitis, drug induced liver injury, rhabdomyolysis, or acute viral hepatitis). Bilirubin levels can be as high as 30 mg/dL with severe coagulopathy, leukocytosis, anemia, and new onset of renal failure is seen in patients with hepatorenal syndrome<sup>[40,87]</sup>. Severe

alcohol withdrawal can be a life-threatening when patients develop delirium tremens, seizures, coma, and cardiac arrest. Treatment with hemodynamic stabilization, airway protection, and benzodiazepines are necessary<sup>[88]</sup>. There is a higher prevalence of patients having alcohol withdrawal in alcoholic hepatitis compared to alcoholic cirrhosis<sup>[3]</sup>. Multiple electrolytic disturbances have been identified in patients with alcoholic hepatitis, such as hypokalemia, hypophosphatemia, and hypomagnesaemia among others. Supplementation with thiamine, folic acid, and correction of glucose, potassium, magnesium, and phosphate is recommended<sup>[23]</sup>.

## DIAGNOSIS

Alcoholic hepatitis is mainly a clinical diagnosis. If there is confirmed abstinence for more than 2 mo or the patient reports less than 4 drinks daily on average, alcoholic hepatitis is less likely. Liver biopsy is considered to a gold standard for diagnosis of alcoholic hepatitis, however they are not considered to be routinely performed for AH evaluation in United States. In a review of 11 randomized controlled trials requiring biopsy proven AH, 1409 of 1668 (84.5%) of the liver biopsies confirmed histologic alcoholic hepatitis with increased diagnostic accuracy of 96% when total bilirubin was > 80  $\mu$ mol/L (> 4.7 mg/dL). The authors concluded that a histologic diagnosis was not necessary for diagnosis and management of AH based on these parameters<sup>[89]</sup>. Nevertheless, if clinical diagnosis is not clear or appears multifactorial, a liver biopsy can be considered. Caution must be executed when there is severe portal hypertension and coagulopathy. If the benefits outweigh the risks, a transjugular approach can determine the wedge hepatic venous gradient and portal pressures and is recommended when a patient has severe coagulopathy or ascites<sup>[90]</sup>. Other causes of liver disease, including decompensated alcoholic cirrhosis, sepsis, and biliary obstruction must be ruled out. Abdominal imaging usually shows steatosis and/or cirrhosis with splenomegaly, which is non-specific in alcoholic hepatitis<sup>[91]</sup>.

Cardinal histologic findings of alcoholic hepatitis include ballooning hepatocytes, Mallory-Denk bodies, and neutrophilic infiltration in the setting of macrovesicular steatosis with fibrosis and lobular distortion<sup>[92]</sup>.

## MORTALITY PREDICTORS/PROGNOSIS

Clinical scoring systems have been developed to predict outcomes in patients with alcoholic hepatitis and guide treatment. Maddrey's discriminant function, Glasgow score, and MELD score help determine if corticosteroids need to be initiated, while the Lille score evaluates if they need to be continued.

The Maddrey's score incorporates the serum bilirubin and prothrombin time to produce a discriminant function score (DF). A DF > 32 is characterized as severe alcoholic hepatitis and has high short-term mortality of

approximately 50%. Patients with a DF > 32 may benefit from corticosteroid therapy. A DF < 32 is classified as mild or moderate in severity with mortality rate of 10%. Corticosteroid treatment is not beneficial in this patient group<sup>[93]</sup>.

The MELD score predicts mortality in alcoholic hepatitis and survival in cirrhotic patients. MELD score performs as well as the DF in 30-d mortality prediction. Corticosteroid therapy reduces short term mortality in patients with MELD score of > 11 or bilirubin > 8 mg/dL with ascites<sup>[94]</sup>. A retrospective study determined that an increase in MELD  $\geq 2$  within the first week of hospitalization is independently associated with in-hospital mortality<sup>[95]</sup>. A study by Dunn *et al.*<sup>[96]</sup> found that a MELD  $\geq 21$  has a 75% sensitivity and specificity to predict mortality with an estimated 90-d mortality of 20% for patients with this score. A MELD  $\geq 21$  can be applied to treatment guidelines for corticosteroid administration.

The Lille score monitors the change in total bilirubin after the first week of corticosteroids to identify the response of patients with severe alcoholic hepatitis. Patients with Lille score > 0.45 indicates poor response to corticosteroids and predicts a 6-mo survival of < 25%. Non-responders are recommended to stop corticosteroids due the risk of infection<sup>[97]</sup>. Recently, a study showed that Lille score on day 4 was as good as day 7 to predict 90-d mortality and reduces unnecessary steroid exposure<sup>[98]</sup>. A meta-analysis of five randomized clinical trials with prednisolone treated subjects with severe alcoholic hepatitis showed an improved survival benefit when sub-classified based on Lille score. Complete responders (Lille score  $\leq 0.16$ ), partial responders (Lille score 0.16-0.56), and null responders (Lille score  $\geq 0.56$ ) has 28-d survival rates of 91%, 79% and 53%, respectively. Corticosteroids had a significant effect on 28-d survival in subjects with Lille score  $\leq 0.56$ <sup>[99]</sup>. Side effects of steroids include infections, hypokalemia, osteopenia, and weight gain. Fungal infections, especially Aspergillosis are common in the steroid treated group<sup>[100]</sup>.

Another prognostic score is the Glasgow alcoholic hepatitis score (GAHS), which incorporates age, serum bilirubin, blood urea nitrogen, prothrombin time, and peripheral white blood cell count. Patients with a DF  $\geq 32$  and a GAHS < 9 did not show benefit from treatment with corticosteroids. For those patients with a GAHS  $\geq 9$ , there was a significant improvement in survival for patients who received corticosteroids. Day 28 survival was 78% for those treated with corticosteroids compared to 52% for the placebo group<sup>[101]</sup>.

Altamirano and his group published the Alcoholic Hepatitis Histologic Score system in order to predict the 90-d mortality. The degree of fibrosis, degree of neutrophil infiltration, type of bilirubinostasis, and presence of megamitochondria were independently associated with 90-d mortality. The factors identified patients with a low (0-3 points), moderate (4-5 points), or high (6-9 points) mortality within 90 d (3%, 19% and 51%, respectively).

The disadvantage of this scoring system is that it requires a liver biopsy, which is not routinely performed in the majority of alcoholic hepatitis patients<sup>[102]</sup>.

Factors associated with increased mortality from alcoholic hepatitis include: Older age, acute kidney injury, elevated bilirubin level, coagulopathy, leukocytosis, alcohol consumption > 120 g/d, infection, hepatic encephalopathy, upper gastrointestinal bleed, and bilirubin to gamma glutamyl transferase ratio > 1<sup>[103-106]</sup>.

### Metabolomic profiling

Metabolomic profiling is recently constructed to identify biochemical markers in liver-related disease<sup>[107]</sup>. In a study by Rachakonda *et al.*<sup>[108]</sup>, metabolomic profiles were able to differentiate alcoholic cirrhotics vs severe alcoholic hepatitis patients with 100% accuracy. The features related to the pathogenesis of alcoholic hepatitis were confirmed by several findings in this study. Severe alcoholic hepatitis was associated with enhanced triglyceride lipolysis, impaired mitochondrial fatty acid beta oxidation, upregulation of omega oxidation, and decreased plasma membrane remodeling. Although there was an increase in measured bile acids found in severe alcoholic hepatitis, intestinal dysbiosis was suggested due to low deoxycholate and glycodeoxycholate levels. Other changes seen in severe alcoholic hepatitis include increased glucose consumption by the pentose phosphate pathway, altered tricarboxylic acid cycle activity, and enhanced peptide catabolism. Altered levels of small molecules related to glutathione metabolism and antioxidant vitamin depletion were observed<sup>[108]</sup>. Another study performed by Rachakonda *et al.*<sup>[109]</sup> showed that patients with severe alcoholic hepatitis were found to have higher levels of serum resistin and plasma activation inhibitor-1 levels with decreased serum leptin levels. Levels of inflammatory cytokines, such as tumor necrosis factor  $\alpha$ , IL-6, IL-8, and IL-15 were higher in patients with severe alcoholic hepatitis. IL-6 levels of  $\geq 38.66$  pg/mL were found to have significantly decreased mean survival rates<sup>[109]</sup>.

## BIOMARKERS

The development of biomarkers sensitive to the detection of alcoholic hepatitis can be helpful for prognostication. Selected-ion flow tube mass spectrometry breathe testing was able to identify increased levels of acetaldehyde, trimethylamine, acetone, and pentane in patients with alcoholic hepatitis with underlying cirrhosis compared to those with liver cirrhosis and acute decompensation from etiologies other than alcohol. These biomarkers represent breakdown products of ethanol metabolism in alcoholic hepatitis. Given the small sample size, larger studies will need to be performed for validation of results<sup>[110]</sup>.

Other markers, such as procalcitonin, lipopolysaccharide, liver progenitor cell proliferation, soluble TNF receptor 1, microRNA profiling, and IL-22 serum

levels are being studied for clinical application towards prognostication of alcoholic hepatitis<sup>[104,111-115]</sup>.

## ABSTINENCE AND MEDICATIONS TO PREVENT RECIDIVISM

The most important primary intervention for alcoholic hepatitis management is abstinence counseling<sup>[116]</sup>. Abstinence can improve survival in patients with alcoholic liver disease by improving histologic features of hepatocyte injury with reduction of portal hypertension and progression into cirrhosis<sup>[5]</sup>. Two thirds of patients abstaining from alcohol have significant improvement within 90 d<sup>[117]</sup>. A 30% decrease in survival rate is seen in patients with compensated cirrhosis who continue to use alcohol compared to those who are abstinent<sup>[118,119]</sup>. Continued interventions, such as combination psychotherapy with cognitive behavioral therapy, peer driven support counseling, motivational enhancement therapy, and comprehensive medical care can reduce recidivism<sup>[120]</sup>. Risk of recidivism is as high as 67% to 81% over the course of one year<sup>[121]</sup>.

Medications to maintain abstinence have been investigated. FDA approved medications are disulfiram, naltrexone, and acamprosate<sup>[122]</sup>. Disulfiram was first approved in 1983<sup>[123]</sup>. Other agents have been explored due to poor tolerability and lack of evidence to support its efficacy<sup>[124]</sup>. Disulfiram is not recommended for use in cirrhotic patients as the literature describes cases of fulminant hepatitis requiring liver transplant<sup>[125]</sup>. Naltrexone is an opioid antagonist used to decrease alcohol cravings, however it can cause hepatocellular injury<sup>[126]</sup>. Nalmefene works in a similar mechanism of action to naltrexone, but does not have the risk of hepatocellular injury and has a longer half-life<sup>[127]</sup>. Acamprosate is structurally similar to gamma amino butyric acid and is associated with reducing alcohol withdrawal symptoms based on 15 controlled trials. As a maintenance medication, it can decrease the relapse rate and relapse severity compared to placebo<sup>[128]</sup>. In a recent randomized, double-blind study in the United States, there was no evidence of efficacy for acamprosate compared to placebo among alcohol-dependent individuals recruited from a primary care setting<sup>[129]</sup>. These patients did not receive extensive multidisciplinary counseling. In the COMBINE trial, there was no substantial benefit for patients treated with acamprosate vs naltrexone or intensive abstinence counseling. The PREDICT study is a randomized clinical trial conducted in Germany, which compared its data to the COMBINE study. The primary outcome examined the first occurrence of heavy drinking. PREDICT found neither acamprosate nor naltrexone to supply any additional benefit compared with placebo<sup>[130]</sup>.

There are few medication options to prevent recidivism in advanced chronic liver disease. Baclofen is  $\gamma$  aminobutyric acid B-receptor antagonist, which is minimally metabolized in the liver. It is one of the few treatments studied in cirrhotic patients. Addolorato *et al.*<sup>[131]</sup> performed a randomized double-blinded placebo-controlled in alcoholic-

dependent cirrhotics with baclofen 10 mg three times daily for 12 wk in the treatment arm. Seventy-one percent of maintained abstinence compared to 29% in the placebo group. Baclofen may be beneficial to achieving and maintaining abstinence safely in Child-Pugh class A, B and C cirrhotic patients<sup>[131]</sup>. Gamma hydroxyl butyrate may be well tolerated in patients with decompensated cirrhosis with alcohol withdrawal symptoms due to the short half-life of 4-6 h. Further studies need to be performed before recommendations on efficacy and safety can be made<sup>[132]</sup>. None of the medications discussed have been studied in the context of alcoholic hepatitis and remains a challenge to medical practitioners.

## TREATMENT

### Nutritional supplementation

Patients with alcoholic hepatitis and cirrhosis have nutritional deficiencies and sarcopenia. Protein calorie malnutrition is associated with short and long term mortality<sup>[133]</sup>. Vitamin A, Vitamin D, thiamine, pyridoxine, folate, and zinc are common vitamin deficiencies seen in alcoholics<sup>[134]</sup>. Early studies from the Veterans' Association found 100% of the 363 alcoholic hepatitis patients had protein calorie malnutrition<sup>[135]</sup>. The degree of malnutrition is associated with the severity of liver disease. AASLD and EASL guidelines recommend enteral nutritional therapy in AH patients, however the evidence remains controversial<sup>[2,136]</sup>. Moreno *et al.*<sup>[137]</sup> randomized 136 biopsy confirmed severe alcoholic hepatitis patients to receive either intensive enteral nutrition *via* feeding tube plus methylprednisolone or conventional nutrition plus methylprednisolone for 14 d. There is no significant difference in the six-month survival between the groups with 44.4% deaths in the intensive enteral nutrition arm and 53.1% of the controls. The study results were likely affected by being underpowered. The mortality rate at one and six months are lower in the intensive enteral nutrition group compared to the control, but the results are not statistically significant. Of note, 48.5% of the patients had the enteral tube discontinued prematurely. Five patients had serious adverse events related to enteral nutrition, such as aspiration pneumonia, hyperglycemia, and hepatic encephalopathy exacerbation. Nevertheless, this study implies that patients receiving < 21.5 kcal/kg per day have a significantly lower survival rate with increased risk of infection and hepatorenal syndrome at 6 mo compared to those with better nutritional rates. Patients with nutritional requirements of  $\geq 65$  g/d of lipids and  $\geq 77.6$  g/d of protein have better six-month survival rates<sup>[137,138]</sup>. Further investigation needs to be pursued to delineate the role of nutrition in AH patients.

### Corticosteroids

Patients with mild alcoholic hepatitis (DF < 32) have a 10% mortality rate when not treated with prednisolone.

Supportive care is warranted<sup>[139]</sup>. Multiple treatment options have been studied, however only prednisolone have remained the mainstay of therapy<sup>[91,136]</sup>. Corticosteroids have a wide range of immune modulatory functions including suppression of pro-inflammatory transcription factors: NFκB and activator protein 1 (AP-1), which lower circulating levels of TNF-α and IL-8<sup>[140,141]</sup>. Prednisolone use is indicated in patients with DF > 32 or hepatic encephalopathy, but contraindicated in active infection, gastrointestinal bleeding, acute pancreatitis, or renal failure<sup>[142,143]</sup>.

Studies examining the combination of prednisolone and pentoxifylline treatment produced mixed results<sup>[144,145]</sup> or showed no added benefit of pentoxifylline<sup>[146,147]</sup>. The Steroids or Pentoxifylline for Alcoholic Hepatitis trial is the largest randomized clinical trial to date, which examined the short and long term mortality of patients with severe alcoholic hepatitis. Results show no reduction in all cause mortality at 28 d for patients treated with prednisolone or pentoxifylline. However, there is a non-significant mortality benefit at 28 d in the prednisolone treated group, which is not seen at 3 and 12 mo<sup>[148]</sup>. Corticosteroids may have some benefit within the first month, but cannot be generalized to a provide long term value.

The meta-analysis of 22 randomized clinical trials performed by Singal *et al.*<sup>[90]</sup> show a reduction in short-term mortality in patients with severe alcoholic hepatitis treated with steroids vs placebo. Corticosteroids with N-acetylcysteine (NAC) compared to corticosteroids alone may be effective in improving short-term mortality<sup>[149]</sup>. More recently, Thursz *et al.*<sup>[150]</sup> performed a meta-analysis of 9 randomized clinical trials comparing the use of corticosteroids, pentoxifylline, or both for the treatment of severe alcoholic hepatitis. They found that corticosteroid treatment improved 28 d survival compared to pentoxifylline and control group. There is no added benefit of treatment with combination group of corticosteroids and pentoxifylline<sup>[151]</sup>.

### Pentoxifylline

Pentoxifylline inhibits tumor necrosis factor, a cytokine responsible for the inflammatory cascade initiation seen in alcoholic hepatitis. One out of four randomized controlled trials showed a mortality rate of 25% in pentoxifylline treated patients with DF > 32 compared with 46% in the placebo group. The benefit seen was mostly to prevent hepatorenal syndrome<sup>[151]</sup>. It can be an alternative for patients who have contraindications to steroids or early renal failure, however is not recommended as a first line agent.

### N-acetylcysteine

Oxidative stress produced from alcoholic hepatitis depletes glutathione levels. NAC is an antioxidant substance, which is a pro-drug to the precursor of glutathione. Moreno *et al.*<sup>[137]</sup> produced a randomized clinical trial of NAC vs placebo, which shows no significant difference<sup>[129]</sup>.

In 2006, Phillips *et al.*<sup>[152]</sup> found that corticosteroids are superior to NAC for short-term survival. Nguyen-Khac *et al.*<sup>[153]</sup> examined the use of NAC with corticosteroids in a 2011 randomized clinical trial. They found patients with combination therapy have improved one-month survival compared to patients treated with corticosteroids. There are fewer cases of infections and hepatorenal syndrome in the combination treatment arm. Nevertheless, there is no significant difference in survival at 6 mo<sup>[153]</sup>. Further studies are needed to evaluate the efficacy of NAC.

### Other anti-TNF alpha inhibitors

Anti-TNF alpha inhibitors, such as infliximab and etanercept is not recommended for the treatment of alcoholic hepatitis. Although early pilot studies of corticosteroids and infliximab show an improvement in the Maddrey score within the first month, later studies have shown anti-TNF alpha inhibitors are associated with increased death from infections<sup>[113,154,155]</sup>.

### Liver transplantation

Liver transplantation may be considered as a last option for patients with alcoholic hepatitis when medical treatment has failed or is contraindicated. Most liver transplant centers require a minimum abstinence of six months prior to donor allocation consideration. Given the donor organ scarcity, the risk of recidivism is feared for patients with alcoholic hepatitis undergoing liver transplantation<sup>[156]</sup>.

Data regarding the 6-mo rule as a predictor of long-term sobriety remains controversial<sup>[157]</sup>. Based on a systematic review, there is no difference in early alcohol use in patients transplanted for alcoholic liver disease vs non-alcoholic liver disease at: 6 mo (4% vs 5%) and 12 mo (17% vs 16%). At 7 years post-OLT, 32% of the patients with alcoholic liver disease reports using alcohol. Although comparable rates of any alcohol use are reported in patients transplanted for alcoholic liver disease and non-alcoholic liver disease, the risk of heavy drinking appears much higher in alcoholic liver disease patients<sup>[158]</sup>. There is a wide variation among post-liver transplant alcohol relapse rates reported in the literature, ranging from 20% to 50%. Heavy drinking rates range from 10% to 20%<sup>[159]</sup>. The duration of pre-transplant abstinence does not appear to correlate with post-transplant survival<sup>[160]</sup>, however studies for long term follow-up of the graft in patients transplanted for alcoholic hepatitis with continued alcohol abuse requires further investigation.

Mathurin *et al.*<sup>[161]</sup> reports the results of a multicenter European trial which carefully selected corticosteroid refractory AH patients whom were deemed to have a low risk of recidivism after liver transplantation. The episode of AH is deemed as the patient's first liver decompensating event. Other inclusion criteria includes: Close and supportive family members, absence of severe coexisting or psychiatric disorders, and a covenant to adhere to life-long alcohol abstinence. The study reports



no alcoholic relapse within the initial 6-mo follow-up period. Three of 26 patients transplanted for refractory alcoholic hepatitis later resumed drinking alcohol: One at 720 d, one at 740 d, and one at 1140 d after transplantation. Despite counseling by an addiction specialist, 2 patients remained daily consumers (30 g/d and > 50 g/d), whereas 1 consumed alcohol occasionally (approximately 10 g/wk). None of them had graft dysfunction<sup>[161]</sup>.

Im *et al.*<sup>[162]</sup> applied inclusion criteria similar to Mathurin's European trial for early liver transplantation in severe alcoholic hepatitis in the United States. The low candidate acceptance rate (20%) and the high survival rates for transplanted AH patients compared to controls (89% vs 11%) is comparable to the findings in Mathurin's study. Two patients (25%) had alcohol use post OLT. One patient self-reported a "slip" of 60 g and 15 g of alcohol use at day 84 and 260, respectively. Serial urine ethanol testing and self-reporting were negative thereafter. One patient had alcohol relapse, which is defined as: Four or more drinks daily or at least one drink for 4 or more days in succession after liver transplantation. When the subject with alcohol relapse was further analyzed, it was deemed that the hepatic decompensation was not the patient's first event and the subject had poor insight to disease prior to transplant. Limitations to the study include small sample size ( $n = 9$ ) and short follow-up period (median = 765 d)<sup>[162]</sup>.

A three-year pilot by Lee examined 2 groups of patients selected to receive a liver transplant: Severe alcoholic hepatitis as the first episode of liver decompensation vs alcoholic cirrhotics with  $\geq 6$  mo of abstinence. Early liver transplant provided excellent short-term survival in both groups. There were similar rates of alcohol relapse in both groups: 23.5% vs 29.2%. Although lacking statistical significance, patients transplanted for AH had higher rates of harmful drinking post-transplant compared to the control group (23.5% vs 11.5%,  $P = 0.42$ ). The data was particularly concerning given the two out of the four patients with harming drinking patterns died secondary to recurrent alcohol use (alcohol overdose and medication noncompliance with graft failure, respectively)<sup>[163]</sup>.

Although preliminary results may appear promising, ethical issues pertaining to organ shortage, sociocultural concerns about judicious organ allotment, and recidivism risk remain<sup>[164]</sup>. The feasibility of patient selection through strict psychosocial assessment is limited by resources. An addiction psychiatrist experienced in liver transplant may not be readily available in all centers. Liver transplantation for refractory severe acute alcoholic hepatitis should be judiciously employed in highly selected individuals who are at low risk of recidivism<sup>[165]</sup>.

New therapeutic options for alcoholic hepatitis are needed. Corticosteroid use are helpful in 50% of cases, however they are associated with a higher rate of infections and do not offer long term survival benefit.

Treatments targeting gut dysbiosis, innate immunity, inflammation pathways, and apoptosis are currently being studied (Table 1).

## NEW THERAPEUTIC OPTIONS

### Gut microbiota modification: Probiotics

Animal studies mimicking alcoholic hepatitis have observed changes in microbial translocation and dysbiosis<sup>[166]</sup>. Patients with alcoholic hepatitis have abnormalities in bacterial overgrowth, intestinal mucosal damage, increased gut permeability with bacterial translocation, and resulting endotoxemia<sup>[167]</sup>. The use of probiotics to modify gut bacteria are studied for the treatment of alcoholic hepatitis. Animals studies by Wang *et al.*<sup>[168]</sup> concludes *Lactobacillus rhamnosus* treatment reduced alcohol-induced hepatic inflammation by attenuation of TNF- $\alpha$  production via inhibition of TLR-4 and TLR-5 mediated endotoxin activation. A pilot study with mild alcoholic hepatitis patients who received *Bifidobacterium bifidum* and *Lactobacillus plantarum* 8PA3 for five days shows significantly reduced ALT, AST, lactate dehydrogenase, total bilirubin, and restoration of gut flora compared to placebo. Other studies have showed that alcoholic cirrhotics have cytokine reduction with reduced liver disease severity and hospitalization when treated with probiotic VSL#3<sup>[169,170]</sup>. Rifaximin is studied for the role of bacterial overgrowth in decompensated alcoholic cirrhotics. Rifamixin administered for 28 d decreased endotoxemia in the systemic and splenic circulation with reduction in portal hypertension. Currently, there are clinical trials examining the role of *Lactobacillus rhamnosus*, rifaximin, fecal microbiota transplantation, and antibiotics in AH patients<sup>[99]</sup>.

### Immune modulators

Chronic ethanol stimulation increases the production of inflammatory cytokines and chemokines to induce liver injury. Multiple mechanisms are proposed to modulate the innate immune system. It is not clear if animal and cellular models can be extrapolated for use in humans. Based on animal studies, IL-22 is a hepatoprotective cytokine. Chronic-binge ethanol fed mice treated with recombinant IL-22 protein induced activation of hepatic STAT3 to prevent alcohol-induced steatosis, liver injury, and oxidative stress in a study by Ki *et al.*<sup>[12]</sup>. IL-22 down regulates the expression of fatty acid transport protein. It is found to have antioxidant, apoptotic, proliferative, and antimicrobial properties with minimal side effects<sup>[11]</sup>. IL-17 levels produced by TH17 cells are elevated in patients with alcoholic hepatitis. IL-17 induces neutrophil recruitment and stimulates hepatic stellate cells to secrete chemokines, such as IL-8 and CXCL<sup>[171,172]</sup>. Alcoholic hepatitis patients with expression of these chemokines in the liver are correlated with worsening severity of portal hypertension and patient survival<sup>[173,174]</sup>. Therapeutic agents targeting the reduction of CXCL and IL-17 with IL-22 upregulation can be a new treatment

**Table 1** New potential treatments for alcoholic hepatitis

Treatment	Class	Mechanism of action
Probiotics	Gut microbiota modification	Reduction of bacterial endotoxins and translocation
IL-22 recombinant protein	Immune modulation	Hepatoprotective: Antioxidant, apoptotic, proliferative, and antimicrobial properties
G-CSF	Growth factor	Liver regeneration
Obeticholic acid	Farnesoid X receptor	Improvement in cholestasis
Emricasan	Caspase inhibitor	Apoptosis, inflammation, and fibrosis inhibitor
Anakinra (Pentoxifylline + Zinc)	IL-1 receptor	Decreases hepatic inflammation
SAMe	Glutathione precursor	Decreases oxidative stress
Metadoxine	Antioxidant	Decreases oxidative stress and steatosis
ELAD	Extracorporeal human hepatic cell-based liver treatment	Toxin removal, reduction of inflammation, liver regeneration

IL: Interleukin; G-CSF: Granulocyte colony-stimulating factor; ELAD: Extracorporeal liver assist device; SAMe: S-adenosyl-L-methionine.

strategy<sup>[54,175]</sup>.

### **Liver regeneration: Granulocyte colony-stimulating factor**

Bone marrow-derived stem cells can populate the liver and differentiate into hepatic cells when faced with liver insult. Experimental studies show that granulocyte colony-stimulating factor (G-CSF) promote the mobilization of bone marrow stem cells to ameliorate liver injury and enhance the proliferative capacity of hepatocytes<sup>[176]</sup>. G-CSF mobilizes CD 34+ cells, increases hepatocyte growth factor, and induces proliferation of hepatic progenitor cells within 7 d of administration in patients with alcoholic cirrhosis with biopsy proven alcoholic steatohepatitis<sup>[177]</sup>. In a pilot study, 46 patients with severe alcoholic hepatitis were randomized to receive G-CSF  $\geq 5$   $\mu$ g/kg for 5 d with standard medical therapy (pentoxifylline with nutrition) vs standard medical therapy alone. Findings shows a statistically significant number of peripheral CD 34+ cells and improvement of Child Pugh score, MELD, and discriminant function for up to 3 mo in the G-CSF group. Ninety day survival benefit is seen in G-CSF group compared to placebo<sup>[178]</sup>. The addition of corticosteroids would be helpful in delineating the survival benefit. A clinical trial testing the efficacy of G-CSF in the management of patients with severe alcoholic hepatitis whom have failed corticosteroids is needed.

### **FXR/obeticholic acid**

FXRs are nuclear hormone receptors that participate in bilirubin metabolism. Bile acids are the physiologic ligands of FXRs, which regulate bile acid, carbohydrate, and lipid metabolism. In addition, they modulate liver regeneration after injury. FXR activation is protective against cholestatic and fatty liver injury. In a murine model, mice were fed an ethanol or control diet. FXR impairment is exhibited in the ethanol group. FXR agonist therapy is found to be hepatoprotective, likely from suppression of microsomal CYP2E1 enzyme upregulation<sup>[179]</sup>. FXR activation is shown in other studies to prevent and improve liver fibrosis in mice<sup>[180,181]</sup>.

Obeticholic acid is a selective FXR. A phase 2 clinical

trials shows obeticholic acid improved insulin sensitivity and markers of liver inflammation in patients with diabetes and nonalcoholic fatty liver disease. Phase 2 clinical trials are exploring obeticholic acid in patients with alcoholic hepatitis.

### **Caspase inhibitors**

Alcohol exposure causes hepatocytes to release extracellular vesicles in a caspase-dependent manner to elicit apoptosis and macrophage activation<sup>[182]</sup>. Apoptosis may trigger abnormal liver tissue repair, inflammation, regeneration, and fibrosis<sup>[183]</sup>. Caspase inhibitors may decrease apoptosis and inflammation in a variety of liver diseases. Emricasan is a pan-caspase inhibitor studied in patients with hepatitis C and NASH. In clinical trials, emricasan significantly reduces the aminotransferase activity in non-cirrhotic hepatitis C patients. Similar trends are observed in patients with NASH and hepatitis B, however statistical analysis was not performed on these groups<sup>[184]</sup>. In NASH studies, mice fed a high fat diet demonstrates a five-fold increase in hepatic apoptosis and 1.5-fold and 1.3-fold increase in caspase-3 and -8, respectively. Mice with emricasan administration demonstrates a reduction in inflammation and fibrosis compared to placebo. Based on the positive preliminary data found in murine NASH models, clinical trials evaluating emricasan for benefit in patients with alcoholic liver disease are ongoing. Thus far, a phase 2 clinical trial concluded that Child Pugh A and B cirrhotic patients with baseline MELD  $\geq 15$  who are treated with emricasan showed significant improvement compared to placebo in MELD scores, Child-Pugh scores, bilirubin levels, and INR in preliminary data<sup>[185]</sup>.

### **Combination therapy: Anakinra-blocks IL-1 beta receptor, pentoxifylline and zinc vs methylprednisolone**

Alcohol-induced liver injury activates Kupffer cells, which stimulation production of inflammasomes and IL-1 $\beta$ , which initiate the inflammatory cascade. Effects include liver inflammation, steatosis, injury, and fibrogenesis. Pharmacological inhibition of IL-1 signaling has a hepatoprotective effect. There was recovery from acute-on-chronic alcoholic liver injury<sup>[186]</sup>. Anakinra, an IL-1

receptor antagonist combined with pentoxifylline and zinc is being studied in phase 2 and 3 clinical trials to examine the efficacy against corticosteroids.

### **S-adenosil-L-methionine**

S-adenosil-L-methionine (SAME) is a direct precursor of glutathione, which serves as a major physiologic defense mechanism against oxidative stress. A recent pilot study randomized two groups of twenty patients each with severe alcoholic hepatitis treated with prednisolone 40 mg daily vs prednisolone 40 mg with intravenous SAME 800 mg for 28 d. After the first week, intravenous SAME regimen was converted to oral doses of 1200 mg/d for two months. The response rate measured by the Lille's score is significantly improved in the prednisolone and SAME (95% of patients) compared to the prednisolone only group (65%). Hepatorenal syndrome occurred in 20% patients in the prednisolone group, but none in the combination treatment group. Difference between the groups regarding 28-d mortality could not be inferred. Although not statistically significant, the six-month survival rate is 90% in the prednisolone plus SAME group vs 75% in the prednisolone group. Larger trials are needed to validate the study results<sup>[187]</sup>.

### **Metadoxine**

Metadoxine is an antioxidant, which aids in glutathione metabolism and inhibits hepatic steatosis<sup>[188]</sup>. The addition of metadoxine with corticosteroids is found to improve 30 and 90 d survival rates. The metadoxine and corticosteroid group is found to have a better treatment response based on Lille's score, lower rates of hepatorenal syndrome, and decreased development and/or progression of hepatic encephalopathy compared to the corticosteroid group. There are no significant adverse side effects<sup>[189]</sup>. Another study combined metadoxine with either prednisone or pentoxifylline for 30 d. The group receiving metadoxine combined with prednisolone or pentoxifylline had increased three and six-month survival rate of 50% compared to the 20% survival rate in the prednisolone or prednisone only group. The rates of hepatorenal syndrome and hepatic encephalopathy development are significantly less in the metadoxine group, however infections are not<sup>[190]</sup>. Additional studies with a greater sample size are needed to increase the power of future studies.

### **ELAD**

There are ongoing Phase 3 clinical trials of ELAD for acute severe alcoholic hepatitis<sup>[191]</sup>. Patients with acute renal failure, severe coagulopathy, and MELD > 28 have worse outcomes with ELAD. There are no survival differences between the ELAD over the control group in day 28 and 91. Pre-specified exploratory analysis of 101 patients < age 47 showed an improved 3-mo survival in the ELAD group compared to the control group (81.4% vs 67.2%). When analyzed for patients less than 50 years old, creatinine < 1.3 mg/dL, bilirubin  $\geq$  16 mg/dL, and INR  $\leq$  2.5,

the 3-mo survival rate was 94% in the ELAD group and 68% in the control group. The most recent ELAD trial, VTL-308 incorporates the new inclusion and exclusion criteria<sup>[192]</sup>. The preliminary results are eagerly awaited. There are limitations to the use of ELAD, including high cost and stringent inclusion criteria. Patients are usually monitored in the intensive care use with frequent monitoring and blood draws. Currently, there are limited centers performing ELAD research and the patient selection criteria excludes: Alcohol use > 6 wk, persons > 50 years old, severe coagulopathy, and advanced renal failure.

Many therapies have been studied for alcoholic hepatitis without proven efficacy. Treatment with antioxidants, including vitamin E and silymarin do not have a survival benefit in alcoholic hepatitis or cirrhosis patients. Colchicine, amlodipine, propylthiouracil, anabolic steroids, and insulin and glucagon combinations are not effective in patients with alcoholic hepatitis<sup>[45]</sup>.

## **FUTURE RESEARCH**

Most of the understanding of alcoholic liver disease pathogenesis stem from animal models of alcoholic liver disease recreated *via* ad libitum or intragastric ethanol feeding. Recent publications propose a new model of ad libitum feeding with 40% intake of caloric intake from a Western diet high in cholesterol and saturated fat combined with 60% ethanol *via* intragastric infusion to simulate a "true" model of alcohol hepatitis, where contributing factors such as obesity and alcohol abuse are taken into account. This model recreates findings seen in chronic alcoholic liver disease with superimposed alcoholic hepatitis when a weekly binge dose of ethanol is added. However, the model could not emulate the acute-on-chronic hepatic decompensation seen in alcoholic hepatitis<sup>[193,194]</sup>. The search for molecular targets through genomic studies holds the future direction of answering unsolved questions about alcoholic hepatitis pathogenesis. Further study of IL-22's antioxidant, anti-apoptotic, anti-steatosis, antibacterial, proliferative effect, and other hepatoprotective properties in conjunction with the inflammatory and immunomodulatory function of corticosteroids is underway<sup>[12,195]</sup>. Recent literature highlights the use of biospecimens (*i.e.*, liver tissue, peripheral serum, stool) for *in vitro* and *in vivo* studies as a new approach to finding targets for therapy<sup>[194]</sup>. New findings elucidated under such methods, include impaired bacterial killing from monocyte oxide burst dysfunction and defective T cell function in AH subjects. Although the reversal of defective monocyte oxidative burst is not restored by the IFN-gamma, the negative regulator of Janus Kinase responsible for suppressing cytokine signalling-1 was discovered to have increased expression<sup>[46]</sup>. Restoration of T-cell interferon gamma production, reduction in production of IL-10 producing T cells, and improvement in neutrophil antibacterial function occurs when antibodies against PD1 and TIM3

are blocked<sup>[47]</sup>.

## CONCLUSION

Alcoholic hepatitis is increasingly recognized as a form of acute-on-chronic liver failure in patients with underlying alcohol-related disease<sup>[196,197]</sup>. Patients with severe alcoholic hepatitis remain a challenging population to treat. New treatment options for AH involving gut microbiota modification, immune modulation, promotion of liver regeneration, apoptosis inhibitors, farnesoid receptors, and ELAD appear promising thus far, however the research is still in the preliminary phases. Currently, early liver transplantation for severe AH failing standard medical therapy is not universally implemented and further investigation is warranted. Solving the complex pathophysiology of alcoholic hepatitis through translational studies with clinical application is challenging. The study of new animal model simulating "true" AH and use of genomic analysis to provide molecular targets are emerging into present day practice. The utilization of clinical trials fuelled by constant evolving concepts discovered *via* translational research will help determine the endpoints and safety of the new therapeutic options to bridge the gap of a disease with high morbidity and mortality.

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## Role of circulating microRNAs in liver diseases

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### Abstract

MicroRNAs (miRNAs) are small RNAs regulate gene expression by inhibiting the turnover of their target mRNAs. In the last years, it became apparent that miRNAs are released into the circulation and circulating miRNAs emerged as a new class of biomarkers for

various diseases. In this review we summarize available data on the role of circulating miRNAs in the context of acute and chronic liver diseases including hepatocellular and cholangiocellular carcinoma. Data from animal models are compared to human data and current challenges in the field of miRNAs research are discussed.

**Key words:** Liver disease; Acute liver failure; MicroRNA; Liver fibrosis; Hepatocellular carcinoma; Autoimmune hepatitis; Cholangiocarcinoma

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**Core tip:** In this article, we aim to review the role of circulating microRNAs (miRNAs), a class of small non-coding RNAs involved in various pathological processes, in the context of liver disease. The focus is on current and future applications of miRNAs as potential diagnostic and prognostic biomarkers in the field of acute liver failure, liver fibrosis and cirrhosis, autoimmune liver disease as well as liver cancer.

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### INTRODUCTION

MicroRNAs (miRNAs) are small RNAs that do not encode for proteins, but regulate gene expression<sup>[1]</sup>. MiRNAs are transcribed by the RNA polymerase II or RNA polymerase II<sup>[2-4]</sup>. The resulting 500-3000 nucleotides long transcripts (pri-miRNAs) are cleaved in a second step by the "microprocessor complex" into approximately 70 nucleotides long precursor miRNAs (pre-miRNA), which are actively exported from the nucleus into the cytoplasm. Finally, pre-miRNAs are processed by the

RNase III endonuclease “Dicer” into approximately 22 nucleotides long double stranded miRNAs, which bind to the Argonaute protein and are integrated into the “RNA-induced silencing complex”. Within this complex, miRNAs bind the 3′ or 5′ untranslated region of the target mRNAs, leading to a transcriptional or translational repression of the target mRNA<sup>[2,4-6]</sup>. Alterations in miRNA expression profiles were described in organ development, aging, and cell death<sup>[7]</sup>, as well as in the pathophysiology of complex diseases such as inflammation, fibrosis and cancer<sup>[8-13]</sup>.

Besides their role in the regulation of gene expression, miRNAs have been described in body fluids, where they might serve as biomarkers<sup>[14-17]</sup>. Based on their extraordinary stability, their less complex chemical structure and their lack of post-processing modifications, circulating miRNAs were suggested as “optimal” serum based biomarkers<sup>[18]</sup>. Circulating miRNAs can be either bound to serum proteins and lipoproteins or be encircled in extracellular vesicles including exosomes, microvesicles or apoptotic bodies<sup>[17,19]</sup>. As exosomes can be released by various hepatic cells (*e.g.*, hepatocytes and Kupffer cells) and can be transferred to other recipient cells to regulate expression profiles in these cells, they were suggested to play an important role in hepatic cell-cell-communication and in the pathophysiology of different liver diseases. Findings that miRNAs encircled in these vesicles are well protected from degradation furthermore highlight the potential of exosomal miRNAs to serve as potent biomarkers<sup>[20-22]</sup>. With respect to the concept of “liquid biopsy” which has recently been suggested as a novel detection tool for malignant diseases<sup>[23,24]</sup>, miRNA might thus function as a potential “liquid biopsy” not only for malignant but also benign liver disease.

In this review, we evaluated studies indexed in Medline between 2006 and 2016. The terms “microRNA”, “liver”, “liver failure”, “fibrosis”, “cirrhosis”, “hepatocellular carcinoma”, “cholangiocarcinoma”, “autoimmune hepatitis”, “primary sclerosing cholangitis”, “primary biliary cholangitis”, “biomarker”, “diagnostic”, “prognostic” and combinations of these terms were used.

## ACUTE LIVER FAILURE

Acute liver failure (ALF) is characterized by a massive loss of liver cell function based on various etiologies (*e.g.*, drug intoxication, viral or autoimmune hepatitis (AIH), Wilson’s disease or Budd-Chiari syndrome) without preexisting liver disease<sup>[25,26]</sup>. Despite significant improvements regarding therapeutic options (*e.g.*, liver transplantation), ALF has remained a challenging clinical condition with mortality rates of about 50%<sup>[27]</sup>. In this context, biomarkers allowing early diagnosis or estimation of patients’ fate might be helpful for the guidance of therapy<sup>[28,29]</sup>. However, routinely used serum biomarkers for liver injury such as AST and ALT are not liver specific and only have a limited prognostic value<sup>[30-32]</sup>. Therefore, new biomarkers are urgently needed to further improve patients’ individual treatment options and overall survival

in the context of acute liver injury.

In a pilot study on the potential of miRNAs as ALF biomarkers, Wang *et al.*<sup>[18]</sup> demonstrated that liver specific miR-122 and miR-192 were elevated in sera of mice after acute Acetaminophen (APAP) intoxication compared to controls. Of note, miR-122 and miR-192 serum levels were increased in a dose- and exposure duration-dependent manner and were detectable significantly earlier than the classic serum aminotransferases<sup>[18]</sup>. Consistently, circulating miR-122 and miR-192 levels were elevated in patients with APAP-induced ALF compared to healthy controls<sup>[33]</sup>. Moreover, miR-122 serum levels returned earlier to normal when compared to ALT, indicating that circulating miR-122 might have a shorter half-life in comparison to ALT<sup>[33]</sup>. High throughput sequencing of miRNAs in sera of patients with APAP overdose revealed 36 miRNAs to be elevated compared to healthy controls. Besides the already described miR-122 and miR-192, miR-483, miR-194 and miR-210 were additionally found to be increased in the sera of these patients<sup>[32]</sup>. Antoine *et al.*<sup>[29]</sup> demonstrated in a large cohort of patients with APAP-induced ALF that increased miR-122 serum levels are detectable very early after liver intoxication when serum ALT levels are still unaffected. Furthermore, levels of circulating miR-122 enabled the prediction of liver injury development with a high accuracy<sup>[29]</sup>.

An increasing number of studies have investigated circulating miRNAs regarding their prognostic potential for acute liver injury. Just recently, Russo *et al.*<sup>[34]</sup> applied a microarray based expression analysis using a panel of 1733 miRNAs and 1658 pre-miRNAs in sera of 78 drug-induced liver injury (DILI) patients. These patients showed elevated serum levels of miR-122, miR-1246, -4270, -4433, -4463, -4484, -4532 and pre-miR-4767 as well as decreased serum levels of miR-455-3p, -1281 and pre-miR-4274 compared to healthy controls. Out of these, miR-122, miR-4463 and miR-4270 had a prognostic value as decreased serum levels correlated with the decrease of DILI patients within 6 mo. In this study, low albumin (less than 2.8 g/L) and low miR-122 serum levels (less than 7.89 relative fluorescent units) had a sensitivity of 100% and a specificity of 57% for the prediction of death in DILI patients<sup>[34]</sup>. The prognostic value of miRNA profiles were further investigated in a retrospective study on patients with ALF caused by viral hepatitis, toxic liver injury, Budd-Chiari syndrome, Wilson’s disease, AIH or indeterminate etiology<sup>[26]</sup>. In this study, serum levels of miR-122, miR-21 and miR-221 were found to be significantly increased in patients that showed a spontaneous recovery from ALF compared to non-recovered patients<sup>[26]</sup>. Increased levels of circulating miR-122, miR-21 and miR-221 in patients with a spontaneous recovery from ALF were further associated with increased hepatocyte proliferation and liver tissue regeneration due to decreased expression of the respective miRNAs target genes in the liver like heme-oxygenase-1 (miR-122), programmed cell death 4 (miR-21), p27 and p57 (miR-221)<sup>[26]</sup>.



**Table 1** Summary of circulating miRNAs as diagnostic biomarkers in various liver diseases

Medical condition	miRNA	Serum levels	# of patients	Method for determination	Ref.	
Acute liver failure (drug induced)	miR-122	↑	53	qPCR	Starkey Lewis <i>et al</i> <sup>[33]</sup>	
		↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> <sup>[32]</sup>	
		↑	129	qPCR	Antoine <i>et al</i> <sup>[29]</sup>	
		↑	78	miRNA microarray	Russo <i>et al</i> <sup>[34]</sup>	
	miR-192	↑	53	PCR	Starkey Lewis <i>et al</i> <sup>[33]</sup>	
		↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> <sup>[32]</sup>	
		↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> <sup>[32]</sup>	
	miR-483	↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> <sup>[32]</sup>	
	miR-194	↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> <sup>[32]</sup>	
	miR-210	↑	6	RNA sequencing, qPCR	Krauskopf <i>et al</i> <sup>[32]</sup>	
	miR-4532	↑	78	miRNA microarray	Russo <i>et al</i> <sup>[34]</sup>	
	miR-455-3p	↓	78	miRNA microarray	Russo <i>et al</i> <sup>[34]</sup>	
	miR-1281	↓	78	miRNA microarray	Russo <i>et al</i> <sup>[34]</sup>	
	Liver fibrosis (CHC)	miR-122	↑	53	qPCR	Cermelli <i>et al</i> <sup>[37]</sup>
miR-34a		↑	53	qPCR	Cermelli <i>et al</i> <sup>[37]</sup>	
Liver fibrosis (NAFLD)		miR-122	↑	34	qPCR	Cermelli <i>et al</i> <sup>[37]</sup>
		↑	28	qPCR	Salvoza <i>et al</i> <sup>[38]</sup>	
		miR-34a	↑	34	qPCR	Cermelli <i>et al</i> <sup>[37]</sup>
↑		28	qPCR	Salvoza <i>et al</i> <sup>[38]</sup>		
Liver cirrhosis	miR-513-3p	↑	67	miRNA microarray, qPCR	Roderburg <i>et al</i> <sup>[40]</sup>	
	miR-571	↑	67	miRNA microarray, qPCR	Roderburg <i>et al</i> <sup>[40]</sup>	
	miR-29	↓	67	miRNA microarray, qPCR	Roderburg <i>et al</i> <sup>[41]</sup>	
AIH	miR-21	↑	46	miRNA microarray, qPCR	Migita <i>et al</i> <sup>[47]</sup>	
	miR-122	↑	46	miRNA microarray, qPCR	Migita <i>et al</i> <sup>[47]</sup>	
PSC	miR-1281	↑	40	miRNA microarray, qPCR	Voigtländer <i>et al</i> <sup>[48]</sup>	
	miR-126	↑	40	miRNA microarray, qPCR	Voigtländer <i>et al</i> <sup>[48]</sup>	
	miR-200c	↓	30	miRNA microarray, qPCR	Bernuzzi <i>et al</i> <sup>[49]</sup>	
PBC	miR-505-3p	↓	10	RNA sequencing, qPCR	Ninomiya <i>et al</i> <sup>[50]</sup>	
	miR-197-3p	↓	10	RNA sequencing, qPCR	Ninomiya <i>et al</i> <sup>[50]</sup>	
	miR-122	↑	207	RNA sequencing, qPCR	Tan <i>et al</i> <sup>[51]</sup>	
	miR-141	↑	207	RNA sequencing, qPCR	Tan <i>et al</i> <sup>[51]</sup>	
	miR-26b	↑	207	RNA sequencing, qPCR	Tan <i>et al</i> <sup>[51]</sup>	
	HCC	miR-21	↑	101	qPCR	Xu <i>et al</i> <sup>[58]</sup>
		↑	90	qPCR	Ge <i>et al</i> <sup>[62]</sup>	
		↑	121	qPCR	Tomimaru <i>et al</i> <sup>[64]</sup>	
	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> <sup>[68]</sup>		
	miR-121	↑	101	qPCR	Xu <i>et al</i> <sup>[58]</sup>	
	miR-223	↑	101	qPCR	Xu <i>et al</i> <sup>[58]</sup>	
	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> <sup>[68]</sup>		
miR-16	↓	90	qPCR	Ge <i>et al</i> <sup>[62]</sup>		
↓	40	qPCR	El-Abd <i>et al</i> <sup>[63]</sup>			
miR-122	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> <sup>[68]</sup>		
miR-26a	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> <sup>[68]</sup>		
miR-192	↑	457	miRNA microarray, qPCR	Zhou <i>et al</i> <sup>[68]</sup>		
CCA	miR-21	↑	25	RNA sequencing, qPCR	Correa-Gallego <i>et al</i> <sup>[74]</sup>	
	↑	94	qPCR	Kishimoto <i>et al</i> <sup>[75]</sup>		
	miR-221	↑	25	RNA sequencing, qPCR	Correa-Gallego <i>et al</i> <sup>[74]</sup>	
	miR-150	↑	15	qPCR	Wang <i>et al</i> <sup>[77]</sup>	
	miR-224	↑	30	qPCR	Huang <i>et al</i> <sup>[76]</sup>	

miRNA: MicroRNA; CHC: Chronic hepatitis C; NAFLD: Non-alcoholic fatty liver disease; qPCR: Quantitative RT-PCR; AIH: Autoimmune hepatitis; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis; HCC: Hepatocellular carcinoma; CCA: Cholangiocarcinoma; ↑: High circulating levels; ↓: Low circulating levels.

In summary, measurement of circulating miRNAs might represent important serum biomarkers for ALF and help to improve the prediction of patients' prognosis even at an early time point after liver injury. Table 1 summarizes potential diagnostic biomarker for ALF.

## LIVER FIBROSIS AND CIRRHOSIS

Liver fibrosis and liver cirrhosis represent the most common end-points of chronic liver diseases such as alcoholic steatohepatitis, non-alcoholic steatohepatitis,

and viral hepatitis, which are all associated with a high morbidity and mortality. Currently, histology is considered the gold standard for the diagnosis and staging of liver fibrosis and/or cirrhosis. However, this procedure is related to a number of problems, including the risks for serious complications during liver biopsy, sampling errors and biases, variabilities in histopathologic interpretation and significant financial costs. Thus, alternative non-invasive strategies for the evaluation of liver fibrosis/cirrhosis are of increasing interest. In this context, besides other markers, circulating miRNAs have been

considered by many authors as promising serum based biomarkers with a potential for being used in clinical routine.

In the past, an overwhelming amount of data supporting a role for miRNAs in the development and progression of chronic liver diseases into liver cirrhosis and finally hepatocellular carcinoma (HCC) was presented (reviewed e.g., in<sup>[35]</sup>). Based on these data, the group of El-Ahwany analyzed serum levels of different miRNAs with an established role in the activation of hepatic stellate cells (HSC) in sera of 66 subjects with early stage liver fibrosis and 65 subjects with late-stage fibrosis<sup>[36]</sup>. Forty healthy subjects served as normal controls. In line to their role in the activation of HSC, serum concentrations of miR-138, miR-140, miR-143, miR-325, miR-328 and miR-349 were significantly elevated in patients with fibrosis compared to healthy controls. ROC analysis revealed a sensitivity and specificity of miR-138 of 89.3% and 71.43% for prediction of early stage fibrosis and of 89.3% and 93.02% for prediction of late stage fibrosis, respectively, demonstrating that analyses of circulating miRNAs might be helpful to detect even early stages of liver fibrosis. Besides these miRNAs, several groups demonstrated that levels of miR-34a, one of the best investigated miRNAs in the context of chronic liver diseases, are elevated in patients with liver fibrosis<sup>[37-39]</sup>. In a large cohort of patients, Cermelli *et al.*<sup>[37]</sup> described elevated levels of miR-34a in patients with both hepatitis C (CHC)- and NAFLD-dependent liver fibrosis. Interestingly, levels of miR-34 were independent of the viral load but reflected the stage of disease in both disease entities. In this study, miR-34a correlated with AST/ALT levels, stage of fibrotic disease, inflammatory activity and serum lipids in NAFLD patients, highlighting that levels of circulating miRNAs might reflect specific aspects in the pathophysiology of chronic liver diseases<sup>[37]</sup>. In line with this assumption, we described elevated levels of miR-513-3p and miR-571 in patients with alcohol- or hepatitis C-induced liver cirrhosis. However, only serum level of miR-571 reflected the disease severity in liver cirrhosis, while miR-513-3p was independent on the stage of fibrosis or inflammatory activity in these patients<sup>[40]</sup>. Besides these up-regulated miRNAs, a down-regulation of circulating miR-29 was found in patients with chronic liver injury and liver fibrosis. Levels of miR-29 correlated with the stage of liver fibrosis, MELD score and disease entity<sup>[41]</sup>. In the context of alcohol induced liver injury, microarray based screening of exosomal miRNAs revealed an up-regulation of miRNA-192, miRNA-122, miRNA-30a, miRNA-744, miRNA-1246, miRNA 30b and miRNA-130a in blood sera of chronic alcohol-fed mice compared to healthy controls<sup>[42]</sup>. Moreover, ROC curve analyses indicated a diagnostic potential of miRNA-192, miRNA-122, and miRNA-30a for the identification of alcohol-induced liver injury<sup>[42]</sup>.

Recently, the group of Matsuura *et al.*<sup>[43]</sup> attempted to determine whether circulating miRNAs might be used to estimate disease progression in chronic hepatitis C

patients. One hundred and thirty CHC patients were prospectively followed. In this study, reduced plasma levels of the let7-family reflected a more advanced fibrosis stage whereas elevated concentrations of miR-122-5p were indicative for an increased inflammatory activity, but not for the degree of liver fibrosis<sup>[43]</sup>. In another large cohort of CHC-patients, Trebicka *et al.*<sup>[44]</sup> demonstrated that circulating miR-122 levels positively correlated with an enhanced inflammatory activity but negatively with liver fibrosis, which was most probably due to the loss of liver cells (as the major source of miR-122) during chronic liver injury. Interestingly, miR-122 serum levels were associated with the survival of CHC-cirrhosis patients independent of the MELD score, sex and age<sup>[45]</sup>, underscoring the potential of this liver specific miRNA in the diagnosis of liver fibrosis and cirrhosis.

In summary, circulating miRNAs might represent diagnostic and prognostic biomarkers in patients with liver fibrosis or cirrhosis.

## AUTOIMMUNE LIVER DISEASE

Although autoimmune liver diseases have gained rising importance in the field of hepatology due to its increasing incidence over the last decades<sup>[46]</sup>, only very few studies have evaluated the involvement of circulating miRNAs in AIH, primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC).

To our knowledge only one study investigating circulating miRNAs in patients with AIH exists to date. In this study, serum samples of 46 type-1 AIH patients were screened for 2555 miRNAs using a microarray system and compared to patients with chronic hepatitis C and healthy controls. Circulating levels of miR-21 and miR-122 were significantly higher in AIH patients compared to both control groups. Interestingly, the authors observed a strong decrease of miR-21 and miR-122 levels after treatment with glucocorticoids, indicating a potential role of these miRNA not only as a diagnostic marker but also as a marker to assess treatment response<sup>[47]</sup>.

In PSC patients, serum levels of miR-1281 and miR-126 were shown to be significantly increased compared to healthy controls. Importantly, the elevation of these miRNAs in PSC patients was also significantly higher compared to CCA patients, arguing that miR-1281 and miR-126 might reflect disease-specific processes of PSC that do not or to a lesser extend occur during malignant transformation of bile duct cells into CCA<sup>[48]</sup>. Moreover, Bernuzzi *et al.*<sup>[49]</sup> described miR-200c as significantly down-regulated in patients with PSC in large screening approach including 667 miRNAs.

In PBC patients, a deep sequencing approach revealed circulating levels of miR-505-3p and miR-197-3p as significantly decreased when compared to healthy controls<sup>[50]</sup>. However, this study was performed in a very small cohort of patients ( $n = 10$ ) and needs further validation. In another study, Tan *et al.*<sup>[51]</sup> establish a diagnostic serum miRNA panel in a cohort of 207 PBC

patients using a stepwise logistic regression model. The panel, consisting of miR-122, miR-141 and miR-26b, had an AUC of 0.905 for the discrimination between PBC patients and healthy control, which was superior to established biomarkers for PBC such as AP and ANA.

In summary, the role of circulating miRNA in autoimmune liver disease has so far only been analyzed in a very limited number of studies with comparatively small cohort sizes. Thus, further studies are needed to make a clear statement on the potential role of serum miRNAs as a biomarker for AIH, PSC and PBC.

## LIVER CANCER

Circulating miRNAs have also become of increasing interest as biomarkers for hepatic and hepatobiliary malignancies. The following section reviews the emerging role of circulating miRNAs in the field of HCC and cholangiocarcinoma (CCA).

### HCC

HCC represents the most common primary tumor of the liver and shows a steadily increasing incidence rate in most areas of the world<sup>[52,53]</sup>. Despite being the sixth most common type of cancer worldwide, HCC is the second leading cause of cancer related death among men worldwide, corroborating the dismal prognosis of this disease<sup>[54]</sup>. Even in medically developed countries such as the United States, HCC patients face a 1-year and 5-year survival rate of less than 50% and 10%, respectively<sup>[55]</sup>. Since early detection of HCC is essential to provide patients with a potentially curative therapeutic approach and established tumor markers such as AFP feature a limited diagnostic potential especially at an early stage of disease, circulating miRNAs as biomarkers for HCC might help to improve the disease's poor prognosis.

As the most abundantly expressed miRNA in human liver tissue<sup>[56]</sup>, miRNA-122 was found to be up-regulated in serum samples of HCC patients, showing a sensitivity and specificity of 81.6% and 83.3%, respectively when compared to healthy controls<sup>[57,58]</sup>. Nevertheless, as shown before, circulating levels of miR-122 were also described for different non-malignant hepatic diseases<sup>[59]</sup>, arguing for a rather unspecific characteristic of this miRNA. Interestingly, expression levels of miR-122 were decreased in HCC tissue samples<sup>[60]</sup>, suggesting a potential mechanism of miRNA secretion from HCC cells into the bloodstream.

Moreover, serum levels of exosomal miR-18a, miR-221, miR-222 and miR-224 were significantly higher whereas exosomal miR-101, miR-106b, miR-122 and miR-195 were significantly lower in patients with HCC compared to patients with chronic hepatitis B or liver cirrhosis<sup>[61]</sup>. Furthermore, circulating levels of miR-16 were shown to be down-regulated in patients with HCC, correlating with tumor size and were further able to discriminate HCC from chronic HCV patients<sup>[62,63]</sup>. In contrast non-malignant liver conditions such as NAFLD and chronic hepatitis C showed increased miR-16 serum levels<sup>[37]</sup>, making the

down-regulation of serum miR-16 levels in HCC a fairly specific marker for liver cancer. Serum levels of miR-21 represent a further promising tool for the diagnosis of HCC. Tomimaru *et al.*<sup>[64]</sup> showed that circulating levels of miR-21 can reliably distinguish between HCC patients and healthy controls as well as patients with chronic hepatitis and are superior to the diagnostic potential of AFP. They also found a decrease of miR-21 serum levels after tumor resection, underlining the potential specificity of this miRNA for HCC<sup>[64]</sup>. However, elevated levels of circulating miR-21 were also described in patients with different other types of gastrointestinal cancer<sup>[65,66]</sup>. A recently published meta-analysis on circulating levels of miR-21 described a pooled sensitivity of 81.2% with a specificity of 84.8% for the diagnosis of HCC<sup>[67]</sup>.

Given the fact that the diagnostic power of a single miRNA is limited, various panels consisting of more than one circulating miRNAs have been evaluated as well. Using a microarray to screen 723 miRNAs, Zhou *et al.*<sup>[68]</sup> found a panel of seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) that distinguished between HCC and healthy controls (AUC = 0.941), chronic hepatitis B (AUC = 0.842) and liver cirrhosis (AUC = 0.884) even at an early stage of disease (BCLC 0) in three independent cohorts of 934 participants. In comparison to AFP, another miRNA panel of seven miRNAs (miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505) was shown to have a superior AUC regarding the diagnosis of small-size (AUC = 0.833 vs AUC = 0.727) and early-stage (AUC = 0.824 vs AUC 0.754) HCCs. This panel did also have the ability to detect AFP-negative HCC patients<sup>[69]</sup>. Similar results were obtained for a panel consisting of miR-15b and miR-130 that showed a sensitivity of 98.2% with a specificity of 91.5% for the diagnosis of HCC and had detection sensitivity of 96.7% for patients with low AFP serum levels (< 20 ng/mL)<sup>[70]</sup>.

Circulating miRNAs might also help to assess patients' outcome and their likelihood to benefit from different treatment options (surgery, systemic treatment, locally ablative treatment) in order to find a personalized therapy for individual patients. For instance, serum levels of miR-221 correlated with tumor size and tumor stage and patients with high levels of circulating miR-221 showed a significantly reduced overall survival compared to patients with lower miR-221 levels<sup>[71]</sup>. Likewise, high serum levels of miR-122 were found to independently predict a poor overall survival in a cohort of 122 HCC patients<sup>[72]</sup>.

### CCA

Although CCA represents a rare type of cancer in most parts of the world, it shares a very unfavorable prognosis with a 5-year survival rate of less than 5% for advanced disease stage<sup>[73]</sup>. Again, early detection of CCA is necessary to offer patients a surgical tumor resection, which is the only potentially curative treatment option, but the established tumor markers CA19-9 and CEA have a

restricted diagnostic power. Besides the available data on their potential role as a biomarker for HCC, miRNAs have also been evaluated in few studies as a diagnostic tool for CCA.

Based on a CCA tissue expression analysis, which revealed 262 regulated miRNAs in tumor samples, circulating levels of miR-21 and miR-221 were found to be significantly elevated in patients with intrahepatic CCA, showing a high discrimination ability of miR-21 between patients and healthy controls (AUC = 0.94)<sup>[74]</sup>. Nevertheless, these results are limited due to a small number of analyzed patients ( $n = 25$ ). MiR-21 was further evaluated in a cohort of 94 patients with biliary tract cancer (BTC) and showed an AUC of 0.93 and 0.83 for the differentiation between BTC and healthy controls and BTC and non-malignant bile duct disease, respectively<sup>[75]</sup>. Interestingly, serum levels of miR-21 decreased after surgical tumor resection<sup>[75]</sup>. In another rather small study including a total of 30 CCA patients, Bernuzzi *et al.*<sup>[49]</sup> identified circulating miR-483-5p and miR-194 as dysregulated in CCA patients. Furthermore, serum levels of miR-483-5p and miR-222 were able to discriminate between PSC and CCA patients. Other circulating miRNAs that were shown to be dysregulated in CCA patients are miR-224<sup>[76]</sup> and miR-150<sup>[77]</sup>.

Some studies have also evaluated a potential use of miRNAs as a prognostic tool for CCA. Analyzing 103 patients with CCA, Cheng *et al.*<sup>[78]</sup> described decreased serum levels of miR-106 as a predictor for poor survival. Moreover, elevated levels of circulating miR-26a correlated with disease stage and were reported to be an independent prognostic marker for CCA patients.

In summary, circulating miRNAs are of increasing interest for the diagnosis and prognosis of liver cancer. Although reliable data on serum/plasma miRNAs in the field of CCA are limited, circulating miRNAs are likely to play a decisive role for an early detection and the prediction of survival for both analyzed types of liver cancer in future. However, as the diagnostic and prognostic power of a single miRNA is limited, panels of different miRNA are needed to exceed the established biomarkers for liver cancer. In this context, larger studies will help to further evaluate and verify potential miRNAs for these purposes.

## CONCLUSION

Circulating miRNAs represent a promising new tool for the diagnosis and prediction of prognosis for various acute and chronic liver diseases. Despite their obvious potential as biomarkers, there are several problems that prevent the use of circulating miRNAs as diagnostic tools in clinical routine. Most importantly, despite years of intensive research no consensus on optimal protocols for standardization of sample collection, data normalization and analysis was reached until now. As qPCR and microarray based measurements naturally depend on the design of miRNA specific primers or microarray probes, similarities between different miRNAs might result in

further difficulties regarding the comparison between studies. Moreover, data normalization issues mainly arise from the lack of a valid intrinsic RNA housekeeping gene for human serum samples and high inter-platform differences in miRNA quantification efficacy contribute to a poor comparability between studies. Finally, most studies are carried out as single center study including only a small number of patients. Therefore, next generation sequencing might have an important impact on the validation of miRNA profiles, as it allows mostly sequence independent, parallel measurement and detection of overall numbers of a broad spectrum of different miRNAs (reviewed *e.g.*, in<sup>[79]</sup>). Thus, only if these present limitations can be overcome, circulating miRNAs might take the next step to be finally implemented in diagnostic algorithms or be used to estimate the clinical fate of patients with acute or chronic liver diseases.

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Retrospective Study

# Diagnosis of morbid obesity may not impact healthcare utilization for orthotopic liver transplantation: A propensity matched study

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**Data sharing statement:** Technical appendix, statistical code, and dataset available from the corresponding author at [khalid.mumtaz@osumc.edu](mailto:khalid.mumtaz@osumc.edu).

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## Abstract

### AIM

To study mortality, length of stay, and total charges in morbidly obese adults during index hospitalization for orthotopic liver transplantation.

### METHODS

The Nationwide Inpatient Sample was queried to obtain demographics, healthcare utilization, post orthotopic liver transplantation (OLT) complications, and short term outcomes of OLT performed from 2003 to 2011 ( $n = 46509$ ). We divided patients into those with [body mass index (BMI)  $\geq 40$ ] and without (BMI  $< 40$ ) morbid obesity. Multivariable logistic regression analysis was performed



to characterize differences in in-hospital mortality, length of stay (LOS), and charges for OLT between patients with and without morbid obesity after adjusting for significant confounders. Additionally, propensity matching was performed to further validate the results.

## RESULTS

Of the 46509 patients who underwent OLT during the study period, 818 (1.8%) were morbidly obese. Morbidly obese recipients were more likely to be female (46.8% *vs* 33.4%,  $P = 0.002$ ), Caucasian (75.2% *vs* 67.8%,  $P = 0.002$ ), in the low national income quartile (32.3% *vs* 22.5%,  $P = 0.04$ ), and have  $\geq 3$  comorbidities (modified Elixhauser index; 83.9% *vs* 45.0%,  $P < 0.001$ ). Morbidly obese patient also had an increase in procedure related hemorrhage ( $P = 0.028$ ) and respiratory complications ( $P = 0.043$ ). Multivariate and propensity matched analysis showed no difference in mortality (OR: 0.70; 95%CI: 0.27-1.84,  $P = 0.47$ ), LOS ( $\beta$ : -4.44; 95%CI: -9.93, 1.05,  $P = 0.11$ ) and charges for transplantation ( $\beta$ : \$15693; 95%CI: -51622-83008,  $P = 0.64$ ) between the two groups. Morbidly obese patients were more likely to have transplants on weekdays (81.7%) as compared to those without morbid obesity (75.4%,  $P = 0.029$ ).

## CONCLUSION

Morbid obesity may not impact in-hospital mortality and health care utilization in OLT recipients. However, morbidly obese patients may be selected after careful assessment of co-morbidities.

**Key words:** Deceased donors; Outcome; Complications; Economics; Selection criteria

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**Core tip:** Morbid obesity is a relative contraindication to orthotopic liver transplantation. Previous studies, mostly in the pre-MELD era, suggested worsened outcomes in these patients. As the prevalence of obesity continues to increase, so will the number of patients who are morbidly obese requiring liver transplantation. Utilizing the Nationwide Inpatient Sample which is the largest publicly available database in the United States, we did not find any difference in mortality, or healthcare utilization when comparing those with and without morbid obesity receiving liver transplantation. Our findings suggest that in highly selected patients, morbid obesity may not be a significant contraindication to transplantation.

Peck JR, Latchana N, Michaels A, Hanje AJ, Hinton A, Elkhammas EA, Black SM, Mumtaz K. Diagnosis of morbid obesity may not impact healthcare utilization for orthotopic liver transplantation: A propensity matched study. *World J Hepatol* 2017; 9(12): 595-602 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i12/595.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i12.595>

## INTRODUCTION

There has been a great deal of attention given to the outcomes of orthotopic liver transplantation (OLT) in obese patients, with varying reports on morbidity and mortality. A study by Nair *et al*<sup>[1]</sup> investigated graft and patient survival in obese patients receiving OLT in the United States between 1988 through 1996 using the United Network for Organ Sharing database. They found that primary graft non-function and immediate 1-year and 2-year mortality were higher in morbidly obese individuals. They also found increased 5-year mortality in morbidly [body mass index (BMI) of 35.1-40 kg/m<sup>2</sup>] obese patients. Contrary to that, Pelletier *et al*<sup>[2]</sup>, reported no increased risk of post-transplant mortality in obese or morbidly obese patients recruited from 2001 to 2004. The disparities between the aforementioned studies by Nair *et al*<sup>[1]</sup> and Pelletier *et al*<sup>[2]</sup> can likely be attributed to the Nair *et al*<sup>[1]</sup> study occurring in the pre-MELD era as compared to within or just before the application of MELD by Pelletier.

Greater peri-operative morbidity and increased post-operative length of stay appears to be a fairly consistent finding in the morbidly obese patients in various studies<sup>[3-5]</sup>. A few studies do report increased wound related and infectious complications in patients with morbid obesity after transplantation<sup>[3,6]</sup>. In one study, obese patients surprisingly did not require prolonged ventilation support as compared to non-obese patients<sup>[6]</sup>.

Studies have also shown socioracial disparities in OLT utilization. In addition to race, women, older patients, individuals with non-commercial insurance, individuals in certain geographic locations (as defined by donor service areas), and those with alcoholic liver disease have been shown to receive lower rates of transplantation<sup>[7]</sup>.

Large population based studies from United States on health care utilization and short term outcomes of liver transplantation in morbidly obese patients were not found. We hypothesized that provided selected carefully morbidly obese patients undergoing liver transplantation may not have different healthcare utilization and short term outcomes. We studied the health care utilization, in-hospital morbidity, mortality and direct charges for care in morbidly obese patients receiving OLT in the United States during 2003-2011.

## MATERIALS AND METHODS

### Database information

The Nationwide Inpatient Sample (NIS) is the largest publicly available database in the United States. It contains data from over 8 million hospital stays each year, and allows users to track and analyze trends and outcomes of health care. The NIS database is the largest all-payer inpatient care database in the United States, representing an approximately 20% stratified sample of 1044 non-

federal hospitals in 47 states<sup>[8]</sup>.

The information was collected from the NIS database from years 2003 to 2011 among all adult (age > 18 years) in-patients with a procedure code for liver transplantation as determined by International Classification of Disease-Clinical Modification, Ninth Revision, (ICD-CM) codes. According to weighted estimate, 47185 adult patients were identified who underwent liver transplantation with ICD-CM procedure code 50.59 (other liver transplantation, *i.e.*, non-auxiliary).

The NIS database has limited clinical variables, but it provides a large sample size representative of the United States. Moreover, it is reliable in terms of hard end-points such as inpatient mortality and hospital length of stay. Another unique feature of this database is information on the direct charges for hospital stay, which have not been studied in the past among obese liver transplant recipients. Additional data collected including healthcare utilization were, age, gender, race, income (National Quartile), type of insurance, type of hospital (rural/urban non-teaching vs urban teaching), hospital size, hospital region, and Modified Elixhauser index based on pre-OLT comorbid medical conditions<sup>[9]</sup>. This index counts the number of comorbidities present from a list of 29. We modified it by removing liver failure and morbid obesity.

We divided the patients into those with morbid obesity (BMI  $\geq 40$ ) and with a BMI < 40. The following ICD-9 codes were used for morbid obesity, 278.01, V85.4, V85.41, V85.42, V85.43, V85.44 and V85.45. Patients without one of the previous codes present were assumed to have a BMI under 40. We chose a BMI cutoff of 40 as previous studies have shown that when compared to lower BMIs, there is a higher sensitivity and specificity when accounting for correct documentation<sup>[10]</sup>. Variables studied among two groups were the pre-OLT comorbidities and post-OLT complications. We divided the post OLT complications into two distinct categories, *i.e.*, systemic and technical. Systemic complications included those which were among broader groups of events for which timing was indeterminate (*i.e.*, cardiovascular complications, Post-LT infections, *etc.*). Technical complications were felt to be related to the actual surgery itself<sup>[11]</sup>.

### Outcomes and predictors

We studied outcomes including mortality during the hospitalization for OLT, length of hospital stay, total direct charges for care (without professional fees) among patients with and without morbid obesity. The NIS quantifies inpatient discharges and does not link patients across hospital discharges. As such, patients with multiple discharges may have been counted multiple times if they had multiple hospitalizations where the procedure code for OLT was documented.

The major pre-, intra, and postoperative complications were identified using ICD-9-CM diagnostic codes (appendix 1). As the ICD-9-CM coding system does not include transplant-specific codes for many of the

postoperative variables that are of particular interest, the best available codes were used.

This study was exempted from review by The Ohio State University Institutional Review Board.

### Statistical analysis

SAS 9.3 (SAS Institute, Cary, NC) was used to perform all analyses, employing appropriate survey estimation commands and strata weights. Weighted frequencies and percentages were calculated for all categorical variables; means and 95% CIs were calculated for continuous variables. Differences between patients with and without morbid obesity (BMI  $\geq 40$ ) were analyzed using  $\chi^2$  tests or student's *t*-tests, as appropriate. Variables significantly associated with morbid obesity on univariate analysis were included in all multivariate models. We performed a multivariate logistic regression for mortality, while multivariate linear regression was used for length of stay and total hospital charges.

Propensity scores were calculated using a multivariate logistic regression model for morbid obesity containing all demographic variables (Age, Gender, Race, Income, Insurance, Hospital Location, Teaching Status, Size, and Region), and comorbid conditions (29 Elixhauser comorbidities excluding obesity and liver failure).

Patients with and without morbid obesity were then matched 1:1 using a greedy matching algorithm with a caliper of 0.2 times the standard deviation of the propensity scores. One hundred and forty-three pairs were formed. One hundred and forty-three of the original 145 (unweighted number) patients with morbid obesity were matched with a control. Note that our cohort contains 168 patients with morbid obesity; however, only 145 of the 168 were eligible for matching due to missing data primarily within the race variable.

The gmatch macro written by the Mayo Clinic was used for the matching. The statistical methods of this study were reviewed by Alice Hinton from the Ohio State University (<http://www.mayo.edu/research/departments-divisions/departments-health-sciences-research/division-biomedical-statistics-informatics/software/locally-written-sas-macros>).

## RESULTS

### Demographics

After weighting, the NIS represented 46509 patients who underwent liver transplantation from 2003 through 2011. Of these patients, 818 (1.8%) were morbidly obese. The demographic and hospital characteristic variables are shown in Table 1. The groups were similar with regards to age, type of insurance, type and region of hospital. There were more females among the morbidly obese group (46.8%) as compared to without morbid obesity (33.4%),  $P = 0.002$ . There were more transplant recipients belonging to white race (75.2% vs 67.8%,  $P = 0.002$ ) and low national income quartile (32.3% vs 22.5%,  $P = 0.04$ ) among morbidly obese patients as

**Table 1** Demographic and hospital characteristics in morbidly obese and non-morbidly obese patients who underwent a liver transplant

	No morbid obesity <i>n</i> = 45691		Morbid obesity <i>n</i> = 818		<i>P</i> -value
Age (mean, CI)	53.23	(52.84, 53.61)	53.33	(52.05, 54.61)	0.87
Gender					0.002
Male	30444	66.64%	435	53.21%	
Female	15242	33.36%	383	46.79%	
Race					0.002
White	25668	67.81%	544	75.15%	
Black	2975	7.86%	32	4.49%	
Hispanic	5638	14.90%	127	17.60%	
Other	3571	9.43%	20	2.75%	
Income (National Quartile)					0.04
Low	9947	22.46%	258	32.30%	
Moderate	11190	25.27%	213	26.63%	
High	11816	26.69%	167	20.87%	
Very high	11324	25.58%	161	20.20%	
Type of insurance					0.11
Medicare	11817	25.99%	246	30.10%	
Medicaid	6487	14.27%	74	8.99%	
Private	24983	54.95%	441	53.93%	
Other	2179	4.79%	57	6.97%	
Type of hospital					0.95
Rural/urban	233	0.51%	< 10	0.48%	
non-teaching					
Urban teaching	45069	99.49%	814	99.52%	
Hospital size					0.25
Small/medium	6492	14.33%	88	10.74%	
Large	38809	85.67%	730	89.26%	
Hospital region					0.43
Northeast	7865	17.21%	118	14.42%	
Midwest	9953	21.78%	206	25.24%	
South	15116	33.08%	319	39.02%	
West	12757	27.92%	174	21.32%	
Admission day					0.02
Week day	34444	75.39%	668	81.72%	
Weekend	11247	24.62%	149	18.28%	
Modified elixhauser index <sup>1</sup>					< 0.01
< 3	25123	54.98%	131	16.06%	
≥ 3	20568	45.02%	686	83.94%	

<sup>1</sup>After excluding liver failure and obesity.

compared to those without morbid obesity. In addition, morbidly obese transplant recipients had significantly more comorbid conditions with  $\geq 3$  conditions ( $n = 686$ ; 83.9%) on the modified Elixhauser index than those without morbid obesity ( $n = 20568$ ; 45.0%),  $P < 0.001$ . Lastly, morbidly obese patients were more likely to have transplants on weekdays (81.7%) as compared to those without morbid obesity (75.4%,  $P = 0.028$ ).

### Post OLT complications

Table 2 shows the various post OLT complications in patients who underwent liver transplantation. Among systemic post OLT complications, there were significantly more respiratory complications in morbidly obese patients (4.87% vs 1.05%,  $P = 0.04$ ) after transplant. Contrary to that, hemorrhage complicating a procedure

was significantly higher in non-morbidly obese patients (11.80% vs 7.04%,  $P = 0.03$ ) as compared to morbidly obese patients. However, all other post OLT complications were equally distributed in the two groups. Similarly, hepatic artery thrombosis ( $P = 0.05$ ), anastomotic biliary leaks ( $P = 0.08$ ), and accidental laceration during a procedure ( $P = 0.06$ ) were more frequent in non-morbidly obese, though they did not reach statistical significance. Overall, complication rates were equally distributed in the two groups.

### Multivariate analysis

Table 3 shows the adjusted odds ratio (aOR) for mortality and  $\beta$ -coefficients for length of stay and charges for liver transplantation in the non-morbidly obese and morbidly obese groups. Non-morbidly obese patients had a 5.27% mortality whereas the mortality among morbidly obese transplant recipients was 4.83% (aOR: 0.98; 95%CI: 0.50-1.92,  $P = 0.95$ ). The average length of stay in non-morbidly obese patients was 20.9 d and in morbidly obese patients it was 18.7 d ( $\beta$ : -3.90; 95%CI: -7.94-0.14,  $P = 0.06$ ). The average total charges for transplantation was \$342324 and \$378452 in non-morbidly obese and morbidly obese patients, respectively ( $\beta$ : \$612; 95%CI: -54780-56004,  $P = 0.98$ ). Data was adjusted for gender, race, income, modified Elixhauser comorbidity index, weekend admission, and diabetes.

### Propensity based analysis

In order to further endorse our findings, a matched cohort on the basis of morbid obesity status was then created using propensity scores. The propensity score analysis was not able to account for the weighting in the dataset. Before weights were taken into account 168 of the OLT patients were morbidly obese. Of the 168 patients 143 (85%) were matched 1:1 with a non-morbidly obese patient on the basis of propensity scores. Thus, in this cohort, there were a total of 286 patients divided equally into two groups based on morbid obesity status (143 patients each in morbidly obese and non-morbidly obese groups). After propensity matching, no differences between pre- and post OLT variables in the two groups were statistically significant (appendix 2). This allowed analysis of outcomes based on morbid obesity status alone, thereby reducing selection bias based on various other characteristics. Analysis showed no significant difference in mortality (OR: 0.70; 95%CI: 0.27-1.84,  $P = 0.47$ ), LOS ( $\beta$ : -4.44; 95%CI: -9.93-1.05,  $P = 0.11$ ) or charges for transplantation ( $\beta$ : \$15693; 95%CI: -51622-83008,  $P = 0.64$ ) between two groups (Table 4).

## DISCUSSION

In this Nationwide Inpatient Sample database study we found that the diagnosis of morbid obesity may not have a significant impact on the health care utilization in the liver transplant cohort. We found that 1.8% of

**Table 2** Complications of patients who underwent a liver transplant

	No morbid obesity <i>n</i> = 45691		Morbid obesity <i>n</i> = 818		<i>P</i> -value
Systemic complications					
Any	20546	44.97%	394	48.20%	0.5253
Post LT infection	13308	29.13%	297	36.26%	0.2103
Cardiovascular complication	781	1.71%	25	3.05%	0.3858
Infections, surgical wound	2035	4.45%	35	4.29%	0.9301
Cardiac complications	1972	4.32%	49	6.00%	0.2737
Peripheral vascular complications	152	0.33%	0	0.00%	--
Respiratory complications	481	1.05%	40	4.87%	0.0433
Digestive system complications	95	0.21%	≤ 10	1.12%	0.2376
Other postoperative infection	2035	4.45%	35	4.29%	0.9301
Pulmonary insufficiency following surgery	269	0.59%	≤ 10	0.57%	0.9654
Unspecified intestinal obstruction	145	0.32%	0	0.00%	--
Stroke	149	0.33%	0	0.00%	--
Postoperative shock	69	0.15%	≤ 10	0.57%	0.4556
Post LT complication	9927	21.73%	142	17.40%	0.1441
Technical complications					
Any	16044	35.11%	263	32.27%	0.4206
Hepatic artery thrombosis	8940	19.57%	113	13.80%	0.0531
History of exploratory laparotomy exploratory laparotomy	221	0.48%	≤ 10	0.57%	0.8483
Anastomotic leak of biliary tree	1442	3.16%	49	6.00%	0.0837
Perforation of the intestine	148	0.32%	0	0.00%	--
Hemorrhage complicating a procedure	5390	11.80%	58	7.04%	0.0278
Accidental laceration during a procedure	965	2.11%	≤ 10	0.67%	0.0611
Iatrogenic pulmonary embolism and infarction	169	0.37%	20	2.49%	0.0862
Iatrogenic pneumothorax	691	1.51%	≤ 10	1.14%	0.6429
Hematoma	3487	7.63%	65	7.94%	0.8931
Seroma complicating a procedure	74	0.16%	≤ 10	1.15%	0.2145
Disruption of wound	25	0.06%	0	0.00%	--
Disruption of internal operation wound	179	0.39%	0	0.00%	--
Disruption of external operation wound	378	0.83%	20	2.43%	0.1632

Variables are expressed as weighted frequency (percentage) and differences between the groups are analyzed with  $\chi^2$  tests. LT: Liver transplantation.

**Table 3** Results of multivariate linear/logistic regression for mortality, length of stay and charges for liver transplantation in study cohort

Outcomes	No morbid obesity <i>n</i> = 45691 (%)	Morbid obesity <i>n</i> = 818 (%)	Adjusted OR/ $\beta$ -coefficient (95%CI)	<i>P</i> -value
Mortality	2407 (5.27%)	39 (4.83%)	0.98 (0.50-1.92)	0.95
Length of stay in days, mean (CI)	20.9 (18.7-23.1)	18.7 (15.5-22)	-3.9 <sup>1</sup> (-7.94-0.14)	0.06
Total charges, mean (CI)	342324 (305778-378870)	378452 (320453-436452)	612 <sup>1</sup> (-54780-56004)	0.98

<sup>1</sup> $\beta$ -coefficients. Data was adjusted for gender, race, income, modified Elixhauser comorbidity score, weekend admission, and diabetes.

patients who underwent liver transplantation from 2003 to 2011 were morbidly obese, *i.e.*, BMI  $\geq 40$ . Moreover, morbidly obese transplant recipients were more likely to be females, Caucasian, low national income quartile, and had OLT surgeries on weekdays; they also had more pre-transplant comorbid conditions based on the modified Elixhauser index. The majority of post-OLT complications, except procedure related hemorrhage and respiratory complications were equally distributed in all transplant recipients. Despite these differences, in pre- and post-liver transplant issues, no difference in mortality, LOS or charges for transplantation was observed in the two groups.

In our study the incidence of morbidly obese OLT recipients is equal to previous studies by Nair *et al*<sup>[1]</sup> but less than Pelletier *et al*<sup>[2]</sup>. However, the prevalence

of morbid obesity reported in the general population is approximately 6.4%. This discrepancy is likely due to the plausible super-selective nature of transplantation candidacy. Obese candidates are at a higher risk for mortality may now be more readily identified and carefully selected. Whereas obesity in itself is not an indication for invasive pre-cardiac screening, obesity-related comorbidities such as coronary artery disease, hypertension, and dyslipidemia may warrant cardiac catheterization or additional testing<sup>[12]</sup>. This allows for detection of morbidly obese individuals with severe cardiac disease which precludes liver transplantation.

We found no statistically significant difference between healthcare utilization in our cohort of morbidly obese and non-morbidly obese patients. Previous studies have shown that individuals referred for OLT were more likely to have



**Table 4 Analysis of outcomes in the propensity matched sample**

Outcomes	No morbid obesity <i>n</i> = 143	Morbid obesity <i>n</i> = 143	Adjusted OR/ $\beta$ -coefficient (95%CI)	<i>P</i> -value
Mortality	10 (7.04%)	< 10 (4.93%)	0.70 (0.27-1.84)	0.47
Length of stay in days, mean (CI)	24.1 (19.5-28.7)	19.6 (16.8-22.5)	-4.44 (-9.93-1.05) <sup>1</sup>	0.11
Total charges, mean (CI)	388530 (344027-33033)	395518 (349932-441105)	15693 (-51622-83008) <sup>1</sup>	0.64

<sup>1</sup> $\beta$ -coefficients.

private insurances<sup>[13]</sup>. As would be expected, the majority of individuals who receive liver transplantation also had private insurances (55% and 54% for non-morbidly obese and morbidly obese, respectively); however, there was no overall difference between the two groups among utilization of Medicaid, Medicare, and others ( $P = 0.11$ ). The vast majority of both groups of liver recipients were transplanted at urban teaching hospitals ( $> 99\%$ ,  $P = 0.95$ ), similar to trends reported in other studies<sup>[14]</sup>. There also was no statistically significant difference between groups for hospital size ( $P = 0.24$ ) or hospital region ( $P = 0.43$ ). Current guidelines from the American Association for the Study of Liver Disease consider morbid obesity a relative contraindication to liver transplantation<sup>[15]</sup>. Previously reported data on outcomes in morbidly obese transplant recipients has been contradictory, with some studies showing equivalent outcomes<sup>[16,17]</sup> while others showed increased post-operative complications<sup>[18]</sup> and decreased survival<sup>[1]</sup>. Importantly, we found that there was no statistically significant increase in mortality for morbidly obese liver transplant recipients. This contrasts data from previous studies which suggest higher rates of mortality in morbidly obese patients after transplant. The differences reported in peri-operative mortality and morbidity in studies can potentially be explained by heterogeneity amongst the obese and morbidly obese patients. Also the sample size and effect of era may be responsible for the variability in outcomes.

Obesity has been shown to be protective in patients in many settings, including the intensive care unit and in patients with severe sepsis<sup>[19,20]</sup>. There are multiple hypotheses for the improved outcomes seen in obese patients in these settings. It has been demonstrated that obesity leads to loss of tissue homeostasis and development of an inflammatory response characterized by an accumulation of pro-inflammatory type-1 phenotype macrophages<sup>[21,22]</sup>. However, critical illness instigates the accumulation of alternatively activated M2 macrophages with a more anti-inflammatory role<sup>[22]</sup>. It has also been observed that critically ill obese patients with ARDS have reduced levels of inflammatory cytokines<sup>[23]</sup>. The shift to an anti-inflammatory milieu may partially explain a protective role of obesity in LT patients. Another possible explanation relates to the nutritional reserves possessed by obese patients, which may help them tolerate the increased metabolic demands of critical illness<sup>[24]</sup>.

We also found no statistically significant difference in either length of stay or total hospital charge. We

hypothesize that multiple factors may be influencing this outcome. First, selection criteria is more stringent since the development of the MELD system. In addition, as the prevalence of obesity in the United States continues to increase, surgeons and other physicians are more experienced in the nuances of providing care for these patients. Lastly, it is also possible that our short-term outcomes are not reflective of the long-term outcomes in these patients.

Our study did have some important limitations. First, this was a retrospective study based on diagnostic codes and utilizing a database. As we previously mentioned, there were no variable data points, and all our collected information was dependent upon documentation of the presence or absence of pathology.

Another limitation is that we only investigated outcomes during the index hospitalization of transplantation. We did not have data for re-admissions and long term outcomes of transplantation. Though we assume the majority of poor outcomes would happen during or shortly post-operatively, it would be interesting to follow the outcomes over a longer period of time and see if any meaningful differences occur.

An important consideration in the data we used is its dependence upon diagnostic coding and accurate documentation for validity, and was therefore vulnerable to selection bias. Previous papers have theorized that accurate reporting of obesity as comorbidity has historically been inferior to recent reporting. As obesity has been increasingly recognized as a public health epidemic, health care providers would be more likely to accurately document obesity<sup>[25]</sup>.

Lastly, our method of data collection did not allow for stratifying patients by disease severity, etiology of cirrhosis, or donor factors based on donor risk index. Therefore, survival analyses may be of constrained generalizability due to these limitations.

In conclusion, patients with morbid obesity undergoing OLT have increased respiratory complications and  $\geq 3$  comorbidities based on modified Elixhauser comorbidity index. Based on NIS database we found that health care utilization during admission for OLT is similar in morbidly obese and non-morbidly obese patients. Keeping in mind the limitations of NIS database, morbidly obese patients may be selected for OLT carefully after assessing their comorbidities. Further studies are needed to evaluate long term outcomes in these patients in era of MELD score based allocation of liver, which may

affect how patients are selected for transplantation in the future.

## COMMENTS

### Background

There have been varying reports on the morbidity and mortality in obese patients undergoing orthotopic liver transplantation (OLT). Consistently, studies have shown greater peri-operative morbidity as well as increased post-operative length of stay. Studies have also shown socioracial disparities in OLT utilization. Despite this, there have not been any large population based studies from United States on health care utilization and short term outcomes of liver transplantation in morbidly obese patients. The authors hypothesized that provided selected carefully morbidly obese patients undergoing liver transplantation may not have different healthcare utilization and short term outcomes.

### Research frontiers

The need for liver transplantation continues to rise, as is the prevalence of obesity. The results of this study contribute to clarifying that carefully selected morbidly obese patients may be acceptable candidates for liver transplantation.

### Innovations and breakthroughs

In this study, there was no difference in mortality, length of stay, or charges between morbidly obese and non-morbidly obese individuals receiving liver transplantation. This differs from previous reports.

### Applications

This study suggests that morbidly obese patients may be selected for liver transplantation after carefully assessing their comorbidities.

### Peer-review

This is a very interesting study performed on a great United States database based on more than 46000 patients undergoing liver transplantation. The retrospective study indicated that morbid obesity might not impact in-hospital mortality and health care utilization in OLT recipients.

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Retrospective Study

# Passive expansion of sub-maximally dilated transjugular intrahepatic portosystemic shunts and assessment of clinical outcomes

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**Author contributions:** Hsu MC, Weber CN and Nadolski GJ designed the study, performed the data analysis, and wrote the manuscript; Stavropoulos SW, Clark TW, Trerotola SO, Shlansky-Goldberg RD and Soulen MC performed the majority of the procedures and were involved in editing the manuscript.

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## Abstract

### AIM

To assess for passive expansion of sub-maximally dilated transjugular intrahepatic portosystemic shunts (TIPS) and compare outcomes with maximally dilated TIPS.

### METHODS

Polytetrafluoroethylene covered TIPS (Viatorr) from July 2002 to December 2013 were retrospectively reviewed at two hospitals in a single institution. Two hundred and thirty patients had TIPS maximally dilated to 10 mm (mTIPS), while 43 patients who were at increased risk for hepatic encephalopathy (HE), based on clinical evaluation or low pre-TIPS portosystemic gradient (PSG), had 10 mm TIPS sub-maximally dilated to 8 mm (smTIPS). Group characteristics (age, gender, Model for End-Stage Liver Disease score, post-TIPS PSG and clinical outcomes were compared between groups, including clinical success (ascites or varices), primary patency,



primary assisted patency, and severe post-TIPS HE. A subset of fourteen patients with smTIPS underwent follow-up computed tomography imaging after TIPS creation, and were grouped based on time of imaging (< 6 mo and > 6 mo). Change in diameter and cross-sectional area were measured with 3D imaging software to evaluate for passive expansion.

## RESULTS

Patient characteristics were similar between the smTIPS and mTIPS groups, except for pre-TIPS portosystemic gradient, which was lower in the smTIPS group (19.4 mmHg  $\pm$  6.8 *vs* 22.4 mmHg  $\pm$  7.1,  $P = 0.01$ ). Primary patency and primary assisted patency between smTIPS and mTIPS was not significantly different ( $P = 0.64$  and  $0.55$ , respectively). Four of the 55 patients (7%) with smTIPS required TIPS reduction for severe refractory HE, while this occurred in 6 of the 218 patients (3%) with mTIPS ( $P = 0.12$ ). For the 14 patients with follow-up computed tomography (CT) imaging, the median imaging follow-up was 373 d. There was an increase in median TIPS diameter, median percent diameter change, median area, and median percent area change in patients with CT follow-up greater than 6 mo after TIPS placement compared to follow-up within 6 mo (8.45 mm, 5.58%, 56.04 mm<sup>2</sup>, and 11.48%, respectively,  $P = 0.01$ ).

## CONCLUSION

Passive expansion of smTIPS does occur but clinical outcomes of smTIPS and mTIPS were similar. Sub-maximal dilation can prevent complications related to over-shunting in select patients.

**Key words:** Variceal hemorrhage; Portal hypertension; Transjugular intrahepatic portosystemic shunts; Ascites; Sub-maximal dilation; Underdiluted; Passive expansion; Hepatic encephalopathy

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**Core tip:** Sub-maximal dilation of transjugular intrahepatic portosystemic shunts (TIPS) is a method to reduce the risk of over-shunting and hepatic encephalopathy. The current study is a retrospective review to compare clinical outcomes of sub-maximally dilated TIPS (smTIPS) with maximally dilated TIPS (mTIPS) and assess for passive expansion of smTIPS. The study demonstrated that passive expansion of smTIPS does occur, however shunts may not fully expand and expansion may occur even after 6 mo. Clinical outcomes of smTIPS and mTIPS were similar, suggesting sub-maximal dilation may be an acceptable method to prevent complications related to over-shunting in select patients.

Hsu MC, Weber CN, Stavropoulos SW, Clark TW, Trerotola SO, Shlansky-Goldberg RD, Soulen MC, Nadolski GJ. Passive expansion of sub-maximally dilated transjugular intrahepatic portosystemic shunts and assessment of clinical outcomes. *World J Hepatol* 2017; 9(12): 603-612 Available from: URL:

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## INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is an established treatment for the sequelae of portal hypertension, particularly variceal hemorrhage and refractory ascites. Two major complications can arise following TIPS placement: Shunt dysfunction and hepatic encephalopathy (HE)<sup>[1-3]</sup>. Shunt dysfunction occurs from stenosis and the consequent rise in portosystemic gradient (PSG) resulting in relapse of clinical manifestations of portal hypertension<sup>[1,4-6]</sup>. In the era of bare metal stents, TIPS dysfunction was a major problem that led to relatively low primary patency rates, typically less than 50% at one year<sup>[1,5,7]</sup>. However, expanded polytetrafluoroethylene (PTFE) covered TIPS have improved patency rates and clinical outcomes compared to bare metal TIPS<sup>[1-4,8-10]</sup>. Primary patency rates at two years have now been shown to range from 62%-89%<sup>[7,10-14]</sup>.

Despite these advances, HE remains a pertinent post-procedural complication as portosystemic shunt physiology can trigger or worsen HE<sup>[1,2,8]</sup>. New or progressive post-TIPS HE of any severity has been shown to occur in 5%-35% of patients, while severe post-TIPS HE that does not respond to medical management and requires TIPS reduction or occlusion, occurs in up to 7% of patients<sup>[3,10,15,16]</sup>.

Given the potential conflicting relationship between portal decompression and HE, efforts have been made to develop techniques to balance the desired therapeutic effect while minimizing over-shunting<sup>[3,17]</sup>. One such technique is to sub-maximally dilate a 10 mm TIPS<sup>[18,19]</sup>. Sub-maximal dilation theoretically allows for further dilation of the TIPS in the event that the initial portal decompression is insufficient while avoiding over-shunting<sup>[6,16,18]</sup>. However, this technique would only be effective if the sub-maximally dilated TIPS do not expand significantly over time. Published data suggest the continued outward radial force of the TIPS stent may lead to passive expansion to its nominal diameter, limiting the value of initial gradient calibration<sup>[6,19,20]</sup>. The current study is a retrospective review to compare clinical outcomes of sub-maximally dilated TIPS (smTIPS) with maximally dilated TIPS (mTIPS) at a single large academic institution and assess for passive expansion of smTIPS in a sub-set of patients with follow-up cross-sectional imaging.

## MATERIALS AND METHODS

Approval from the Institutional Review Board was obtained for this retrospective study, which was carried out in full compliance with the Health Information Portability and Accountability Act. An interventional radiology database (Hi-IQ, Conexsys, Lincoln, RI) was used to identify all TIPS placed using an expanded PTFE-covered

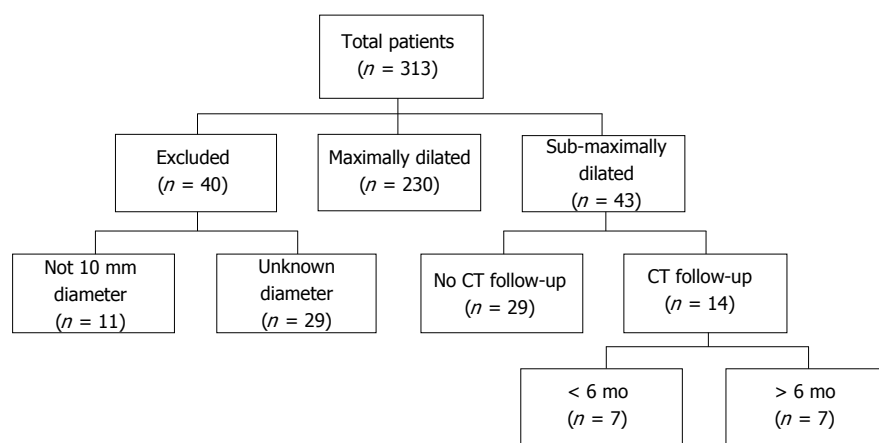


Figure 1 Patient selection. CT: Computed tomography.

stent graft (Viatorr) between July 2002 and December 2013 at two hospitals in a single institution ( $n = 313$ ). The electronic medical record was used to obtain patient characteristics, including age, gender, pre-TIPS Model for End-Stage Liver Disease (MELD) score, and pre-TIPS PSG, and retrospectively reviewed to assess for measurements of clinical outcomes, including post-TIPS PSG, clinical success, primary patency, primary assisted patency, and severe post-TIPS HE.

### Procedure

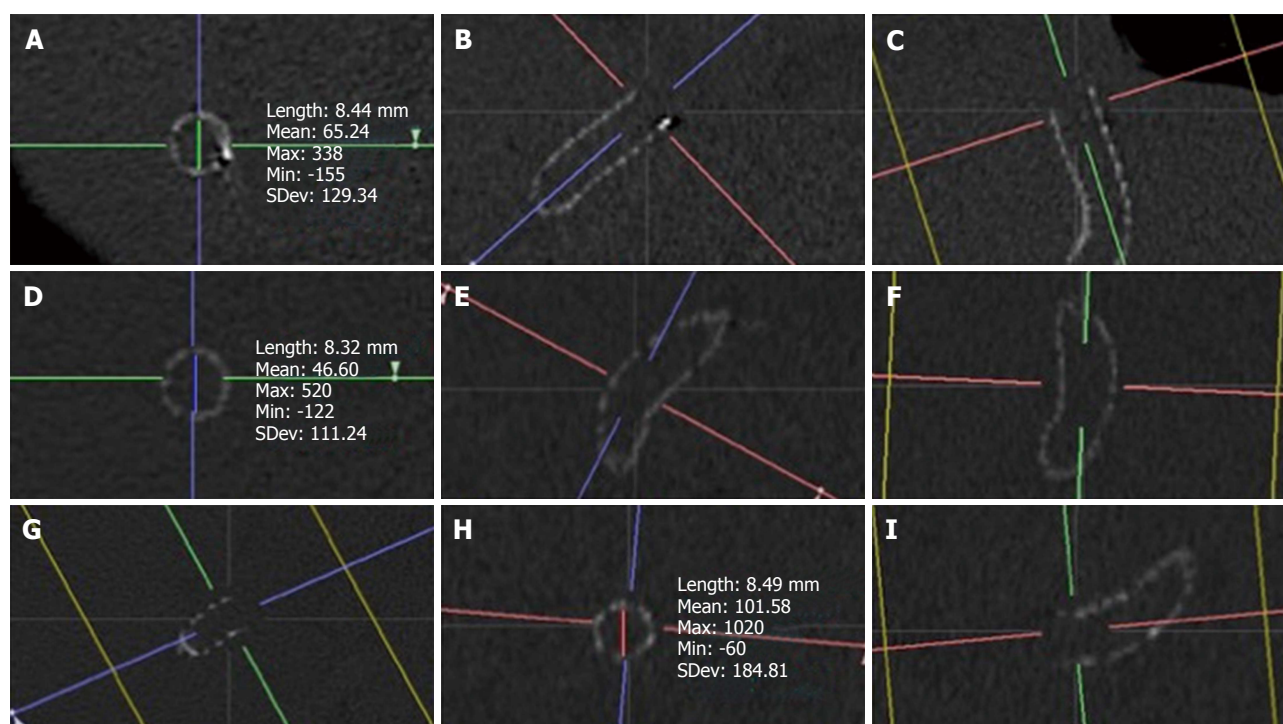
All TIPS creation was performed as previously described<sup>[12]</sup>. In patients who were considered vulnerable to post-TIPS HE based on (1) past medical history of HE on clinical evaluation by the referring hepatologist or interventional radiology service; or (2) low pre-TIPS PSG that could result in over-shunting post-TIPS as determined by the performing interventional radiologist, a modified TIPS creation procedure was performed. The modified TIPS creation involved initial placement of a nominal 10 mm TIPS stent that was sub-maximally dilated to 8 mm (smTIPS). Following initial dilation with the 8 mm balloon, the PSG was measured and post-TIPS portography was repeated at the same injection rate as the initial portogram (8–10 mL/s for 2 s for all cases). If the PSG normalized ( $\leq 12$  mmHg) and there was no venographic evidence of elevated gradient (*i.e.*, persistently filling varices), then the procedure was ended. Otherwise, the smTIPS stent was further dilated with a 10 mm balloon, PSG measured, and portography repeated (mTIPS). Coil embolization of persistently filling varices following TIPS creation with normalized PSG was performed in patients who initially presented with variceal hemorrhage.

Decision for angioplasty, thrombectomy, or stent placement during TIPS revision was based on venographic findings and PSG measurements. All patients received HE prophylaxis with lactulose<sup>[21]</sup>. In cases of severe post-TIPS HE refractory to medical management (protein restriction, lactulose, and/or rifaximin), TIPS reduction was performed with coaxial deployment of a FLAIR stent within the existing TIPS, or with a stent graft with parallel balloon-expandable stent as previously described<sup>[15]</sup>. All patients were instructed to maintain

a protein-restricted diet. Patients with ascites were instructed to follow a fluid-restricted, low sodium diet.

Inclusion criteria were patients with maximally dilated 10 mm PTFE-covered TIPS or 10 mm PTFE-covered TIPS sub-maximally dilated to 8 mm, as confirmed in the medical record (Figure 1). Of the 313 patients who underwent TIPS creation during the study period, forty patients were excluded due to placement of PTFE-covered TIPS of other nominal sizes ( $n = 11$ ) or patients with post-TIPS stent deployment angioplasty diameters that were not confirmed in the medical record ( $n = 29$ ). The remaining 273 patients had confirmed TIPS created with 10 mm nominal diameter stent, of which 230 patients had mTIPS created and 43 patients underwent creation of smTIPS. In the group of patients with smTIPS, any computed tomography (CT) imaging follow-up was identified from the medical record ( $n = 14$ ) and reviewed with TeraRecon (TeraRecon, Foster City, CA), which is an advanced 3D imaging processing software. Using this imaging software, two orthogonal planes were obtained before measuring the diameter of the TIPS stent at the hepatic venous end, mid-stent, and the portal venous end (Figure 2). These values were then averaged to obtain a composite measure of TIPS diameter.

For the purposes of the current study, clinical success was defined based on the indication for TIPS placement. In patients who had TIPS placed for varices, clinical success was defined as absence of further episodes of variceal hemorrhage or development of varices requiring intervention. Patients in the varices group with less than one month of follow-up were excluded from the clinical success analysis ( $n = 6$  for smTIPS;  $n = 21$  for mTIPS). For patients with refractory ascites requiring TIPS placement, clinical success was categorized as complete response (absence of large-volume paracentesis within six months post-TIPS creation) or partial response (greater than 50% decrease in frequency of large-volume paracentesis). Patients in the ascites group with less than six months of follow-up were excluded from the clinical success analysis ( $n = 17$  for smTIPS;  $n = 55$  for mTIPS). Primary patency was defined as the time from TIPS creation until revision for identified stenosis, elevated PSG ( $> 12$  mmHg), or recurrent symptoms. Primary



**Figure 2** TeraRecon measurement of transjugular intrahepatic portosystemic shunt stent at hepatic venous end, mid-stent, and portal venous end. A: Axial image at the hepatic venous end with cross-sectional diameter; B: Coronal image at the hepatic venous end with orthogonal plane designation; C: Sagittal image at the hepatic venous end with orthogonal plane designation; D: Axial image at mid-stent with cross-sectional diameter; E: Coronal image at mid-stent with orthogonal plane designation; F: Sagittal image at mid-stent with orthogonal plane designation; G: Axial image at the portal venous end with orthogonal plane designation; H: Coronal image at the portal venous end with cross-sectional diameter; I: Sagittal image at the portal venous end with orthogonal plane designation.

assisted patency was defined as the time from TIPS creation until shunt occlusion requiring recanalization. Severe post-TIPS HE was defined as encephalopathy refractory to conservative medical management requiring TIPS reduction.

### Statistical analysis

Statistical calculations were performed with GraphPad Prism software (version 6.05; GraphPad Software; La Jolla, CA). Unless otherwise indicated, all data were reported as mean  $\pm$  SD. Categorical variables were compared using Fisher's exact test. Continuous variables were compared using unpaired two-tailed Student's *t*-test and Mann-Whitney test for data with a parametric and non-parametric distribution, respectively. Primary and primary assisted patency rates were estimated with the Kaplan-Meier method. Patients were censored at the time of death or liver transplantation. Patency rates between smTIPS and mTIPS groups were compared with the log-rank test. Severe post-TIPS HE was analyzed on an intention-to-treat basis resulting in 12 patients from the mTIPS group, originally dilated to 8 mm but subsequently maximally dilated to normalize the post-TIPS PSG, being included in the smTIPS group. A *P*-value less than 0.05 was considered significant for all analyses.

## RESULTS

Patient characteristics and post-TIPS PSG are presented

in Table 1. There were 150 males and 80 females who underwent mTIPS creation with a mean age of 54.5 years  $\pm$  0.7 (range, 20-81). Of the 43 patients that had smTIPS created, 23 were male with a mean age of 56.5 years  $\pm$  2.3 (range 10-83). There was no statistically significant difference between the two patient populations based on gender or age (*P* = 0.17 and 0.29, respectively). The mean pre-TIPS MELD score in patients with mTIPS was 13.5  $\pm$  0.3 (range, 6-28) while it was 13.6  $\pm$  0.6 (range, 6-25) for patients with smTIPS, which was not significantly different (*P* = 0.82). The mean pre-TIPS PSG was higher for patients with mTIPS (22.4 mmHg  $\pm$  7.1; range, 9-73) compared to those with smTIPS (19.4 mmHg  $\pm$  6.8; range, 8-45), which was statistically significant (*P* = 0.01). Following TIPS placement, the median PSG was 8 mmHg for both mTIPS and smTIPS (range, 2-20 and 1-13, respectively) with a mean percent decrease in PSG of 61.0%  $\pm$  12.4 (range, 0-89) and 59.1%  $\pm$  15.9 (range, 0-95), respectively. These were not statistically different (*P* = 0.13 and 0.53, respectively). The patients with post-TIPS PSG above the goal of 12 mmHg had a mean pre-TIPS PSG of 33  $\pm$  13.2 (range 20-73) and experienced a mean percent decrease in PSG following TIPS creation of 48.3%  $\pm$  13.1 (range, 30.8-82.2) compared to those patients with post-TIPS PSG at or below the goal of 12 mmHg who had a mean pre-TIPS PSG of 21.1 mmHg  $\pm$  5.7 (range, 8-53) and mean percent decrease in PSG of 61.6%  $\pm$  12.6 (range, 0-95) (*P* < 0.01 and < 0.01) (Table 2).

Of the 43 patients with smTIPS, there were 14



**Table 1** Demographics and clinical characteristics

	Sub-maximally dilated	Maximally dilated	P value
Total patients	43	230	NA
Male	23	150	0.17
Female	20	80	
Mean age (yr)	56.5 ± 2.3 (range 10-83)	54.5 ± 0.7 (range 20-81)	0.29
Mean MELD	13.6 ± 0.6 (range 6-25)	13.5 ± 0.3 (range 6-28)	0.82
Mean pre-TIPS PSG (mmHg)	19.4 ± 6.8 (range 8-45)	22.4 ± 7.1 (range 9-73)	0.01
Median post-TIPS PSG (mmHg)	8 (range 1-13)	8 (range 2-20)	0.13
Mean percent change in PSG (%)	59.1 ± 15.9 (range 0-95)	61.0 ± 12.4 (range 0-89)	0.53

MELD: Model for end-stage liver disease; TIPS: Transjugular intrahepatic portosystemic shunt; PSG: Portosystemic gradient; NA: Not applicable.

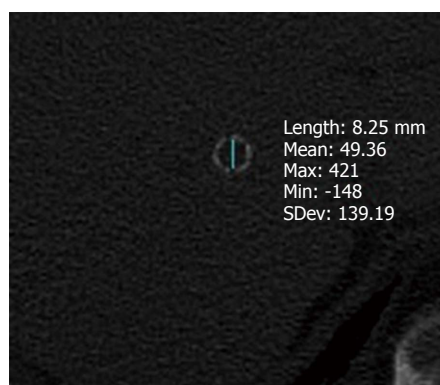
**Table 2** Mean pre-transjugular intrahepatic portosystemic shunt portosystemic gradient and percent change in portosystemic gradient in patients with post-transjugular intrahepatic portosystemic shunt portosystemic gradient above and below 12 mmHg

	> 12 mmHg	≤ 12 mmHg	P value
Mean pre-TIPS PSG (mmHg)	33 ± 13.2 (range 20-73)	21.1 ± 5.7 (range 8-53)	< 0.01
Mean percent change in PSG (%)	48.3 ± 13.1 (range 30.8-82.2)	61.6 ± 12.6 (range 0-95)	< 0.01

TIPS: Transjugular intrahepatic portosystemic shunt; PSG: Portosystemic gradient.

**Table 3** Measurements of 8 mm transjugular intrahepatic portosystemic shunt stents on computed tomography imaging follow-up

	Median diameter (mm)	Median percent diameter change	Median area (mm <sup>2</sup> )	Median percent area change
< 6 mo ( <i>n</i> = 7)	8.05 (range 7.84-8.43)	0.67%	50.94	1.34%
> 6 mo ( <i>n</i> = 7)	8.45 (range 8.23-8.72)	5.58%	56.04	11.48%
P-value	0.01	0.01%	0.01	0.01%

**Figure 3** Mid-stent measurement of sub-maximally dilated transjugular intrahepatic portosystemic shunt 103 d (< 6 mo) following creation.

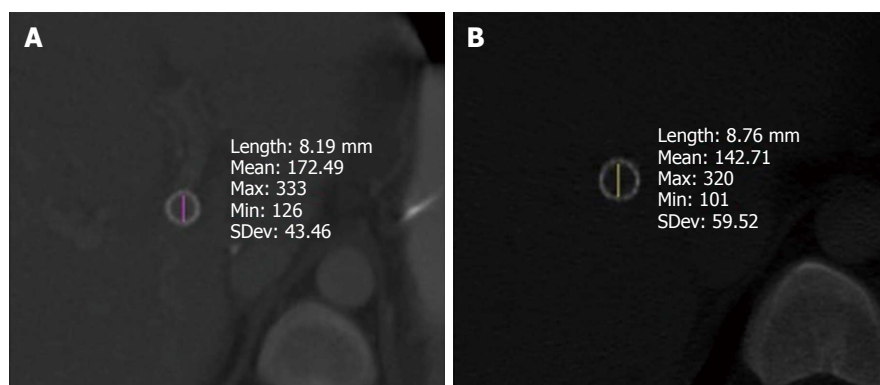
patients who had CT imaging follow-up (Table 3). Median time to last imaging follow-up was 373 d. The diameter and cross-sectional area of initial TIPS placement was assumed to be 8 mm and 50.27 mm<sup>2</sup>, corresponding to the diameter and area of the balloon used for dilation. Seven patients had last CT imaging follow-up within 6 mo (range, 4-172 d) and 7 patients had last CT imaging follow-up after 6 mo (range, 573-2131 d). The 7 patients with imaging follow-up within 6 mo had a median diameter, percent diameter change, area, and percent area change of 8.05 mm (range, 7.84-8.43 mm), 0.67%, 50.94 mm<sup>2</sup>, and 1.34%, respectively. The patients that

had last imaging follow-up after 6 mo had a median diameter, percent diameter change, area, and percent area change of 8.45 mm (range, 8.23-8.72 mm), 5.58%, 56.04 mm<sup>2</sup>, and 11.48%, respectively. When comparing these two subgroups, there was a statistically significant increase in diameter, percent diameter change, area, and percent area change (*P* = 0.01) (Figures 3 and 4).

Post-TIPS clinical success is summarized in Table 4. Nine of 14 patients (64%) who had smTIPS placed for refractory ascites experienced complete clinical success and 11 of 14 patients (79%) experienced at least partial clinical success. Similarly, 63 of the 98 patients (64%) who underwent mTIPS placement for refractory ascites experienced complete clinical success and 89 of 98 patients (91%) had at least partial clinical success. There was no statistically significant difference in complete or partial clinical success between patients with smTIPS or mTIPS (*P* = 1 and *P* = 0.17, respectively). For variceal bleeding, 7 of 9 patients (78%) with smTIPS and 64 of 75 patients (85%) with mTIPS experienced clinical success, which was not significantly different (*P* = 0.62).

Kaplan-Meier survival curves depicting primary and primary assisted patency rates for smTIPS and mTIPS are shown in Figures 5 and 6, respectively. Primary patency for smTIPS and mTIPS was 85% ± 9.1% and 76% ± 5.9%, respectively, at one year, and 77% ± 13 and 70% ± 6.9%, respectively, after two years. Primary assisted patency for smTIPS and mTIPS was 95% ± 5% and





**Figure 4** Mid-stent measurement of sub-maximally dilated transjugular intrahepatic portosystemic shunt (A) 182 d and (B) 573 d following creation (> 6 mo) in the same patient.

**Table 4** Clinical Success of transjugular intrahepatic portosystemic shunt *n* (%)

Sub-maximally dilated		Maximally dilated		<i>P</i> value
Complete Clinical Success of TIPS for Ascites				
Yes	9 (64)	63 (64)		1
No	5 (36)	35 (36)		
Partial Clinical Success of TIPS for Ascites				
Yes	11 (79)	89 (91)		0.17
No	3 (21)	9 (9)		
Clinical Success of TIPS for Varices				
Yes	7 (78)	64 (85)		0.62
No	2 (22)	11 (15)		

TIPS: Transjugular intrahepatic portosystemic shunt.

95%  $\pm$  3%, respectively, at one year, and 88%  $\pm$  13% and 94%  $\pm$  4%, respectively, after two years. There was no statistically significant difference between primary or primary assisted patency between the two groups ( $P$  = 0.64 and 0.55, respectively). Four of the 55 patients (7%) with smTIPS required TIPS reduction for severe refractory HE, while this occurred in 6 of the 218 patients with mTIPS (3%) using an intention-to-treat analysis, although not statistically significant ( $P$  = 0.12) (Table 5). In both smTIPS and mTIPS, the MELD scores and post-TIPS PSG were not significantly different between patients who experienced severe post-TIPS HE and those who did not (Table 6).

## DISCUSSION

Despite improved patency rates and reduced need for shunt revision with PTFE-covered TIPS, HE remains a problem following TIPS placement with some speculation that improved patency rates may increase the incidence of HE<sup>[1-4,8,9]</sup>. HE arises when compounds derived from the intestine that require hepatic detoxification bypass the hepatic vascular bed in the setting of a portosystemic shunt, and subsequently enter systemic circulation. These compounds, typically nitrogenous in composition, travel to the central nervous system and disturb neurotransmission, which leads to eventual alterations in consciousness and

**Table 5** Severe post- transjugular intrahepatic portosystemic shunt hepatic encephalopathy *n* (%)

Severe post-TIPS HE	Sub-maximally dilated	Maximally dilated	<i>P</i> value
Yes	4 (7)	6 (3)	0.12
No	51 (93)	212 (97)	

TIPS: Transjugular intrahepatic portosystemic shunt; HE: Hepatic encephalopathy.

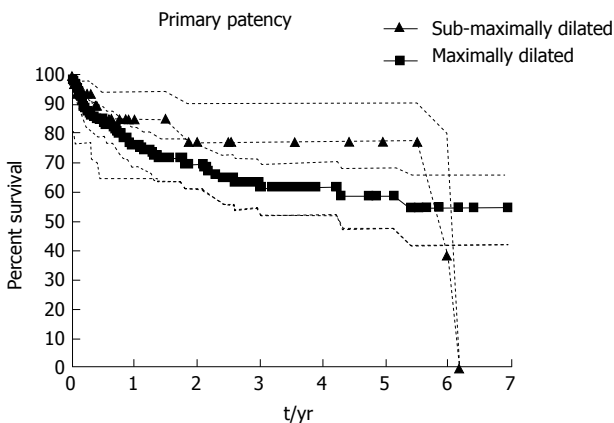
behavior that manifest as HE<sup>[22]</sup>. This pathogenesis is further supported with the evidence that HE occurs with spontaneous portosystemic shunts, even in the absence of hepatic dysfunction or TIPS<sup>[23,24]</sup>. Prior investigations have also shown that an increased volume of shunted blood, decreased portal hepatic perfusion, and a lower PSG following TIPS placement correlate with higher rates of HE<sup>[5,17,18,25]</sup>.

With knowledge of the pathogenesis of HE, different techniques have been studied in an effort to balance the desired therapeutic effect while minimizing over-shunting and increased risk of HE, such as smaller diameter TIPS or altering the goal in PSG reduction for patients with HE<sup>[3,17]</sup>. Another technique is sub-maximal dilation of TIPS, which allows for further staged dilation, if necessary, and theoretically minimizes over-shunting<sup>[6,16,18,26]</sup>. However, passive expansion of the TIPS may limit the effectiveness of this technique with prior evidence, in both peripheral circulation and TIPS, that suggests this phenomenon should be taken into consideration. Late expansion of bare metal nitinol stents was demonstrated after 6 mo in peripheral arteries of an animal model<sup>[27]</sup>. Haskal *et al*<sup>[20]</sup> showed that after immediate recoil of Wallstent TIPS stents after placement, passive expansion to nominal diameter occurred at follow-up venography three to six months later. Pieper *et al*<sup>[19]</sup> studied 29 patients with Viatorr TIPS sub-maximally dilated to a mean of 64% of their nominal area, and found passive expansion to 88% during follow-up, with significant expansion occurring within 6 mo. Finally, Gaba *et al*<sup>[28]</sup> evaluated 41 patients with 10 mm nominal Viatorr TIPS sub-maximally dilated to 8

**Table 6** Model for End-Stage Liver Disease and post-transjugular intrahepatic portosystemic shunt portosystemic gradient for patients with and without severe post-transjugular intrahepatic portosystemic shunt hepatic encephalopathy

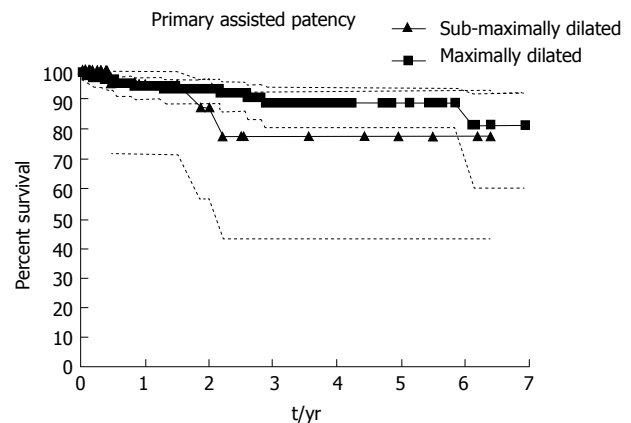
	Mean MELD with HE	Mean MELD without HE	P value	Median post-TIPS PSG with HE (mmHg)	Median post-TIPS PSG without HE (mmHg)	P value
Sub-maximally dilated	13.3 ± 2.9 (range 11-17)	13.7 ± 4.3 (range 6-25)	0.85	7.5 (range 6-8)	8 (range 1-13)	0.67
Maximally dilated	15.8 ± 4.3 (range 12-24)	13.4 ± 4.1 (range 6-28)	0.16	10 (range 4-11)	8 (range 2-20)	0.36

MELD: Model for End-Stage Liver Disease; HE: Hepatic encephalopathy; TIPS: Transjugular intrahepatic portosystemic shunt; PSG: Portosystemic gradient.



<b>P = 0.64</b>	Sub-maximally dilated		Maximally dilated	
	Patency (%)	No. at risk	Patency (%)	No. at risk
1 yr	85 ± 9.1	13	76 ± 5.9	96
2 yr	77 ± 13	10	70 ± 6.9	66
3 yr	77 ± 13	7	62 ± 8.3	39
4 yr	77 ± 13	6	62 ± 8.3	29
5 yr	77 ± 13	4	59 ± 10	17
6 yr	38 ± 42	2	55 ± 11	10

**Figure 5** Primary patency rates of sub-maximally dilated (8 mm) vs maximally dilated (10 mm) transjugular intrahepatic portosystemic shunt. On Kaplan-Meier analysis, yearly patency rates through 6 years of follow-up after transjugular intrahepatic portosystemic shunt creation are demonstrated with 95%CI and number at risk.



<b>P = 0.55</b>	Sub-maximally dilated		Maximally dilated	
	Patency (%)	No. at risk	Patency (%)	No. at risk
1 yr	95 ± 5	14	95 ± 3	115
2 yr	88 ± 13	10	94 ± 4	75
3 yr	78 ± 22	7	89 ± 6	48
4 yr	78 ± 22	6	89 ± 6	29
5 yr	78 ± 22	4	89 ± 6	20
6 yr	78 ± 22	3	89 ± 6	13

**Figure 6** Primary assisted patency rates of sub-maximally dilated (8 mm) vs maximally dilated (10 mm) transjugular intrahepatic portosystemic shunt. On Kaplan-Meier analysis, yearly patency rates through 6 years of follow-up after transjugular intrahepatic portosystemic shunt creation are demonstrated with 95%CI and number at risk.

mm, and demonstrated passive expansion with follow-up CT median stent diameter of 9.8 mm at a median of 76 d post TIPS creation without difference in incidence of post-TIPS HE in smTIPS vs mTIPS.

In the current study, continued passive expansion of smTIPS was observed in the subgroup of patients with cross-sectional imaging follow-up. Additionally, a significant difference in the increase in median diameter and area was observed when comparing the patients who had last imaging follow-up after 6 mo vs those within 6 mo. No patients in this subpopulation suffered severe refractory post-TIPS HE. While this change was statistically significant, the magnitude of expansion was not to the same degree as suggested by prior studies, and it also occurred over a longer time period (> 6 mo)<sup>[19,28]</sup>. The delayed and less extensive passive expansion observed in this study, although difficult to explain, may be secondary to dilation of the portosystemic tract with an 8 mm balloon prior to placement of the TIPS stent-graft. While the diameter of the balloon used to create the TIPS tract is not always described in prior investigations, a 10 mm

balloon has been used previously<sup>[4]</sup>. It is hypothesized that dilating the tract to only 8 mm may lead to a greater initial counterforce on the stent from the elasticity of the surrounding liver parenchyma and new TIPS tract with minimal potential space, which leads to both slower and less passive expansion. In comparison, dilating the tract to 10 mm may hypothetically allow for a larger initial potential space for more immediate passive expansion of a TIPS sub-maximally dilated to 8 mm. Moreover, it is conceivable that more fibrotic livers with decreased compliance may differentially limit the extent of passive expansion, although this analysis was beyond the scope of this study.

In order to better understand whether or not passive expansion of the TIPS over time is clinically relevant, we compared a variety of outcomes in patients with smTIPS and mTIPS. The post-TIPS PSG demonstrated adequate portal decompression with a median PSG of 8 mmHg in both groups ( $P = 0.13$ ) and no significant difference in mean percent change in PSG ( $P = 0.53$ ). Overall, the observed rate of severe post-TIPS HE was low (4%), and

not significantly different between mTIPS and smTIPS ( $P = 0.12$ ), suggesting the step-wise approach to TIPS creation by assessing PSG following sub-maximal dilation may be effective in minimizing unnecessary over-dilation and thus, over-shunting. These findings are similar to prior reports<sup>[28]</sup>. The lack of an observable difference between the groups may be due to passive expansion allowing for an equilibrium to gradually develop as increasing amounts of blood are shunted through the liver, thus, minimizing severe refractory HE<sup>[26]</sup>. Additionally, there was no significant difference in median post-TIPS PSG or mean MELD between patients who suffered severe post-TIPS HE and those who did not for patients with smTIPS or mTIPS. Finally, no significant difference in primary and primary assisted patency or clinical success for both ascites and varices occurred between the two groups.

These results are somewhat contradictory to a prior study comparing nominal 8 mm and 10 mm TIPS which found increased rates of recurrent portal hypertensive complications in the 8 mm group, leading to early termination of the study<sup>[3]</sup>. A possible explanation for the conflicting results may be related to the small, but not insignificant amount of passive expansion demonstrated with smTIPS. Based on Poiseuille's Law, volumetric flow rate is proportional to change in diameter to the fourth power, as well as change in pressure. It is postulated that despite a decrease in the change in pressure across the TIPS stent from passive expansion, the 5.6% increase in diameter observed in patients with CT imaging > 6 mo would disproportionately cause an increase in volumetric flow rate. As such, gradual passive expansion may slowly increase the amount of shunted blood and decrease the recurrence of portal hypertensive complications, yielding similar clinical success between the two groups obtained in the present study. Furthermore, the nominal 8 mm TIPS group in the same study had a higher incidence of shunt dysfunction, a majority without angiographically evident stenosis, than the smTIPS group in the current study, suggesting that a fixed, smaller diameter TIPS may provide insufficient portosystemic decompression and that passive expansion may be more efficacious in patients deemed to be at risk of post-TIPS HE<sup>[3]</sup>. Previously, the only mechanism to improve TIPS shunting in patients with nominal 8 mm TIPS was to place a parallel TIPS, as no further expansion was possible. The current study highlights a technique that would allow for further TIPS dilation in patients that show signs of inadequate portal decompression following initial creation of smTIPS, potentially obviating the need for a second parallel TIPS.

This study has several important limitations, including its retrospective design and data collection from a single center. The small size of the smTIPS group ( $n = 43$ ) relative to the mTIPS group raises the possibility of a Type I error. As a tertiary center, identification of undocumented TIPS intervention or clinical follow-up at outside institutions is limited. There was more severe refractory post-TIPS HE in the smTIPS group vs the mTIPS group (7% vs 3%), although not statistically significant ( $P = 0.12$ ).

While this finding was not expected, it reflects selection bias between the two groups. Patients who underwent creation of smTIPS had a statistically significant lower mean pre-TIPS PSG compared to mTIPS ( $P = 0.01$ ). This was not surprising given that patients deemed to be higher risk for HE following TIPS creation, which included a low pre-TIPS PSG, were preferentially selected to have smTIPS created to reduce the risk of over-shunting, as determined by the operating physician. Furthermore, even though shunt physiology is a known contributing factor for HE, the pathophysiology of HE is multifactorial and includes other precipitating factors such as hepatic decompensation, noncompliance with dietary restrictions, sepsis, and medications. Additional independent risk factors include older age, elevated serum creatinine, low serum sodium and low albumin; however, these clinical data were difficult to corroborate from a retrospective review spanning 10 years<sup>[2]</sup>. Only a minority (33%) of the patients with smTIPS had subsequent CT exams during the follow-up period. It is conceivable that this may not be representative of the entire subgroup. Additionally, patients did not undergo repeat angiographic TIPS evaluation following CT evidence of passive expansion, which would allow for repeat PSG measurement to determine the true hemodynamic consequences of passive expansion.

In conclusion, in patients with smTIPS there was passive expansion of 10 mm Viatorr TIPS stent-grafts even after 6 mo, however, not all reached their nominal diameter. The clinical outcomes, including incidence of severe post-TIPS HE, between sub-maximally and maximally dilated 10 mm Viatorr TIPS were similar. These findings suggest sub-maximal dilation may be an acceptable method to prevent complications related to over-shunting in select patients.

## COMMENTS

### Background

Transjugular intrahepatic portosystemic shunt (TIPS) is an established treatment for the sequelae of portal hypertension, particularly variceal hemorrhage and refractory ascites. Despite improved patency rates and reduced need for shunt revision with polytetrafluoroethylene-covered TIPS, hepatic encephalopathy (HE) remains a problem following TIPS placement with some speculation that improved patency rates may increase the incidence of HE. HE arises when compounds derived from the intestine that require hepatic detoxification bypass the hepatic vascular bed in the setting of a portosystemic shunt, and subsequently enter systemic circulation. One technique to balance portal decompression while minimizing over-shunting is sub-maximal dilation of TIPS. While sub-maximal dilation theoretically allows for further dilation of the TIPS in the event that the initial portal decompression is insufficient while avoiding over-shunting, published data suggest the continued outward radial force of the TIPS stent may lead to passive expansion to its nominal diameter and limit the value of initial gradient calibration.

### Research frontiers

As sub-maximal dilation of TIPS has gained increased clinical use, there have been more studies investigating the presence and effect of passive expansion in both peripheral circulation and TIPS. Late expansion of bare metal nitinol stents was demonstrated after 6 mo in peripheral arteries of an animal model. Haskal *et al* showed that after immediate recoil of Wallstent TIPS stents after placement, passive expansion to nominal diameter occurred at follow-

up venography three to six months later. Pieper *et al* studied 29 patients with Viatorr TIPS sub-maximally dilated to a mean of 64% of their nominal area, and found passive expansion to 88% during follow-up, with significant expansion occurring within 6 mo. Finally, Gaba *et al* evaluated 41 patients with 10 mm nominal Viatorr TIPS sub-maximally dilated to 8 mm, and demonstrated passive expansion with follow-up computed tomography median stent diameter of 9.8 mm at a median of 76 d post TIPS creation without difference in incidence of post-TIPS HE in smTIPS vs mTIPS.

### Innovations and breakthroughs

While the aforementioned studies focused on establishing the presence of passive expansion, there is a lack of published data investigating the clinical outcomes of sub-maximally dilated TIPS with maximally dilated TIPS in addition to the presence of passive expansion. While the study showed passive expansion does occur, not all shunts fully expanded to nominal diameter and expansion even occurred after 6 mo, unlike prior studies. More importantly, the comparison of clinical outcomes of smTIPS vs mTIPS showed no significant difference in primary patency, primary assisted patency, clinical success, or post-TIPS HE.

### Applications

In patients who are at high risk for post-TIPS hepatic encephalopathy, based on pre-TIPS encephalopathy or low pre-TIPS portosystemic gradient, sub-maximal dilation may be an effective method to balance adequate portal decompression with the risk of over-shunting and hepatic encephalopathy with the knowledge that passive expansion following placement does not appear to affect clinical outcomes.

### Terminology

Sub-maximally dilated TIPS - TIPS stent grafts that are not fully dilated to nominal diameter following deployment.

### Peer-review

The study is to compare clinical outcomes of smTIPS with mTIPS. The results suggest the method may be of significance.

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## Hepatic complications induced by immunosuppressants and biologics in inflammatory bowel disease

My-Linh Tran-Minh, Paula Sousa, Marianne Maillet, Matthieu Allez, Jean-Marc Gornet

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### Abstract

The incidence of inflammatory bowel diseases (IBD) is

rising worldwide. The therapeutic options for IBD are expanding, and the number of drugs with new targets will rapidly increase in coming years. A rapid step-up approach with close monitoring of intestinal inflammation is extensively used. The fear of side effects represents one the most limiting factor of their use. Despite a widespread use for years, drug induced liver injury (DILI) management remains a challenging situation with Azathioprine and Methotrexate. DILI seems less frequent with anti-tumor necrosis factor agents and new biologic therapies. The aim of this review is to report incidence, physiopathology and practical guidelines in case of DILI occurrence with the armamentarium of old and new drugs in the field of IBD.

**Key words:** Drug induced liver toxicity; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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**Core tip:** The therapeutic options for inflammatory bowel disease (IBD) are expanding, and the number of drugs will rapidly increase in coming years. The fear of side effects represents one the most limiting factor of their use. Despite a widespread use for years, drug induced liver injury (DILI) management remains a challenging situation with Azathioprine and Methotrexate. DILI seems less frequent with anti-tumor necrosis factor agents and new biologic therapies. The aim of this review is to report incidence, physiopathology and practical guidelines in case of DILI occurrence with the armamentarium of old and new drugs in the field of IBD.

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## INTRODUCTION

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) mainly involve the intestinal tract. They may be associated with many extra intestinal manifestations<sup>[1]</sup>. Among them, hepatobiliary manifestations are frequent and often linked with immune disorders (primary sclerosing cholangitis, auto immune hepatitis, overlap syndrome and IgG4 associated cholangiopathy) or drug induced liver injury (DILI)<sup>[2]</sup>. Approximately 30% of IBD patients will present abnormalities of liver function tests (LFT) during the course of the disease<sup>[3]</sup>. Over the decades, immunosuppressants (thiopurines, methotrexate, calcineurine inhibitors) and anti-tumor necrosis factor (TNF) agents, took an increasing part in the armamentarium of IBD<sup>[4]</sup>. More recently, integrin antagonists and interleukine 12/23 inhibitors have emerged in patients refractory or intolerant to anti-TNF therapy<sup>[5]</sup>. The safety profile of these drugs is an important issue that may limit their use. Acute and/or chronic hepatic injuries directly induced by the treatment or consequently to occurrence or reactivation of an infection have been described with almost all of these treatments. This article reviews the literature regarding hepatic complications of immunosuppressants and biologics in IBD.

### Thiopurines

Thiopurines including azathioprine (AZA) and 6-mercaptopurine (6-MP) have been shown to be effective for induction and maintenance of remission in IBD<sup>[6,7]</sup>. Combination therapy with infliximab plus azathioprine is more likely to induce clinical remission than those receiving azathioprine or infliximab alone in both CD and UC<sup>[8,9]</sup>. Addition of AZA/6-MP can eliminate antibodies to infliximab in serum and restores clinical response of infliximab in IBD patients<sup>[10]</sup>. Some studies have also suggested that thioguanine (TG) could be used as an alternative for patient's refractory or intolerant to AZA or 6-MP<sup>[11]</sup>. AZA and 6-MP have frequent side effects which usually occur within four to six weeks after introduction and concern up to 25% of patients with an annual risk of 7% per patient-year of treatment<sup>[12,13]</sup>. Depending on its definition, thiopurines hepatotoxicity frequency can vary between 0% and 17%<sup>[14,15]</sup>. In a large study of 786 patients, LFT elevation was observed in 4% of the population<sup>[16]</sup>. In a systematic review of 34 studies including a total of 3485 patients, the mean prevalence of AZA/6-MP induced liver disorders was estimated at 3.4% with no difference between both drugs<sup>[17]</sup>. It has been suggested that the risk of hepatotoxicity was lower in females and higher in CD and active smokers<sup>[13,18]</sup>. Nonalcoholic fatty liver disease (NAFLD) is increased in IBD patients and has been shown to be an independent risk factor for hepatotoxicity in patients exposed to AZA/6-MP<sup>[19]</sup>. In a prospective study, use of corticosteroids was associated with an increased risk of AZA/6-MP induced hepatotoxicity whereas anti-TNF had a protective effect<sup>[20]</sup>. Thus, according to this relatively

high frequency, LFT monitoring is mandatory in exposed patients. Adverse reactions to thiopurines can be divided in two groups: Dose independent and dose dependent. The most frequently reported dose-independent events are rash, fever and arthralgia, pancreatitis and hepatitis. It is thought to be immunological mediated and frequently observed in the first weeks of treatment<sup>[20]</sup>. Dose dependent effects appear later, after months to years, and are correlated with elevated concentration of 6-MMP. Various endothelial cell injuries with resultant raised portal pressures can also developed.

**Physiopathology:** Purine analogues act as a DNA synthesis inhibitor by incorporation of thiopurine nucleotide metabolite into DNA, leading to both cytotoxicity and immunosuppression<sup>[21]</sup>. Thiopurines metabolism go through a complex enzymatic pathway. AZA and 6-MP are prodrugs of 6-thioguanine metabolite (6-TGN), the final effective metabolite. AZA is first absorbed and metabolized in the liver to 6-MP which is metabolized by 3 enzymes including thiopurine S-methyltransferase (TPMT) leading to 6-methylmercaptopurine (6-MMP) formation. 6-MMP is a non-effective metabolite but is involved in thiopurine toxicity, particularly hepatotoxicity. Up to 20% of IBD patients preferentially metabolize thiopurines to 6-MMP. Indeed, high 6-MMP level (up to 5700 pmol/8 × 10<sup>8</sup> erythrocytes) is correlated with a 3-fold increased risk of LFT elevation (18% vs 6%)<sup>[14]</sup>. Various polymorphisms of TPMT gene has been described, leading to different level of enzyme activity: 0.3% of individuals have low or absent TPMT activity, 11% have intermediate activity and 89% have normal activity<sup>[22]</sup>. TPMT polymorphisms has been mainly associated with hematotoxicity especially neutropenia<sup>[23,24]</sup>. It was suggested that high TPMT activity could facilitate hepatotoxicity by the accumulation of 6-MMP. However, in a recent meta-analysis of 10 studies including 1875 patients, TPMT polymorphisms were not associated with hepatotoxicity<sup>[25]</sup>. The mechanisms by which thiopurines cause hepatotoxicity are not well established. A recent study with a proteomic approach suggests that induction of oxidative stress in T-lymphocytes by thiopurines could play an important role<sup>[26]</sup>.

**Acute hepatotoxicity:** Half of thiopurine DILI occur within the first 3 mo usually prematurely after AZA/6MP introduction<sup>[20]</sup>. This acute dose independent toxicity is linked to hypersensitivity and idiosyncratic cholestatic reaction non-mediated by IgE reaction. These effects are unrelated to 6-MMP. Clinical symptoms such as fever, rash or lymphadenopathy, hepatomegaly and other biological abnormalities (atypical lymphocytosis, eosinophilia) may be observed concomitantly with elevated LFT. Most of hypersensitive reactions are hepatitis-like picture with moderate elevation of aspartate aminotransferase and alanine aminotransferase (ALT). More rarely, severe cholestatic hepatitis with jaundice

have also been reported with AZA<sup>[27,28]</sup>.

**Long term hepatic injury:** Nodular regenerative hyperplasia (NRH) is defined by hepatocytes hyperplasia and nodules formation, without fibrosis proliferation separating nodules consecutive to vascular flow variation within liver. It frequently results in portal hypertension (PHT) with its potential complications<sup>[29]</sup>. NRH may be asymptomatic with normal liver tests for many years<sup>[30]</sup>. The diagnosis of NRH remains challenging and mainly depends on histological report. However, the interobserver agreement on the histopathologic diagnosis of NRH is flawed, even when assessed by well-experienced liver pathologists<sup>[31]</sup>. The pathogenesis of NRH in IBD patients is poorly understood but is likely to be multifactorial.

The largest series describing NRH in IBD under thiopurines reported 37 cases in 11 French tertiary centers of the GETAID group. The cumulative risk of NRH was estimated to 0.5% at five years and 1.25% at 10 years. The diagnosis was made after a median time of 48 mo after AZA introduction (range: 6 to 187 mo) and 14 patients (38%) developed PHT during follow-up. Identified risks factors were male sex and stricturing behavior<sup>[28]</sup>. Another study has shown that the high-risk patient group was males with small bowel resection  $\geq$  50 cm either prior to or after AZA initiation<sup>[32]</sup>. However, IBD in itself can be associated with NRH, and was incidentally found in 6% of thiopurine naive IBD patients undergoing bowel resection<sup>[33]</sup>. It has been hypothesized that intestinal surgery might promote obliterative portal venopathy by causing malabsorption of vitamins B12, B6 and folic acid, with resultant hyperhomocysteinemia<sup>[34]</sup>. Some studies have demonstrated that TG treatment (Lanvis<sup>®</sup>) induced more NRH than AZA or 6-MP<sup>[35,36]</sup>. In the study by Dubinsky *et al.*<sup>[35]</sup>, 33% of the patients treated with TG had NRH at liver biopsy. No association was found with duration of TG treatment, cumulative dose, or TG nucleotide levels. Geller *et al.*<sup>[37]</sup> reported systematic liver biopsies in 37 patients exposed to TG during 1 to 3 years. NRH of varying degree was seen in 20 patients (53%). Another study has suggested that low-dose TG maintenance therapy may be safer<sup>[38]</sup>. In 28 patients treated at least 30 mo with TG, they observed no histological sign of HNR in 93% of the cases. This finding is reinforced by a recent study which nicely shows in a murine model that sinusoidal obstructive syndrome induced by TG may be avoided by either inhibition of endothelial activation or simple changes to dosing regimens of TG<sup>[39]</sup>. Nevertheless, regarding the extensive use of newer alternative drugs to thiopurines, TG has been abandoned in clinical practice because of its hepatotoxicity. Natural history of HNR after thiopurines discontinuation remains unclear and either persistent aggravation or improvement have been reported<sup>[11,40]</sup>.

Other vascular disorders associated with thiopurines such as peliosis hepatitis, veno-occlusive disease, hepatoportal sclerosis, sinusoidal dilatation and perisinusoidal fibrosis were also described initially in patients treated for acute leukemia but have been occasionally reported in

IBD patients<sup>[41-44]</sup>. *In vitro* studies with murine sinusoidal endothelial cells and hepatocytes exposed to azathioprine have suggested that the mechanism of hepatotoxicity is sinusoidal endothelial damage associated with glutathione depletion<sup>[45]</sup>.

**Management:** Most of LFT abnormalities resolve spontaneously or after dose reduction. In a large study with long term follow-up, only 3.6% of patients required treatment cessation for hepatotoxicity<sup>[16]</sup>. In another study, 90% of patients normalized their liver test after decreasing dose or treatment withdrawal<sup>[46]</sup>. One of the main questions concerning AZA toxicity management is whether substitution of AZA by 6-MP might affect or decrease hepatotoxicity. In a study of 135 patients with AZA intolerance, 6-MP was well tolerated in almost three quarters of the patients who presented hepatotoxicity (12/17 patients; 71%) suggesting that this option deserves to be tested<sup>[47]</sup>. Some authors have suggested that routine thiopurines metabolite (especially 6-MMP) monitoring may identify subjects at high risk of hepatotoxicity. Administration of 6-MP twice daily instead of once daily has even been proposed to decreased 6-MMP levels to reduce the risk of hepatotoxicity<sup>[46]</sup>. Furthermore, twice daily administration decreases 6-MMP levels without affecting 6-TGN levels may lead to equivalent efficacy<sup>[48]</sup>. Another tool to adapt 6-MMP dosage is coadministration of allopurinol. This drug is a xanthine oxidase inhibitor, an enzyme which metabolizes 6-MP. Xanthine oxidase inhibition leads to increase 6-TGN level by improving drug availability. Since more 6-MP is available for conversion to 6-TGN, a lower dose of thiopurines is sufficient and may avoid toxicity. Safety and effectiveness of long-term allopurinol-thiopurine maintenance treatment in IBD patients has been proven whatever the initial adverse event with increased 6-TGN and decreased 6-MMP concentrations<sup>[49,50]</sup>. In a pilot study of 11 patients with acute thiopurine hepatotoxicity secondarily treated with allopurinol co-therapy with low-dose AZA or MP, 82% of the patients remained in long-term remission with normal liver tests<sup>[51]</sup>. A larger study in 25 patients showed similar results with normalization of LFT in 80% of the cases after switch to a combination treatment<sup>[52]</sup>. It has been shown that 5-ASA daily use results in increased 6-TGN levels and reduced 6-MMP levels with a dose-dependent effect suggesting that salicylates may reduce the risk for hepatotoxic adverse reactions related to AZA/6-MP<sup>[53,54]</sup>. However, there is a lack of prospective data supporting the therapeutic impact of 5-ASA on AZA/6-MP hepatotoxicity prevention. Recently, in a small cohort of 12 patients, no pharmacokinetic interaction was found between adalimumab and thiopurines with comparable concentrations of 6-TGN and 6-MMP before anti-TNF introduction and throughout 12 wk of follow-up<sup>[55]</sup>.

### Methotrexate

Methotrexate (MTX) is an antimetabolite with both anti-proliferative and immunosuppressive activities impairing DNA synthesis *via* inhibition of dihydrofolate reductase,



decreased the production of proinflammatory cytokines and lymphocytes apoptosis<sup>[56]</sup>. Regimens containing MTX are classified as high-dose, intermediate or low dose, determined as dose per unit of body surface area. The management of CD utilized only low dose MTX (< 50 mg/m<sup>2</sup>), usually over a long period of time. In this last group the association between MTX and hepatic dysfunction has been extensively studied. In CD, MTX given intramuscularly once weekly at a dose of 25 mg is effective at inducing and maintaining remission in thiopurine-naïve patients<sup>[57,58]</sup>. Small labelled studies have also suggested efficacy in patients who failed or are intolerant to thiopurines<sup>[59,60]</sup>. Data are more limited and conflicting in UC<sup>[61,62]</sup>. In addition, MTX is widely prescribed in combination with biological therapy to reduce immunogenicity and to maintain clinical response<sup>[63]</sup>. The most common adverse effects involve the gastrointestinal tract such as nausea, vomiting and diarrhea. More serious toxicities such as myelosuppression and abnormal LFT are dose-dependent. Liver toxicity was firstly reported with the use of MTX in psoriasis and inflammatory rheumatic disorders with high initial rate over 25% of the patients. Obesity, alcoholism, diabetes mellitus, previous abnormalities in LFT and a high accumulated dose of MTX were considered as risk factors of liver toxicity in those diseases<sup>[64,65]</sup>. There is a paucity of studies evaluating liver toxicity as a complication of MTX therapy in the setting of IBD, and no gastroenterology societal recommendations on monitoring for hepatic toxicity have been formulated.

**Profile and mechanism of liver injury:** Most of understanding of the hepatotoxic potential of MTX came from its use in non-malignant disease such as rheumatoid arthritis (RA) and psoriasis.

The mechanism by which MTX adversely affect the liver remains unclear. Liver response to inflammation is fibrosis *via* stellate cell, mediated by metabolite accumulation in liver cell and inhibition of folate metabolite leading to a decreased nucleotid synthesis.

Several polymorphisms in enzymes involved in the metabolism of folic acid are related to the toxicity of MTX. The C677T and A1298C polymorphisms in the MTHFR gene were the most reported, however studies have reported conflicting results. Two meta analyses have been performed. One described an association of the C677T polymorphism with increased toxicity whereas the second found no association between either the C677T or the A1298C polymorphisms of MTHFR and toxicity of MTX in RA<sup>[66,67]</sup>.

Methotrexate can induce a variety of non-specific histologic changes including macrovesicular steatosis, stellate cell hypertrophy, portal and lobular inflammation and hepatic fibrosis.

Histological toxicity is assessed according to the Roenigk's classification, a subjective and semi quantitative grading liver injury in four 4 groups<sup>[68]</sup>.

**Grade findings:** (1) Normal; (2) mild fatty infiltration, nuclear variability, or portal inflammation; (3) moderate

to severe fatty infiltration, nuclear variability, or portal inflammation and mild fibrosis; (4) moderate to severe fibrosis; and (5) cirrhosis.

**DILI frequency:** The first case of MTX liver toxicity was described in 1955 in children treated for leukemia. NAFLD syndrome seems to be an independant risk factor associated with DILI under long term low dose methotrexate use<sup>[69]</sup>.

Administration schedule seem to be associated for high, daily dose to liver fibrosis comparing to weekly low dose of MTX. Supplementation with folic acid or folinic acid is associated with reduced incidence of serum transaminase elevation however a relationship between folate depletion and hepatic toxicity has not been fully established<sup>[70,71]</sup>. The reported incidence of liver enzyme abnormalities in subjects with IBD receiving MTX is variable.

The pooled incidence rate of abnormal hepatic aminotransferase levels (defined as more than 2-fold increase over the upper limit of the normal range) in patients treated with methotrexate for IBD was 1.4 per 100 person-months, while the rate of hepatotoxicity (defined as greater than a 2-fold over the upper limit of the normal range) was 0.9 per 100 person-months. The rate of withdrawal from treatment due to these abnormalities was 0.8 per 100 person-months<sup>[72]</sup>.

It is estimated that 15% to 50% of patients receiving a chronic low dose of MTX therapy will develop elevated LFT, usually mild and limited. In most recent studies, incidence seems lower varying from 5%-10% probably due to co-founding risk factors in initial studies such as alcohol intake, obesity, diabetes mellitus, daily dosing and concomitant use of hepatotoxic drugs increasing<sup>[72-74]</sup>.

In a retrospective study by Fournier on 87 IBD patients with a mean duration of 81 wk and a cumulative dose of 1813 mg, 76% of the population kept normal LFT throughout MTX therapy. Among the patients who developed abnormal LFT, underlying risk factors were found in nearly half of the cases. In 11 patients who have received a cumulative dose exceeding 15000 mg, a liver biopsy found no case of moderate or severe fibrosis (Roenigk IIIb or IV) despite abnormal LFT in nine of them. In twenty patients (23%) with abnormal LFT at baseline, spontaneous normalization under MTX was observed in 45% of the cases. Eventually, only 5% of the whole population, needed treatment discontinuation for MTX hepatotoxicity<sup>[74]</sup>.

Another study reporting 20 liver biopsies in patients treated with a cumulative MTX dose of 2633 mg with abnormal LFT in 30% of the cases confirmed the low incidence of severe fibrosis (Roenigk IIIb in 5%)<sup>[75]</sup>. These data suggest that abnormal LFT are poorly correlated with liver histology and confirm the low incidence of severe hepatotoxicity and its uncertain relation with cumulated MTX dose.

End stage liver disease is rare under MTX treatment. In a large retrospective study identifying patient who were listed for liver transplantation over 24 years in the United States, only 117 (0.07%) had MTX related

liver disease with characteristic closed to alcoholic liver disease and NAFLD<sup>[76]</sup>.

**Management:** Patients who undergo MTX therapy should have a careful initial evaluation of historic and physical examination emphasis in alcohol intake, exposure to viral hepatitis, NAFLD risk factors and family history of liver disease.

Regular liver laboratory studies are recommended in patients treated with MTX. Liver biopsy is not recommended routinely during MTX treatment whatever the cumulative dose. However, it should be performed in cases of persistent alteration of transaminases (especially if they do not decrease after reducing the drug dose) and in patients with high accumulated doses, together with other risk factors.

According to RA and psoriasis guidelines<sup>[64,65]</sup>: Laboratory tests for monitoring hepatotoxicity are recommended, every 2 wk initially for 6 wk to 2 mo and then every 2-3 mo; liver biopsy should be performed in selected cases, in case of sustained liver abnormality (especially in case of persistent abnormal LFT despite dose reduction) or high accumulated doses in patients with others risk factors of hepatotoxicity. Treatment needs to be discontinued in cases of severe fibrosis or cirrhosis; adjusting MTX dose could be proposed in case of liver blood elevation and control in 2 and 4 wk.

Transient elastography (Fibroscan) and non-invasive biochemical methods are emerging as new diagnostic tools to evaluate liver fibrosis in various situations<sup>[77]</sup>. In a prospective study in CD patients, the median fibroscan values were similar in 33 treated with cumulative dose of more than 1500 mg and 21 patients naïve of Methotrexate<sup>[78]</sup>. However, this tool could be useful to select patient who should undergo liver biopsy. In a retrospective study of 46 patients treated with MTX for IBD, transient elastography detected six cases of significant fibrosis in patients with normal liver function tests<sup>[79]</sup>. In a case-control study of 518 patients treated with MTX for various inflammatory diseases, 44 patients (8.5%) had FibroScan and/or FibroTest results suggesting severe liver fibrosis. In a multivariate analysis, the 2 factors associated with abnormal markers of liver fibrosis were high body mass index > 28 kg/m<sup>2</sup> and high alcohol consumption. Neither long MTX duration nor cumulative doses were associated with elevated FibroScan or FibroTest results<sup>[78]</sup>. These data suggest that transient elastography should be useful mainly in heavy drinkers or patients with NAFLD risk factors treated with MTX.

### Anti-TNF

TNF- $\alpha$  is a cytokine produced mainly by macrophages that participates in the regulation of inflammation, cell death and proliferation. This cytokine has proinflammatory and immunoregulatory functions and plays a central role in IBD. TNF- $\alpha$  has also effects in the liver, as a mediator of hepatotoxicity and promotor of hepatocyte proliferation and liver regeneration<sup>[80,81]</sup>. There are several anti-TNF agents currently approved for the induction and main-

tenance treatment of IBD, namely infliximab (IFX), adalimumab (ADA), golimumab and certolizumab pegol. Several adverse events have been reported with the use of these agents, such as acute infusion and injection-site reactions, cardiopulmonary and neurologic events, among others<sup>[80]</sup>. The greatest emphasis has been given to the risk of infections and malignancies, but with an increasing use, other side effects are being uncovered, such as immune-mediated diseases<sup>[82,83]</sup>.

**DILI frequency:** In the earlier controlled trials of IFX in RA and CD minor elevation of liver enzymes were reported, but extreme elevations were rare, and there were no cases of jaundice or liver failure<sup>[84,85]</sup>. In a Food and Drug Administration (FDA) post-marketing surveillance program more than 130 cases of liver injury associated with either IFX or etanercept were reported, some of which were fatal or necessitating liver transplantation. This led FDA to issue a safety warning in December 2004 stating that severe hepatic reactions, including acute liver failure, autoimmune hepatitis (AIH) and cholestasis could be caused by IFX<sup>[86]</sup>. In contrast, ADA hepatotoxic potential appears to be low, usually manifesting as an asymptomatic and transient elevation of liver enzymes<sup>[87]</sup>. During ADA controlled Phase 3 trials for CD the rate of liver enzymes elevation was similar to the control-treated patients<sup>[88]</sup>. In a study from Iceland that included patients with IBD, rheumatologic and dermatologic disorders, the absolute risk of DILI associated with IFX was 1 in 120, and with ADA was 1 in 270, but only 11 patients with liver injury were identified in a 5-year period<sup>[89]</sup>. Even though the numbers were small, no statistically significant differences were found between the rates of DILI of the anti-TNF agents studied. Similar rates had been found in a population-based group from the same group, with a 1 in 148 risk of DILI associated with IFX<sup>[90]</sup>. However, as data on the propensity of the anti-TNF to cause drug-induced liver disease comes mainly from case reports and small series it is difficult to estimate the absolute and relative risk of hepatic injury associated with these drugs<sup>[91,92]</sup>. In a retrospective study by Shelton *et al*<sup>[93]</sup> 1753 IBD patients who initiated anti-TNF therapy (1170 IFX, 575 ADA, 8 certolizumab pegol) were analyzed for new onset ALT elevation. One hundred and two patients (6%) had at least one elevated ALT after initiation of the anti-TNF but in 54 of these patients an alternate cause for liver enzymes elevations was found. Of the 48 patients left (45 due to IFX and 3 to ADA), 4 were considered as highly probable of being caused by anti-TNF. There were no differences in the frequency of concomitant immunomodulator use, either thiopurines or methotrexate. In respect to the newest anti-TNF agents, certolizumab and golimumab, to our knowledge there aren't literature reports of DILI. Nevertheless, FDA label for both of them mentions the risk of hepatitis B virus reactivation and elevation on liver enzymes.

**Profile of liver toxicity:** In addition to the risk of reactivation of hepatitis B virus (HBV) infection, anti-

TNF are associated with specific patterns of liver injury. The most common presentation is a hepatocellular injury, found in about 75% of the cases<sup>[89,92,94,95]</sup>. Other presentations are also described, such as a mixed injury pattern with lower peak ALT levels and, more rarely, a cholestatic injury pattern, reported with both IFX and ADA<sup>[94,96-98]</sup>. Overt liver failure sometimes requiring transplantation has rarely been reported<sup>[98-100]</sup>. Immunoallergic features such as eosinophilia and rash don't seem to occur frequently in anti-TNF DILI<sup>[89,98]</sup>. The median latency time to liver enzyme elevation is reported between 13 and 18 wk<sup>[89,93,98]</sup>. Most patients treated with IFX develop liver injury within the fourth infusion, but, rarely, it can occur after several years of treatment<sup>[89,91]</sup>. Histologically, a review by Colina *et al.*<sup>[92]</sup> found necroinflammation in the biopsied cases of DILI caused by IFX reported in the literature, but with uneven characteristics between reports. Bridging and massive necrosis were described in the most severe cases. There were also features normally described in AIH such as piecemeal necrosis in the periportal interface and prominent plasma cells. In two cases ductal damage was reported, one of which was diagnosed as overlap syndrome. Rarely, features associated with toxicity such as eosinophils and neutrophils infiltration and ceroid containing Kupffer cells were seen. One of the features of DILI associated with anti-TNF is the presence of autoimmunity markers in some patients, such as positivity for antinuclear (ANA - often with a homogeneous pattern), anti-double-stranded DNA (anti-DsDNA) and anti-smooth muscle antibodies and/or classic histologic features of AIH, already described for IFX<sup>[83,91-94,101-104]</sup>, etanercept and ADA<sup>[105-107]</sup>. One of the largest series of 34 patients with DILI, have included 26 cases associated with IFX, 6 with ADA and 4 with etanercept<sup>[94]</sup>. Twenty-two of 33 subjects who underwent serologic analysis (67%) were tested positive for anti-nuclear and/or smooth muscle antibodies and presented both later and higher peak levels of alanine aminotransferase than seronegative patients. Of these 22, 17 underwent liver biopsy and 15 subjects had clear features of autoimmunity. The prognosis was good after drug discontinuation, although some patients had benefit from a course of corticosteroids. It is a challenge to distinguish between AIH and drug-induced-AIH as these entities may have similar clinical, biochemical, serological and histological manifestations, with no pathognomonic features<sup>[108]</sup>. In a Weiler-Norman and Schramm editorial a specific nomenclature for immune-mediated DILI in 3 categories was proposed<sup>[109]</sup>. Furthermore, the diseases for which anti-TNF are used may have simultaneous autoimmune disorders and increased autoimmune markers at baseline as part of their immune dysregulation. Lastly, anti-TNF agents can also induce autoantibodies positivity in some patients without the development of liver abnormalities<sup>[110-113]</sup>. In several of the mentioned studies and case series, a proportion of the patients presenting with autoimmune features were treated with corticosteroids. In some of these patients, there was a decrease or disappearance of

autoantibodies with no need of further treatment which suggests an immune-mediated DILI rather than a drug-induced AIH<sup>[89,91,92,94]</sup>. Of note, there are also cases of malignancies described in patients treated with anti-TNF agents, notably case reports of hepatocellular carcinoma in non-cirrhotic patients<sup>[114-116]</sup> and of hepatosplenic T cell lymphoma<sup>[117-121]</sup>. All these patients were in combination treatment with an anti-TNF and a thiopurine, making it difficult to establish the specific role of the anti-TNF agent.

**Hepatotoxicity as a class-effect?** Even though IFX, etanercept and ADA are all anti-TNF agents that directly bind soluble and membrane-bound TNF- $\alpha$ , they are structurally different. IFX is a chimeric IgG1 monoclonal antibody, ADA a fully humanized IgG1 monoclonal antibody and etanercept (not used in IBD but frequently used in rheumatology) is a soluble TNF- $\alpha$  receptor fusion protein<sup>[122]</sup>. This might partially explain why patients with a lack of response to one anti-TNF agent benefit from a switch to another anti-TNF. Also, in the past years, polymorphisms in genes encoding proteins related to TNF- $\alpha$  were identified, explaining to some extent the differences in treatment efficacy and toxicity profile<sup>[123]</sup>. So, even though these drugs were all associated with the development of features of autoimmunity, the capacity in doing so is different for each molecule. In some studies, IFX generated a much higher rate of ANA seroconversion and ANA titer increase than etanercept and ADA<sup>[90]</sup>. Development of autoantibodies has also been described for certolizumab pegol and golimumab<sup>[124,125]</sup>. There are already several cases of successful treatment with another anti-TNF after a prior DILI episode<sup>[90,93-95,126,127]</sup>. This suggests a lack of cross-toxicity within this class of drugs. Etanercept is not a treatment option for IBD, but ADA seems to be a safe alternative in patients who developed liver injury due to IFX and vice-versa.

**Mechanism of liver injury:** The mechanism by which anti-TNF agents induce DILI is still unknown. Even more puzzling is the fact that some patients develop autoimmune diseases for which anti-TNF are a therapeutic option, such as AIH<sup>[109]</sup>. As liver injury can occur after only one infusion and is not related to the dose it seems more likely that the hepatotoxicity of anti-TNF agents is idiosyncratic as opposed to dose-dependent<sup>[93]</sup>. But the complexity of TNF- $\alpha$  role in the liver makes it difficult to draw firm conclusions and several explanations were suggested to date. Genetically predisposed individuals may develop autoimmune diseases triggered by environmental factors. Another possibility is that anti-TNF agents unmask an already existing autoimmune disorder<sup>[83]</sup>. A third explanation relates to the anti-TNF potential in the generation of autoantibodies. The binding of IFX to the transmembrane TNF- $\alpha$  may lead to apoptosis of monocytes and T-lymphocytes with exposition of nucleosomal autoantigens and formation of autoantibodies<sup>[128,129]</sup>. The reduced clearance of nuclear debris due to the downregulation of C-reactive protein may also play a role

by prolonged immune system exposure to intracellular material<sup>[130]</sup>. The structural differences of anti-TNF agents with different binding affinities do membrane TNF- $\alpha$  and different abilities of complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity may explain the different potentials on the induction of autoimmunity<sup>[113,128,129,131]</sup>. Another hypothesis is that anti-TNF agents inhibit the induction of cytotoxic lymphocytes that would suppress auto reactive B cells, therefore promoting humoral autoimmunity<sup>[132]</sup>. All these proposed mechanisms try to explain the immune-mediated DILI caused by anti-TNF agents. However, there are several cases without evidence of autoimmunity, in which direct liver damage may be involved<sup>[133,134]</sup>.

#### Management of DILI associated with anti-TNF:

The optimal management of liver injury induced by anti-TNF therapy is still not consensual. The prognosis is generally good, with most patients presenting with mild elevation in liver enzymes resolving spontaneously with continuation of anti-TNF therapy<sup>[93]</sup>. A consensus statement proposes more restrictive criteria, with avoidance or discontinuation of treatment in patients with transaminases superior to 3 times the upper limit of normal<sup>[135]</sup>. Many authors have since suggested different management algorithms<sup>[91,101,136]</sup>. Ideally, before initiation of treatment, a baseline panel of liver enzymes should be obtained, together with a determination of HBV and HCV status<sup>[137]</sup>. After initiation of treatment, liver enzymes should be monitored periodically, especially during the first three months. When faced with an elevation of liver enzymes, other causes should be excluded, as in any case of suspected DILI. In case of minor elevations of ALT (< 3 times the upper limit of normal), anti-TNF may be continued with close monitoring until resolution. If the enzymes are persistently elevated, superior to 3 times the upper limit of normal or in case of alarm signals such as jaundice, a multidisciplinary approach with refer to an hepatologist and consideration for corticosteroid treatment is advised. A liver biopsy may be useful in this context. If a DILI is documented, anti-TNF withdrawal remains controversial<sup>[91,136]</sup>. Even though advocated by some authors the interest of routine assessment of autoimmune markers prior to the introduction of an anti-TNF agent is not established<sup>[83,91,113,136,138]</sup>. Several studies show that this approach doesn't predict the risk of developing subsequent liver injury or autoimmune events and treatment with anti-TNF can be continued in the presence of an asymptomatic ANA seroconversion<sup>[89,110,112]</sup>. Therefore, routine testing for autoantibodies can't be recommended until further evidence of the clinical implications of these autoantibodies is obtained.

#### New biologic treatments

Natalizumab and vedolizumab are two integrin antagonists approved for the treatment of IBD. Natalizumab is a humanized recombinant monoclonal antibody that blocks  $\alpha 4\beta 1$  and  $\alpha 4\beta 7$  integrin-mediated interactions, preventing migration of leukocytes into the gut and brain<sup>[139]</sup>.

Even though its efficacy in the treatment of CD was demonstrated, natalizumab association with a number of cases of progressive multifocal leukoencephalopathy has limited its use<sup>[140,141]</sup>. Vedolizumab is a humanized monoclonal antibody with specificity to the gut  $\alpha 4\beta 7$  integrin with proven efficacy in the treatment of CD and UC<sup>[142,143]</sup>. Both drugs appeared to have good safety profiles during initial trials. However, on post-marketing surveillance, 6 cases of clinically significant DILI related to natalizumab were reported to FDA, leading to an alteration of its label<sup>[144]</sup>. In all cases, natalizumab was used for the treatment of multiple sclerosis, and liver injury occurred as early as 6 d after the first administration of the drug. Five of the cases had a hepatocellular pattern of injury, and 3 patients had autoimmune features. One patient had recurrence of the increase of liver enzymes upon readministration of natalizumab, providing evidence that natalizumab was responsible for the injury. There were no deaths nor was a liver transplantation needed. Since then, a case of acute liver failure possibly due to drug-induced AIH and a case of fatal fulminant liver failure due to acute HBV infection in patients treated with natalizumab for multiple sclerosis were reported<sup>[145,146]</sup>. There were also cases of elevation of transaminases and/or bilirubin in vedolizumab trials for IBD. Ustekinumab is a fully human monoclonal antibody that blocks the activity of interleukin 12/23 shared p40 subunit. This drug has shown efficacy in the treatment of CD, particularly in patients previously treated with IFX<sup>[147]</sup>. The majority of safety data of ustekinumab comes from dermatologic studies. In PHOENIX 1 and 2<sup>[148,149]</sup>, two studies that evaluated efficacy and safety of ustekinumab in patients with psoriasis, the proportion of patients with liver enzymes abnormalities was low and similar between ustekinumab and control groups. In a small retrospective study including 44 patients with psoriasis treated with ustekinumab, elevation of liver enzymes was mild and uncommon, with no cases of severe DILI<sup>[150]</sup>. Interleukin-12 is involved in the clearance of HBV by suppressing viral replication, which may explain why patients treated with ustekinumab might be at increased risk of HBV reactivation<sup>[151]</sup>. Most pivotal studies of ustekinumab excluded patients infected with HBV and HCV; for this reason its safety in this context is not known. In a retrospective study in patients with psoriasis and concurrent HBV infection treated with ustekinumab, 4 patients infected with HBV received antiviral prophylaxis during treatment, without evidence of virus reactivation<sup>[152]</sup>. Of the 10 patients who didn't receive prophylaxis, 2 fulfilled the criteria for HBV reactivation. In another retrospective study, 3 patients with HCV and 1 patient with HBV under prophylaxis with entecavir were treated with ustekinumab and didn't have an aggravation of the hepatitis<sup>[153]</sup>. Cases of acute HBV infection/HBV reactivation during ustekinumab treatment and, on the other hand, cases where ustekinumab was safely administered despite HBV or HCV infection were reported recently<sup>[154-157]</sup>. Even though a real frequency of hepatic adverse events is not yet known for these drugs, this evidence suggests that all patients considered



for biologic treatment should be screened for hepatitis B and C infection prior to introduction of the drug, and liver function should be monitored periodically for the duration of the treatment.

### Calcineurine inhibitors

Cyclosporine is a potent immunosuppressive drug effective in the treatment of acute severe UC refractory to corticosteroids<sup>[158,159]</sup>. Tacrolimus is a potential alternative to cyclosporine<sup>[160,161]</sup>. One of the main limitations to cyclosporine use in clinical practice is its safety profile, namely nephrotoxicity, neurotoxicity and infections, with a need of frequent monitoring<sup>[158]</sup>. The hepatotoxicity associated with cyclosporine was mainly described in transplant patients. It's generally characterized by a cholestatic pattern due to an impairment of bile formation, probably caused by an interference in the bile secretory apparatus. Liver injury caused by cyclosporine is dose-dependent and can be reduced by a diminution of the dose. Even though the prevalence of liver injury due to cyclosporine was initially estimated to be superior to 50%, this phenomenon was probably due to the use of the drug without blood monitoring, leading to toxic levels of cyclosporine<sup>[162]</sup>. Studies in IBD patients show a much lower prevalence of hepatotoxicity, between 1% to 4%, generally translated by an elevation in liver enzymes<sup>[158,163,164]</sup>. In one study, 19% of patients (21/111) developed abnormal liver function tests, but they were only significantly high in one patient<sup>[165]</sup>. Tacrolimus hepatotoxicity is rare with a similar clinical and biochemical profile to those of cyclosporine. In some cases, there is a lack of cross-reactivity between these two drugs, and one can be used after hepatotoxicity to the other<sup>[162]</sup>. Nonetheless, hepatotoxicity is generally considered as a rare and minor adverse event with these drugs.

### Thalidomide

Thalidomide was initially used to treat morning-sickness associated with pregnancy, until being withdrawn from the market due to its teratogenic effects. Since that, in view of its anti-inflammatory and immunomodulatory properties, it has been reintroduced for the treatment of various diseases including IBD<sup>[166,167]</sup>. Hepatotoxicity with thalidomide is reported as a rare but serious adverse event. In a review of adverse events reported in the first 18 mo of postmarketing surveillance after thalidomide reintroduction in the market, one case of fatal hepatic failure possibly directly related to thalidomide was identified<sup>[168]</sup>. In the latest years, other cases with different degrees of severity were reported, mostly in older females treated with thalidomide for multiple myeloma, some of them with an underlying hepatic disease<sup>[169-172]</sup>. The mechanism of hepatotoxicity of thalidomide remains unclear. The main route of elimination of thalidomide is through non-enzymatic hydrolysis into multiple products in biological fluids and it doesn't seem to undergo significant hepatic metabolism<sup>[173]</sup>.

### New investigational treatments

More recently several molecules have shown promising results in IBD and should obtain medical agreement within the next few years. Mongersen, a new oral SMAD 7 antisense oligonucleotide was superior to placebo for inducing clinical remission at day fifteen and maintained for at least two weeks in CD<sup>[174]</sup>. Increased aminotransferase levels were observed at the dose of 40 mg per day in 5% of the patients but no case was reported at the dose of 10 mg and 160 mg per day.

Tofacitinib, a selective oral inhibitor of the Janus kinase, a family of kinases that mediates signal-transduction activity involving the common gamma chain of the surface receptors for multiple cytokines was superior to placebo for inducing clinical response at week eight in UC<sup>[175]</sup>. At week twelve, adverse events occurring in  $\geq 5\%$  of patients in any tofacitinib group did not include liver toxicity.

Ozanimod, an oral agonist of the sphingosine-1-phosphate receptor subtypes 1 and 5 that induces peripheral lymphocyte sequestration was superior to placebo at a dose of 1 mg per day for inducing clinical remission at eight weeks<sup>[176]</sup>. After exposure to up of 32 wk, aspartate aminotransferase increasing was noted in 2% and 1% of patients treated with 0.5 and 1 mg of Ozanimod respectively. These preliminary data suggest that new therapeutic approaches in IBD induce minor hepatotoxicity.

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## Management of centrally located hepatocellular carcinoma: Update 2016

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### Abstract

Centrally located hepatocellular carcinoma (HCC) is sited in the central part of the liver and adjacent to main hepatic vascular structures. This special location is associated with an increase in the difficulty of surgery, aggregation of the recurrence disease, and greater challenge in disease management. This review summarizes the evolution of our understanding for centrally located HCC and discusses the development of treatment strategies, surgical approaches and recurrence prevention methods. To improve patient survival, a multi-disciplinary modality is greatly needed throughout the whole treatment period.

**Key words:** Centrally located hepatocellular carcinoma; Hepatectomy; Combined treatment; Hepatic vascular occlusion

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**Core tip:** Centrally located hepatocellular carcinoma (HCC) is situated in the deeper portions of the liver and adjoins main vascular structures. Due to this special location, the management of this group of patients is challenging. Low resection rates and high recurrence rates are two major problems that urgently need to be resolved. This review summarizes the evolution of our understanding for centrally located HCC and the development of disease management, and explores the possible strategies to improve overall patient survival.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and the fourth leading cause of cancer death worldwide<sup>[1]</sup>. Traditionally, we describe centrally located HCC as being sited in Couinaud hepatic segments IV, V or VIII<sup>[2]</sup>. These tumors are often adjacent to main hepatic vascular structures and accept a dual blood supply from the right and left hepatic artery branches. Due to this special location, the management of this group of patients is still challenging. Low resection rates and high recurrence rates are two major problems that urgently need to be resolved. In this review, we focus on recently developed centrally located HCC classification, evaluation, surgical techniques and adjuvant treatments, and we explore the possible management strategies to improve overall patient survival.

## DEFINITION AND CLASSIFICATION OF CENTRALLY LOCATED HCC

The traditional definition of centrally located HCC is based on Couinaud's segmental anatomy of the liver. In this system, the liver is divided into eight functionally independent segments. Each segment has separating vascular inflow, outflow and biliary drainage. Segments IV, V and VIII lie in the medial and make up the middle part of the liver. Tumors located in this area are called centrally located HCC. However, in clinical settings, the factor that determines the degree of surgical difficulty is not only the segment location of the tumor but also the proximity of the tumor to major vascular structures. To reflect upon its key clinical characteristics, we previously proposed a clinical definition of centrally located HCC based on the relationship between the tumor and vascular structures. This definition defines centrally located HCC as "carcinoma adjoined hepatic portals, less than 1 cm from major vascular structures (including the main portal branches, the main trunks of the hepatic veins as well as the inferior vena cava) which are usually located in Couinaud segments I, IV, V, VIII, or at the junction of the central segments"<sup>[3]</sup>.

More recently, a new classification system for centrally located HCC was proposed. Focusing on the involvement of resected areas and the anatomical location of tumors relative to the main vascular structures of the liver, this system divided centrally located HCC into four subtypes<sup>[4]</sup>. The first subtype is the tumors that are located in liver segment V or/and IVb. The second subtype is the tumors that are located in liver segment IVa or/and VIII. The tumors that are located in the connection of liver segment V/IVb and liver segment IVa/VIII are categorized in the third subtype. This subtype can be further divided

into those that are superficially located and those that are deeply located. The latter is often closely adjacent to the inferior vena cava. The last subtype specifically describes the large tumors that are located in the middle of two hepatic portals. This classification system may help to plan the extent of resection or assess surgical risk, but its practical significance still needs to be further evaluated. The definition and classification of centrally located HCC, which not only rely on anatomic structures but also tumor behavior and treatment strategies, continue to evolve. We reported a single-center experience of the treatment of centrally located HCC in 2013<sup>[5]</sup>. Since then, many novel retrospective and prospective studies have been performed in this field. With a deeper understanding, the evaluation and management of the disease have also been changed greatly.

## THREE-DEMONSTRAL IMAGING RECONSTRUCTION IN PREOPERATIVE EVALUATION

Centrally located HCC has complex adjacent structures. Consequently, the detailed preoperative evaluation of resectability is necessary. Besides liver function status, a clear image including tumors, blood vessels and bile ducts are essential. This preoperative evaluation is commonly obtained by multiphase contrast-enhanced computed tomography (CT), magnetic resonance imaging or ultrasound. However, because hepatectomy procedures need to be completed in a three-dimensional (3D) setting, planning anatomic resections may be difficult when relying on 2D images. In 1995, van Leeuwen *et al*<sup>[6]</sup> reported depiction of the relationship between tumor and individual segmental anatomy in a 3D format. In 2005, Numminen *et al*<sup>[7]</sup> reported a 3D imaging technique, which was based on the data of multi-detector row CT scanning. So far the clinical value of 3D imaging systems in preoperative evaluations has been confirmed by a series of studies<sup>[8-12]</sup>.

Currently, the 3D morphometric analysis system not only can precisely visualize tumors and adjacent vascular structures such as portal veins, hepatic veins and bile ducts from different directions in a screen but also can calculate the volume of the tumor and its surrounding areas and perform a virtual hepatectomy. Tian *et al*<sup>[13]</sup> reported a 3D morphometric analysis model of liver tumor image reconstruction with customized software for individual patients. This study included 39 patients with centrally located HCC. They all accepted a 3D image reconstruction and morphometric analysis before operation. The results demonstrated that the 3D model provides a quantitative morphometry of tumor masses. The predicted values were also confirmed by intraoperative conditions<sup>[13]</sup>. In clinical practice, 3D image morphometric analysis is often combined with liver function measurements such as Indocyanine Green (ICG) clearance test to determine the appropriate resection area. With the development of imaging techniques,

the usage of 3D imaging reconstruction systems in the surgical evaluation for centrally located HCC will be more and more promising.

## INTRAOPERATIVE VASCULAR OCCLUSION TECHNIQUES

Centrally located HCC is situated in the deeper portions of the liver and adjoins main vascular structures, making hepatectomy difficult and time-consuming. Controlling intraoperative bleeding without excessive hepatic warm ischemia is a critical problem that has long perplexed liver surgeons. In 1908, James Hogarth Pringle described that occlusion of hepatic pedicle could help hemorrhage control<sup>[14]</sup>. Pringle's maneuver was then proposed to minimize blood loss during hepatic surgery. However, clamping of hepatic pedicle means occluding the total inflow of hepatic artery and portal vein. Clamping of hepatic pedicle carries potential hazards for liver function due to hepatic ischemia, while also contributing to intestinal congestion<sup>[15,16]</sup>. In addition, there has been a study that showed that Pringle's maneuver induces hepatic metastasis by stimulating tumor vasculature<sup>[17]</sup>. Especially in some HCC high incidence regions, where most patients have liver cirrhosis, long durations of hepatic pedicle occlusion should be treated with even greater care<sup>[18]</sup>.

To resolve this problem, several selective hepatic vascular approaches have been described, represented by a hemihepatic vascular occlusion technique, which divides hepatic inflow into total right and total left Glisson sheaths<sup>[19]</sup>. In 2012, we proposed a concept named selective and dynamic region-specific vascular occlusion<sup>[20,21]</sup>. Before resecting liver tumors, a careful hepatic pedicle dissection was performed. The left or right hepatic artery and portal vein were dissected, exposed, and encircled with occlusion tapes. If caudate resection was needed, all short hepatic veins were ligated and dissected to free the caudate lobe from the inferior vena cava. For tumors involving the second hepatic portal or the trunk of the hepatic vein, the hepatocaval ligament was divided to make the root of the right hepatic vein stand out. If necessary, the common trunk formed by the middle and left hepatic veins also needed to be isolated to avoid fatal hemorrhage and air embolism. When liver parenchyma was dissected, we dynamically selected different regions for inflow or outflow blood occlusion according to tumor location. We have explored usage of this technique in the hepatectomy of complex centrally located HCC. Our study and other groups' studies showed that selective interruption of the arterial and venous flow to specified regions of the liver can satisfactorily control intraoperative bleeding, while also reducing ischemia-reperfusion injury of the whole liver. Most importantly, selective occlusion can maintain a fluent portal vein blood flow, which potentially avoids intraoperative gastrointestinal congestion and may accelerate postoperative recovery<sup>[20,22-24]</sup>.

Given the complexity of centrally located HCC, there has been an upcoming consensus that the application of hepatic vascular occlusion needs to be more flexible in the hepatectomy. We believe the occlusion techniques not only include dissecting hepatic pedicle, hepatic veins or IVC, but also are embodied in each step of surgical procedure. For example, there is no need to occlude vascular structures when we dissect surface liver parenchyma. In some circumstances, the traditional sutures around the resection area of the liver, or even a simple hand pinching, could be effective to control bleeding. Appropriate occlusion methods can minimize intraoperative bleeding and maximize the protection of liver function. These methods allow surgeons to complete more complicated surgical procedures.

## SURGICAL DETERMINATION AND RESECTION MARGIN

As a special type of HCC, the treatment choice of centrally located HCC is often challenging. Transcatheter arterial chemoembolization (TACE) is often recommended as the primary palliative treatment for unresectable HCC. This treatment is based on the fact that highly vascularized HCCs are mainly supplied by hepatic arteries, while normal liver parenchyma accepts blood supplies from both hepatic arteries and portal veins<sup>[25]</sup>. TACE was frequently performed in patients with centrally located HCC as a combined approach, but the efficacy of the treatment is still controversial. Mostly for unresectable centrally located HCCs, which are often associated with portal vein thrombosis (PVT), TACE in combination with radiotherapy has been reported to be therapeutically beneficial<sup>[26]</sup>. Chen *et al.*<sup>[27]</sup> reported preoperative TACE in 89 patients with large centrally located HCC and compared their recurrence patterns and long-term outcomes. The results showed that preoperative TACE potentially improved resection rate and extended overall patient survival, but preoperative TACE also increased chronic inflammation, perihepatic adhesion and the likelihood of postoperative complications. Radiofrequency ablation (RFA) is another treatment choice for selected patients. Guo *et al.*<sup>[28]</sup> reported 196 patients with centrally located small HCC (diameter < 5 cm), in which 94 patients accepted percutaneous RFA and 102 patients received partial hepatectomy. The results showed that RFA could get similar treatment efficacy as that of partial hepatectomy but with fewer complications in patients with small centrally located HCC. In this study, centrally located HCCs were defined as tumors located at Couinaud's segments IV, V and VIII. For the patient group that we discussed above, the tumor control rate of RFA is often disappointing due to potential injuries to adjacent main vasculatures and risks of bile leakage<sup>[29]</sup>. RFA can also be used to assist liver resection, which showed efficacy of reducing operation time and blood loss<sup>[30,31]</sup>. In addition, tumor ablation can be completed simultaneously in the operation. This new modality is worthy of being

**Table 1** Surgical treatment of centrally located hepatocellular carcinoma

Years	Patients' number	Surgical approaches	Operative variables and outcomes
1993	19	Extended major hepatectomy or irregular hepatectomy (large tumor)	Mean operative blood loss: 1186.6 mL Mean operative time: 7.5 h One year overall survival rate: 84.2% One year recurrence-free survival rate: 73.7% <sup>[65]</sup>
1999	15	Mesohepatectomy	Mean operative blood loss: 2450 mL Hospital stay: 14.9 d Six year overall survival rate: 30% Six year recurrence-free survival rate: 21% <sup>[2]</sup>
2000	18	Mesohepatectomy	Mean operative time: 238 min Mean operative blood loss: 914 mL Hospital stay: 9 d <sup>[66]</sup>
2003	52	Central hepatectomy	Blood transfusion was needed: 1030 ± 1320 mL Bile leak occurred in 4 patients The median overall survival: 51 mo <sup>[35]</sup>
2007	246	Mesohepatectomy (larger tumor)	Mean operative blood loss (without pre-TACE): 420 mL Overall hospital mortality (without pre-TACE): 0.6% Five year overall survival rate (without pre-TACE): 31.7% <sup>[27]</sup>
2008	27	Central bisectionectomy	Median operative time: 330 min Twelve patients had postoperative complications and two died Bile duct injury was the most common complication <sup>[36]</sup>
2012	104	Hemi-/extended hepatectomy and central hepatectomy	Mean blood loss of hemi-/extended hepatectomy and central hepatectomy: 750 mL and 500 mL Five year overall survival rate for hemi-/extended hepatectomy and central hepatectomy: 66.2% and 53.1% Five year recurrence-free survival rate for hemi-/extended hepatectomy and central hepatectomy: 38.9% and 15% <sup>[67]</sup>
2013	292	Mesohepatectomy	Mean operative time: 259 min Mean operative blood loss: 634 mL Hospital stay: 10 d <sup>[68]</sup>
2014	350	Mesohepatectomy	Mean blood loss for large tumor: 950.7 mL Ascites was the most common complication Five year overall survival rate for larger tumor: 30% <sup>[69]</sup>
2014	24	Mesohepatectomy	Mean operative time: 238 min Mean operative blood loss: 480 mL Three year overall survival rate: 46% <sup>[70]</sup>
2014	198	Extended hepatectomy and mesohepatectomy	The biliary leakage incidence after mesohepatectomy: 10.2% Five year overall survival rate for mesohepatectomy: 28.9% Five year recurrence free survival rate for mesohepatectomy: 16.9% <sup>[71]</sup>
2014	119	Hepatectomy with narrow margin	Bile leak occurred in 4 patients Five year overall survival rate: 48.3% Five year recurrence-free survival rate: 27.8% <sup>[3]</sup>
2015	69	Hemi-/extended hepatectomy and central hepatectomy	Mean blood loss of hemi-/extended hepatectomy and central hepatectomy: 522.2 mL and 447.8 mL Hospital stay for hemi-/extended hepatectomy and central hepatectomy: 21.3 and 14.9 d Three year overall survival rate for hemi-/extended hepatectomy and central hepatectomy: 64% and 61% <sup>[72]</sup>
2016	353	Mesohepatectomy	Five year overall survival rate: 40.2% Five year recurrence-free survival rate: 30.7% <sup>[4]</sup>

TACE: Transcatheter arterial chemoembolization.

explored in centrally located HCC treatment. Liver transplantation is an ideal option, but the shortage of liver donors limits its applicability. Only a few patients can fulfill the strict selection criteria of liver transplantation.

Under these circumstances, surgical resection aimed at a total removal of the tumor mass remains the optimal treatment choice for selected patients with centrally located HCC. In early reports, extended major hepatectomy and mesohepatectomy were often recommended (Table 1). The reported overall survival of patients after surgery was much greater than the natural history of the disease<sup>[32,33]</sup>. However, the surgical procedures for centrally located

HCC are still more technically demanding. As is shown in Table 1, the operation time was relatively long and the operative blood loss could be a severe problem, especially before 2000. In recent years, due to the fact that extensive hepatectomy removing the major part of live parenchyma was often difficult to achieve in clinical practice, several non-anatomic approaches of central hepatectomy have been proposed. Surgeons need to weigh the dangers of postoperative liver dysfunction against the radical major resection, especially in patients with chronic hepatic diseases. A surgical group from Japan reported a no-margin resection in HCC patients.

These tumors closely adhered to main hepatic vascular structures and were resected along the surfaces of tumors and vascular structures. There existed no significant differences in patient recurrence free survival and overall survival between this group and those who underwent regular hepatectomy<sup>[34]</sup>. Our group reported 118 patients with centrally located HCC, where the tumor is adherent to major hepatic vessels. These patients underwent comprehensive preoperative assessment. Unfortunately, most of them, especially patients with chronic liver diseases, would not have enough liver functional reserve to accept major hepatectomy based on ICG clearance test and 3D image reconstruction. To completely remove the tumor and preserve remnant liver function, we carefully exposed and resected the tumor from the vascular surface. This surgical approach increased the resection rate for patients with a special type of centrally located HCC. In combination with comprehensive adjuvant therapies, a five-year overall survival rate of 44.9% was reported, which is clearly superior to previously reported palliative strategies<sup>[35-38]</sup>.

For a long time, the safe resection margin is one of the major disputes in the practice of HCC surgery. Several previous studies indicated that a resection margin of more than 1cm is an independent factor of improved recurrence-free survival<sup>[39-42]</sup>. But whether it can benefit all HCC patients is still controversial<sup>[43-47]</sup>. The clinical definition of centrally located HCC emphasizes the vicinity of liver tumor with major vascular structures. It is not easy to obtain a safe ( $> 1$  cm) resection margin for this group of patients. More in-depth studies are needed to explore the possible ways to reduce postoperative recurrence and increase patient survival. It should be noted that HCC is a systematic disease; it would be impractical to prevent recurrence only by extending the resection region. We believe that the individualized surgical approaches, which are based on the patients' condition, liver function, and tumor location, are optimal for patients with centrally located HCC.

## ADJUVANT THERAPIES FOR RECURRENCE PREVENTION

Recurrence disease is one of the main causes of long-term treatment failure for HCC patients. It was reported that the five-year risk of recurrence of HCC after hepatectomy could be as high as 70%<sup>[48]</sup>. Many factors are associated with tumor recurrence, such as tumor size, number, grade, vascular invasion, positive margin, cirrhosis and preoperative treatment<sup>[49-54]</sup>. Surgeons have long been searching for improved adjuvant therapies to reduce recurrence. TACE was investigated most in early studies and showed limited efficacy in preventing recurrence for selected HCC patients. Peng *et al.*<sup>[55]</sup> reported that postoperative TACE enhances the effect of liver resection combined with PVT removal for HCC patients. Another study reported 115 Stage IIIA HCC patients who underwent hepatectomy with adjuvant

TACE or hepatectomy alone. The results indicated that hepatectomy with adjuvant TACE improved patients' recurrence-free and overall survival<sup>[56]</sup>. But for most HCC patients, the primary role of postoperative TACE is to detect and treat early metastasis, rather than extend patient survival<sup>[57]</sup>. Adjuvant intra-arterial injection of iodine-131-labeled lipiodol after resection of HCC also has been reported in recurrence prevention. However, the clinical value of this particular treatment is still uncertain<sup>[58-60]</sup>.

As addressed above, the limited resection margin is a major concern for centrally located HCC. In clinical practice, we observed a higher recurrence rate for this group of patients<sup>[20]</sup>. In 2014, a randomized controlled study explored the safety and efficacy of adjuvant radiotherapy (RT) for centrally located HCC after a narrow margin ( $< 1$  cm) resection<sup>[3]</sup>. The results showed that adjuvant RT for centrally located HCCs after narrow margin hepatectomy was technically feasible and relatively safe. The subgroup analysis demonstrated that postoperative region-specific RT remarkably increased patient recurrence-free survival. Patients with centrally located HCC are often at high risk of recurrence after hepatectomy. It is necessary to pay more attention to postoperative management. Regular follow-up, liver function monitoring, appropriate nutrition support and treatment of chronic liver disease (anti-virus) are important for improving patient survival<sup>[61]</sup>. Some recent studies have shown that integrative strategies, such as herbal medicine, could be effective in maintaining inner environment homeostasis and inhibiting tumor growth<sup>[62-64]</sup>. Integrative medicine focuses on restoring and maintaining a state of complete physical, mental and social well-being and not merely on the eliminating disease or infirmity. It will be interesting to explore these strategies in recurrence prevention. Currently, the development of novel treatment strategies, which incorporate molecular and immunological mechanisms, are underway and hold promise to be used for recurrence control in the future.

## CONCLUSION

Over the past two decades, the management of centrally located HCC has evolved profoundly. Surgical indications, approaches, and techniques are greatly shifting. However, due to the complex procedure of centrally located HCC resection, obtaining high-level clinical evidence of surgical approaches on a large scale is still challenging. Dedicated clinical trials for this population with standardized classification are warranted. Currently, novel treatment options for HCC are constantly emerging. To elucidate which specific therapies or therapeutic combinations may be most beneficial for individual patients, a multi-disciplinary work team involving specialists in surgery, oncology, hepatology, radiology and integrative medicine is greatly needed during the whole treatment period. With more studies being involved, a general guideline for this special type of HCC can be expected and can further contribute to improving patient survival.



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Retrospective Study

# Importance of surgical margin in the outcomes of hepatocholangiocarcinoma

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## Abstract

### AIM

To evaluate the significance of resection margin width in the management of hepatocholangiocarcinoma (HCC-CC).

### METHODS

Data of consecutive patients who underwent hepatectomy for hepatic malignancies in the period from 1995 to 2014 were reviewed. Patients with pathologically confirmed HCC-CC were included for analysis. Demographic, biochemical, operative and pathological data were analyzed against survival outcomes.

### RESULTS

Forty-two patients were included for analysis. The median age was 53.5 years. There were 29 males. Hepatitis B virus was identified in 73.8% of the patients. Most patients had preserved liver function. The median preoperative indocyanine green retention rate at 15 min was 10.2%. The median tumor size was 6.5 cm. Major hepatectomy was required in over 70% of the patients. Hepaticojunctionostomy was performed in 6 patients. No hospital death occurred. The median hospital stay was 13 d. The median follow-up period was 32 mo. The 5-year disease-free survival and overall survival were 23.6% and 35.4% respectively. Multifocality was the only independent factor associated with disease-free survival [ $P < 0.001$ , odds ratio 4, 95% confidence interval (CI): 1.9-8.0]. In patients with multifocal tumor ( $n = 20$ ), resection margin of  $\geq 1$  cm was associated with improved 1-year disease-free survival (40% vs 0%; log-rank,  $P = 0.012$ ).



## CONCLUSION

HCC-CC is a rare disease with poor prognosis. Resection margin of 1 cm or above was associated with improved survival outcome in patients with multifocal HCC-CC.

**Key words:** Hepatocholangiocarcinoma; Hepatocellular cholangiocarcinoma; Survival; Hepatectomy; Resection margin

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**Core tip:** A retrospective review of all patients who had undergone curative resection for hepatocholangiocarcinoma in the last 20 years was performed in a university center. The 5-year disease-free and overall survival were 23.6% and 35.4% respectively. Various patient and disease factors were investigated with respect to their effect to disease free and overall survival using cox regression analysis. Multifocality was the only independent factor associated with disease-free survival ( $P < 0.001$ ). In a subgroup of patient ( $n = 20$ ) who had multifocal tumor, resection margin of  $\geq 1$  cm was associated with improved 1-year disease-free survival (40% vs 0%,  $P = 0.012$ ).

Ma KW, Chok KSH. Importance of surgical margin in the outcomes of hepatocholangiocarcinoma. *World J Hepatol* 2017; 9(13): 635-641 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i13/635.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i13.635>

## INTRODUCTION

Hepatocholangiocarcinoma (HCC-CC) is a rare disease entity contributing to 1%-3% of primary hepatic malignancies<sup>[1-4]</sup>. Histologically, tumor cells of hepatocyte and bile ductal epithelial origins are identified in HCC-CC<sup>[5]</sup>. While "pseudoglandular" structures can as well be observed in other hepatocellular carcinoma (HCC) variants<sup>[6]</sup>, genuine HCC-CC should demonstrate true glandular structures with mucin production<sup>[7]</sup>. Since the first description of HCC-CC in 1949 by Allen and Lisa<sup>[8]</sup>, 3 subtypes of the disease were established: Type 1, double separate tumors - HCC and intrahepatic cholangiocarcinoma (ICC) - in the same liver; type 2, the presence of HCC and ICC in a continuum; type 3, intermingling of HCC and ICC cells<sup>[8]</sup>. In 1985, Goodman *et al*<sup>[9]</sup> revised the classification with new descriptions of 3 types of HCC-CC: The collision type, the transitional type, and fibrolamellar HCC with mucin-producing pseudoglands. Later, the World Health Organization redefined HCC-CC as a distinct tumor with intimate and unequivocal fusion of HCC and ICC cells<sup>[10]</sup>. The disease's clinical outcomes and prognostic factors have barely been studied. The median survival after HCC-CC resection varied from study to study, from 12 to 48 mo<sup>[11-15]</sup>. This disparity may be partially explained by the heterogeneity in diagnostic criteria for

HCC-CC in the studies. The inclusion of HCC variants (which do not contain genuine ICC components) and the collision type of HCC-CC (which is no longer regarded as HCC-CC according to the World Health Organization) probably led to data contamination and resulted in difference in prognosis<sup>[16]</sup>.

The width of resection margin had been shown to affect the oncological outcomes of hepatectomy for HCC<sup>[17-19]</sup> and ICC<sup>[20,21]</sup>. In a prospective randomized trial involving 169 patients by Shi *et al*<sup>[19]</sup>, patients who were randomized to the narrow margin group (1 cm) had significantly inferior 5-year overall survival when compared with patients who had HCC resection with wide margin (2 cm) (49.1% vs 74.9%). For the role of resection margin in ICC, Farges *et al*<sup>[21]</sup> demonstrated a significant correlation between resection margin and median survival in a subgroup of node-negative patients ( $\leq 1$  mm: 15 mo, 2-4 mm: 36 mo, 5-9 mm: 57 mo,  $\geq 10$  mm: 64 mo;  $P < 0.001$ ). In a recent article by our center, patients with early ICC were shown to benefit from resection margin of over 1 cm<sup>[20]</sup>. Nonetheless, the role of resection margin in management of HCC-CC remains to be defined. This retrospective study aimed to elucidate the clinical features of HCC-CC and the impact of resection margin width on patient survival.

## MATERIALS AND METHODS

Data of consecutive patients who underwent hepatectomy for hepatic malignancies in the period from 1995 to 2014 were reviewed. Patients included for analysis were those who: (1) had pathologically confirmed HCC-CC; (2) were not younger than 18 years; and (3) did not receive re-resection for recurrent HCC-CC. Diagnosis of HCC-CC was made by a combination of histological and immunohistochemical staining<sup>[22,23]</sup>, supplemented by electron microscopy examination when necessary<sup>[11]</sup>. Demographic, biochemical, operative and pathological data were analyzed against survival outcomes. Categorical parameters were analyzed with Pearson's  $\chi^2$  test and continuous data were analyzed with the Mann-Whitney  $U$  test. Univariate analysis with bivariate correlation and multivariate analysis with the Cox regression model were performed. In this study, survival outcomes of HCC-CC were compared with the HCC and ICC patients of the same period. The Kaplan-Meier method was used for survival analysis and the log-rank test was used for survival comparison.  $P$ -values of  $\leq 0.05$  were considered statistically significant. The computer software Statistical Product and Service Solutions for Windows (SPSS, Chicago, Illinois, United States) was used for statistical analyses.

### Perioperative care and follow-up protocol

Before hepatectomy, a basic biochemistry test was performed to assess complete blood picture, clotting profile, and liver and renal functions. Levels of tumor markers such as alpha-fetoprotein, carcinoembryonic antigen and cancer antigen 19-9 were recorded. Major

**Table 1** Demographic characteristics and baseline biochemistry of the study population

	No. of patients = 42
Male:female	29:13
Age (yr)	52.5 (26-72)
Hepatitis B virus carrier	31 (73.8%)
Hepatitis C virus carrier	0
Hemoglobin (g/dL)	13.4 (8.6-16.7)
White cell count ( $10 \times 6/L$ )	5.8 (3.5-10.1)
Platelet count ( $10 \times 9/L$ )	185 (89-499)
Creatinine (mmol/L)	84 (61-131)
Total bilirubin (mmol/L)	10 (2-61)
Albumin (g/L)	40 (29-49)
Aspartate transaminase (umol/L)	44 (14-270)
Alkaline phosphatase (umol/L)	92 (26-516)
Prothrombin time (s)	13.5 (10.9-13.5)
Alpha-fetoprotein (u/L)	75.5 (2-219020)
Carcinoembryonic antigen (u/L)	2.3 (0.4-5.9)

Data are presented as median (range) unless otherwise stated.

hepatectomy was defined as resection of more than 3 Couinaud segments. Indocyanine green retention rate at 15 min after injection (ICG-R15) was used to evaluate the sufficiency of liver function for hepatectomy. For major hepatectomy, ICG-R15 of  $\leq 18\%$  was required. For minor hepatectomy, ICG-R15 of  $\leq 22\%$  was required. Patients having planned major hepatectomy were required to undergo computed tomographic volumetric study. The minimum ratio of future liver remnant to standard liver volume was 25% for non-cirrhotic livers<sup>[24,25]</sup>. Our technique of liver resection has been described elsewhere<sup>[24]</sup>. For follow-up, patients were seen at our out-patient clinic every 3 mo in the first 2 years and every 6 mo afterwards. Tumor markers were checked in every visit. Computed tomographic scan was performed 1-3 mo after discharge and then every 6 mo. Adjuvant therapy was not a routine and was offered at the discretion of the surgeon. Recurrence was defined as the presence of radiological or histological evidence of intrahepatic or extrahepatic HCC-CC.

## RESULTS

From 1995 to 2014, 1696 patients underwent hepatectomy for primary liver malignancy. Among them, 50 adult patients had pathologically confirmed HCC-CC (3%). Eight of these 50 patients were excluded because of re-resection. As a result, 42 patients were included for analysis. Their demographic characteristics and baseline biochemistry are shown in Table 1.

### Operative and pathological results

Most of the patients required major hepatectomy, and right hepatectomy was the most commonly performed procedure. Hepaticojejunostomy was performed in 6 patients (Table 2). The median operation time was 414 min (range, 177-1149 min) and the median blood loss volume was 800 mL (range, 5-2400 mL). There was no hospital death. The median length of hospital stay was 13 d

**Table 2** Types of operative procedure performed

	No. of patients (%)
Right/extended right hepatectomy	17 (40.5)
Left/extended left hepatectomy	5 (11.9)
Right trisectionectomy	6 (14.3)
Left trisectionectomy	1 (2.4)
Central bisectionectomy	2 (4.8)
Left lateral sectionectomy	3 (7.1)
Other minor hepatectomy	8 (19.0)

(range, 3-50 d). Three patients developed postoperative complications of Clavien-Dindo grade 3a or above (grade 3a in 1 patient and grade 4 in 2 patients).

Histological examination was performed for all patients. The median tumor size was 6.5 cm (range, 2-23 cm). Twenty patients (47.6%) had multiple (more than 1) tumor nodules. Moderate tumor differentiation (new Edmondson grading) was found in 40% of the patients and 33.3% of the patients had poor tumor differentiation. R0 resection was achieved in 90% of the patients. The median resection margin width was 1 cm (range, 0-6 cm).

### Survival outcomes and related factors

The median follow-up period was 110 mo. Adjuvant treatment was given to 13 patients in the form of transarterial chemo- or radio-embolization, systemic chemotherapy, external radiotherapy, molecular targeted therapy, or a combination of any of these. When it comes to survival outcomes, HCC-CC patients compared unfavorably with HCC patients. The median overall survival was 32 mo in HCC-CC patients and 70 mo in HCC patients (Figure 1A), and the median disease-free survival was 9 mo in the former and 28 mo in the latter (Figure 1B). On the other hand, HCC-CC patients and ICC patients had comparable overall survival (a median of 27 mo in ICC patients) (Figure 1C) while the latter had better disease-free survival (median, 20 mo) (Figure 1D). Recurrence developed in 33 HCC-CC patients (78.6%) (14 had intrahepatic recurrence, 3 had extrahepatic recurrence, and 16 had both).

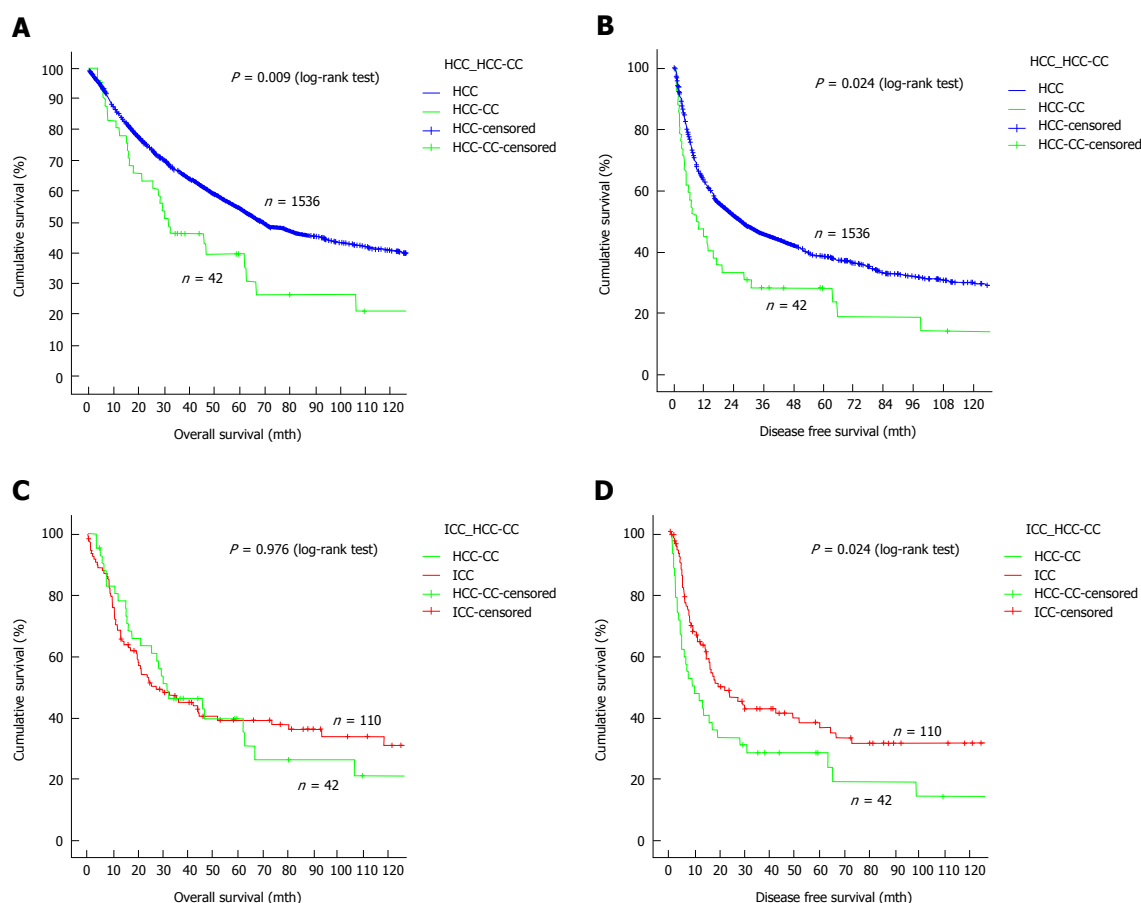
In Cox regression analysis, tumor multiplicity was the only independent factor associated with overall survival [ $P < 0.001$ , odds ratio (OR) 5.26, 95%CI: 2.254-12.290] and disease-free survival ( $P = 0.001$ , OR 4.00, 95%CI: 1.897-8.434) (Table 3). Patients with solitary tumor nodule had a median overall survival of 106 mo whereas those with multiple tumor nodules had a median overall survival of 16 mo ( $P < 0.001$ ) (Figure 2A). The median disease-free survival was 19.2 mo in patients with solitary tumor nodule and 3.1 mo in patients with multiple tumor nodules ( $P < 0.001$ ) (Figure 2B).

Further analyses of the subgroup of patients ( $n = 20$ ) who had multiple tumor nodules were performed. In univariate analysis, disease-free survival had an association with preoperative albumin level ( $P = 0.022$ ) and resection margin width ( $P = 0.013$ ). Multivariate analysis showed

**Table 3** Cox regression analysis for factors affecting overall and disease-free survival

Factor	Overall survival ( <i>P</i> -value)		Disease-free survival ( <i>P</i> -value)	
	Univariate	Multivariate	Univariate	Multivariate
Age	0.269	NS	0.501	NS
Sex	0.513	NS	0.868	NS
HBV status	0.507	NS	0.441	NS
Platelet count	0.389	NS	0.331	NS
Total bilirubin	0.471	NS	0.176	NS
Albumin	0.811	NS	0.663	NS
ICG-R15	0.955	NS	0.749	NS
AFP	0.937	NS	0.308	NS
CEA	0.832	NS	0.716	NS
Operation time	0.239	NS	0.682	NS
Blood loss	0.138	NS	0.037	NS
Resection extent <sup>1</sup>	0.152	NS	0.108	NS
Tumor size	0.845	NS	0.975	NS
Multifocality	< 0.0001	< 0.001	< 0.0001	0.001
Margin width	0.523	NS	0.9	NS
Wide margin ( $\geq 1$ cm)	0.491	NS	0.096	NS
Microvascular invasion	0.373	NS	0.170	NS
Nodal metastasis	0.314	NS	0.229	NS
Adjuvant treatment	0.162	NS	0.052	NS

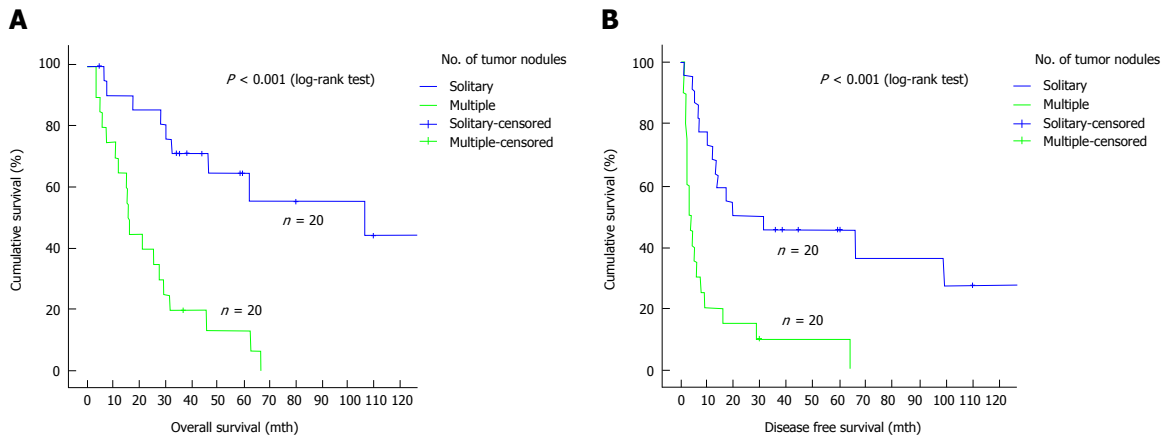
<sup>1</sup>Major *vs* minor. NS: Not significant; HBV: Hepatitis B virus; ICG-R15: Indocyanine green retention rate at 15 min after injection; AFP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen.



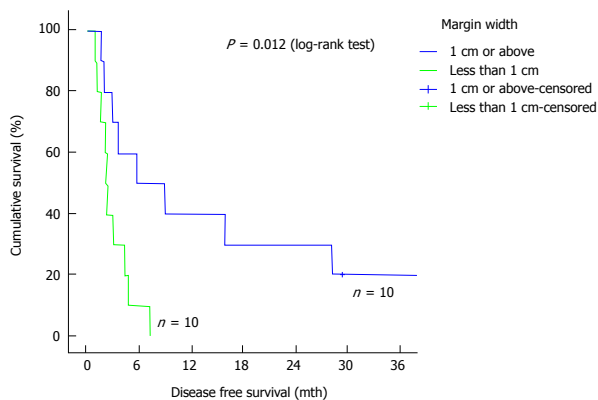
**Figure 1** Survival comparisons between different groups of patients. A: Overall survival of HCC-CC patients and HCC patients; B: Disease-free survival of HCC-CC patients and HCC patients; C: Overall survival of HCC-CC patients and ICC patients; D: Disease-free survival of HCC-CC patients and ICC patients. HCC-CC: Hepatocolangiocarcinoma; ICC: Intrahepatic cholangiocarcinoma.

that resection margin width was the only independent factor affecting disease-free survival. A clear resection

margin of  $\geq 1$  cm could improve 1-year disease-free survival from 0% to 40% ( $P = 0.012$ ) (Figure 3).



**Figure 2** Survival of hepatocholangiocarcinoma patients with solitary vs multiple tumor nodules. A: Overall survival of HCC-CC patients with solitary tumor nodule and with multiple tumor nodules; B: Disease-free survival of HCC-CC patients with solitary tumor nodule and with multiple tumor nodules. HCC-CC: Hepatocholangiocarcinoma.



**Figure 3** Effect of wide resection margin on disease-free survival of patients with multifocal hepatocholangiocarcinoma.

## DISCUSSION

This retrospective study has further illustrated that HCC-CC is a rare and sinister primary hepatic malignancy. The reported incidences of HCC-CC vary greatly. This is probably due to the difference in the pathological definition of the disease. HCC-CC shares the clinicopathological features of HCC and ICC. Male predominance, the existence of background cirrhosis and elevation of alpha-fetoprotein level are hallmarks of HCC. These features are also often seen in HCC-CC. Tumor hypovascularity, involvement of regional lymphadenopathy and poor survival outcomes are common in HCC-CC as well as ICC. This study found that HCC-CC patients had significantly worse overall survival and disease-free survival when compared with HCC patients, which concurs with other reports<sup>[26-29]</sup>. When compared with ICC patients, HCC-CC patients had inferior disease-free survival but were comparable in overall survival. This explains why HCC-CC should be included in the section of carcinoma of the intrahepatic duct in the 7<sup>th</sup> edition of the AJCC cancer staging manual<sup>[30]</sup>. The worse survival outcomes were attributable to its propensity for vascular invasion and

lymph node metastasis<sup>[1,9,31]</sup>.

Despite the availability of the various classification systems for HCC-CC<sup>[8,9,32]</sup>, its prognosis remains difficult. Chantajitr *et al.*<sup>[33]</sup> reported that a cancer antigen 19-9 level of  $\geq 80$  u/mL and the presence of intrahepatic ductal dilatation were independent factors for poor survival. Other studies found that lymphovascular permeation, large tumor size and the presence of tumor satellites were poor prognostic factors<sup>[4,34-37]</sup>. In the current study, tumor multiplicity was the only independent factor associated with inferior disease-free survival and overall survival. This echoes the emphasis on the significance of tumor multiplicity in the staging of ICC in the 7<sup>th</sup> edition of the AJCC Staging<sup>[30]</sup>. The role of adjuvant therapy in HCC-CC management is still unclear. One fourth of the patients in the current study received some form of adjuvant treatment (transarterial chemoembolization, radiotherapy, systemic therapy, *etc.*) at the discretion of the surgeon. Standardization of adjuvant treatment protocol is necessary before the role of adjuvant therapy can be established.

The current study could not demonstrate any benefit of R0 resection for patients with resectable HCC-CC, probably because of the small number of patients with R1 or R2 resection. Since HCC-CC is intrinsically associated with poorer prognostic outcomes when compared with HCC and ICC, small survival advantage conferred by wide resection margin (1 cm or above) could only be shown with a larger study population. However, this survival benefit was demonstrated in the subgroup of patients who had multifocal disease (40% vs 0% disease-free survival at 1 year). Since HCC-CC inherits the tumor biology of HCC and ICC, it has the ability of portal vein invasion and lymphovascular permeation. We therefore postulate that wide resection or even routine anatomical resection would eliminate residual satellite tumor cells or microtumor residing in the same vasculobiliary territory, thereby improving disease-free survival. The retrospective nature of the current study has posed a couple of limitations.



Firstly, missing data on carbohydrate antigen 19-9 made adequate analysis of its influence on survival outcomes impossible. In most of the cases, HCC-CC was diagnosed as HCC and routine blood check for carbohydrate antigen 19-9 was clinically irrelevant. Secondly, the small cohort size predisposed the study to type-II error; some potentially significant factors related to survival outcomes might not be identified by the analysis. However, the study period spanned two decades (1995-2014), which is relatively long. Furthermore, survival comparison between the study cohort and two much larger groups of patients (1536 HCC patients and 110 ICC patients) was performed, which should provide important data reference for future research.

HCC-CC is a rare and sinister primary hepatic malignancy. Patients with solitary tumor had better survival. A resection margin of at least 1 cm improved the disease-free survival of patients with multiple tumor nodules.

## COMMENTS

### Background

Hepatocolangiocarcinoma (HCC-CC) is an uncommon primary hepatic malignancy, contributing to about 1%-3% of all primary liver cancers. Its prognosis is worse than hepatocellular carcinoma (HCC) and similar to that of the intrahepatic cholangiocarcinoma. While resection margin was found to be an important factor associated with long-term oncological outcomes, its role in the management of this rare entity has not been reported.

### Research frontiers

The role of resection margin has been extensively investigated in many cancers, such as oesophageal and colorectal cancers. In HCC and intrahepatic cholangiocarcinoma, wide resection margin was shown to be an independent factor leading to improved survival outcomes. In the context of HCC-CC, previous reports focused mainly on the epidemiology, diagnosis and disease nature, yet, the role of resection margin remained an unexplored area of the disease.

### Innovations and breakthroughs

The rarity of the disease has always been a hurdle for statistical analysis. With the use of a well-maintained patient database in a university surgical center, a HCC-CC population of relatively large sample size were retrieved for analysis.

### Applications

The results of this study showed that HCC-CC is associated with significantly worse overall survival when compared to HCC (9 mo vs 28 mo). Multifocality was found to be the only independent factor associated with inferior disease free survival. Early and regular postoperative surveillance should be offered to this group of patients for early detection of recurrence. In patients with multifocal HCC-CC, attempt should be made to achieve a clear resection margin of 1cm so as to improve the recurrence free survival.

### Terminology

HCC-CC is a rare disease condition and histologically, the features of HCC and cholangiocarcinoma should both be demonstrated in the same tumour mass according to World Health Organization criteria.

### Peer-review

This article is important for clinical management of HCC-CC, with well-designed analysis and trustable conclusions.

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## Bi-directional hepatic hydrothorax

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**Author contributions:** Nellaiyappan M gathered data and drafted the article; Kapetanios A critically reviewed and revised the article.

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### Abstract

A 59-year-old male with alcoholic cirrhosis presented to our hospital with an acutely painful umbilical hernia, and 4 mo of exertional dyspnea. He was noted to be tachypneic and hypoxic. He had a massive right sided pleural effusion with leftward mediastinal shift and gross ascites, with a tense, fluid-filled, umbilical hernia.

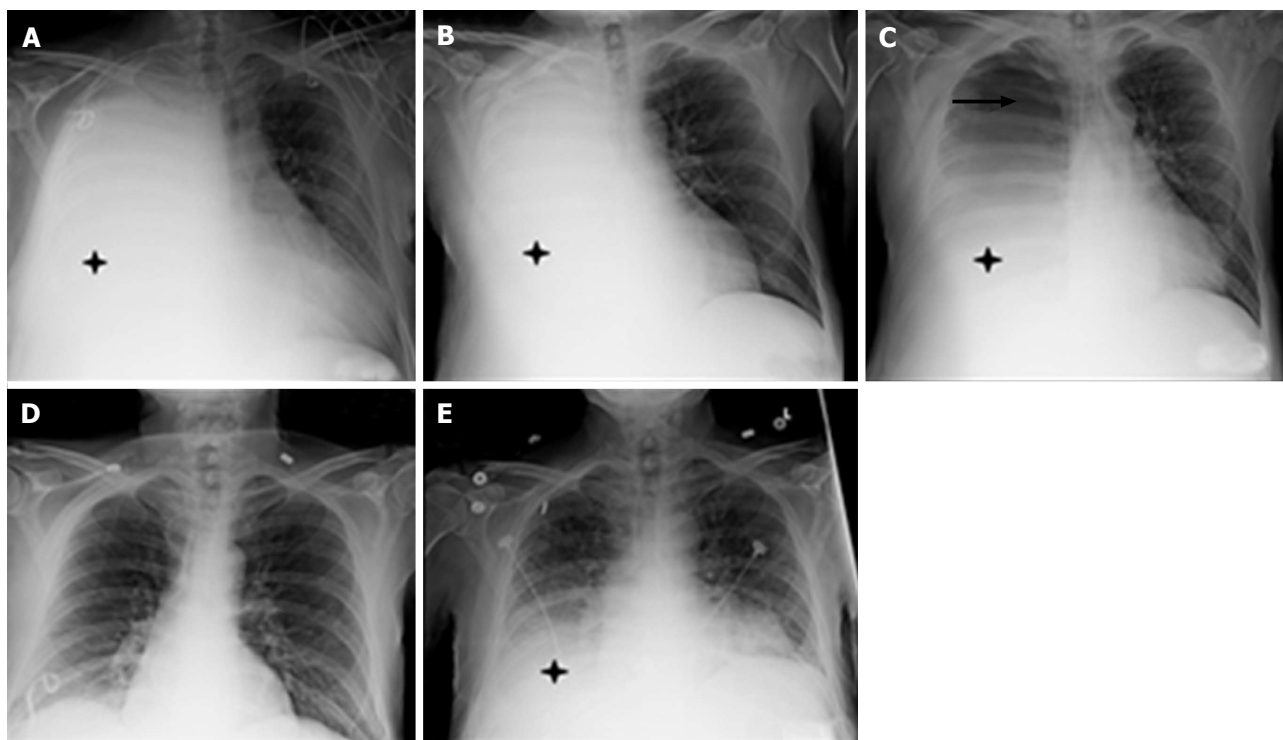
Emergent paracentesis with drain placement and a large volume thoracentesis were performed. Despite improvement in dyspnea and drainage of 15 L of ascitic fluid, the massive transudative pleural effusion remained largely unchanged. He underwent a repeat large volume thoracentesis on hospital day 4. The patient subsequently developed a tension pneumothorax, which resulted in a dramatic reduction in the effusion. A chest tube was placed and serial radiographs demonstrated resolution of the pneumothorax but recurrence of the effusion. The radiographs illustrate the movement of fluid between the peritoneal and pleural cavities. In this case, the mechanism of pleural effusion was confirmed to be a hepatic hydrothorax *via* an unintended tension pneumothorax. Methods to elucidate a hepatic hydrothorax include Tc99m or indocyanine green injection into the ascitic fluid followed by its demonstration above the diaphragm. The unintended tension pneumothorax in this case additionally demonstrates bi-directional flow across the diaphragm.

**Key words:** Hepatic hydrothorax; Bidirectional flow; Iatrogenic pneumothorax

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**Core tip:** Hepatic hydrothorax is usually a clinical diagnosis in patients with cirrhosis and portal hypertension who present with a transudative pleural effusion. The authors herein report an interesting case of radiological confirmation of hepatic hydrothorax through a series of chest radiographs that depict the movement of ascitic fluid between the pleural and peritoneal cavities due to a iatrogenic pneumothorax.

Nellaiyappan M, Kapetanios A. Bi-directional hepatic hydrothorax. *World J Hepatol* 2017; 9(13): 642-644 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i13/642.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i13.642>



**Figure 1** Serial chest radiographs. A: Day 0; B: Day 3; C: Day 4; D: Day 7; E: Day 26. A-C, E: Effusion (asterisks); C: Pneumothorax (arrow).

## TO THE EDITOR

We read with great interest the article titled "A fascinating presentation of hepatic hydrothorax" by Gaduputi *et al*<sup>[1]</sup>. We would like to thank the authors for sharing the clinical images and case details which illustrate the rapid shifts in the hydrothorax in a patient who was on invasive positive pressure ventilatory support. We would like to report an interesting case of hepatic hydrothorax that we encountered in our clinical practice which also demonstrates the mechanics of hepatic hydrothorax. We believe that the images of this common, yet incompletely understood phenomenon will be of interest to your readers at large.

The patient was a 59-year-old male with Child C cirrhosis in the setting of alcohol abuse and chronic hepatitis C who presented to our hospital with an acutely painful umbilical hernia, and 4 mo of exertional dyspnea. He was noted to be tachypneic and hypoxic. He had a massive right sided pleural effusion with leftward mediastinal shift (Figure 1A, day 0, asterisk) and gross ascites with a tense, fluid-filled, umbilical hernia. Emergent paracentesis with drain placement and a large volume thoracentesis were performed. Despite improvement in dyspnea and 15 L of ascitic fluid drainage, the massive transudative effusion remained largely unchanged (Figure 1B, day 3, asterisk). He underwent a repeat large volume thoracentesis on hospital day 4. The patient subsequently developed a tension pneumothorax, with a dramatic reduction in effusion size (Figure 1C, day 4, asterisk, arrow). A chest tube was placed, after which serial radiographs demonstrated resolution of the pneumothorax and recurrence of the effusion (Figure

1D). The radiographs demonstrate the movement of fluid between the peritoneal and pleural cavities (Figure 1C and E). In this case, the mechanism of pleural effusion was confirmed to be a hepatic hydrothorax *via* an unintended tension pneumothorax.

The diagnosis of hepatic hydrothorax should be considered for any patient with unilateral pleural effusion without an obvious cardio pulmonary cause. For cases in which the diagnosis is not obvious based on the clinical picture, methods to elucidate a hepatic hydrothorax include Tc99m labelled sulfur/albumin or indocyanine green injection into the ascitic fluid, followed by its demonstration above the diaphragm<sup>[2,3]</sup>.

The unintended tension pneumothorax in this case also demonstrates bi-directional flow across the diaphragm. As mentioned by Gaduputi *et al*<sup>[1]</sup>, fluid dynamics in hepatic hydrothorax are driven by pressure changes and pressure differences between the pleural, peritoneal cavities. In their patient, mechanical ventilation imparted positive pressure that was transmitted to the intrapleural space thereby causing the hydrothorax to track back to the peritoneal cavity which was relatively less pressurized<sup>[1]</sup>. Similarly, in our patient, a tension pneumothorax imparted positive pressure in the pleural cavity, forcing pleural fluid back into the peritoneal cavity. In both patients, after the source of positive intrapleural pressure was eliminated, the hydrothorax recurred, highlighting bi-directional flow.

While rapid, bi-directional, hepatic hydrothoraces may represent a subset of larger diaphragmatic defects, this phenomenon may be more common than judged by the scant available literature. It is in these cases that an opportunity exists to better delineate the pathophysiology



of hepatic hydrothoraces, and begin to conceive more robust therapeutic options than those currently available to patients. As it stands, hepatic hydrothorax is often a harbinger of further suffering.

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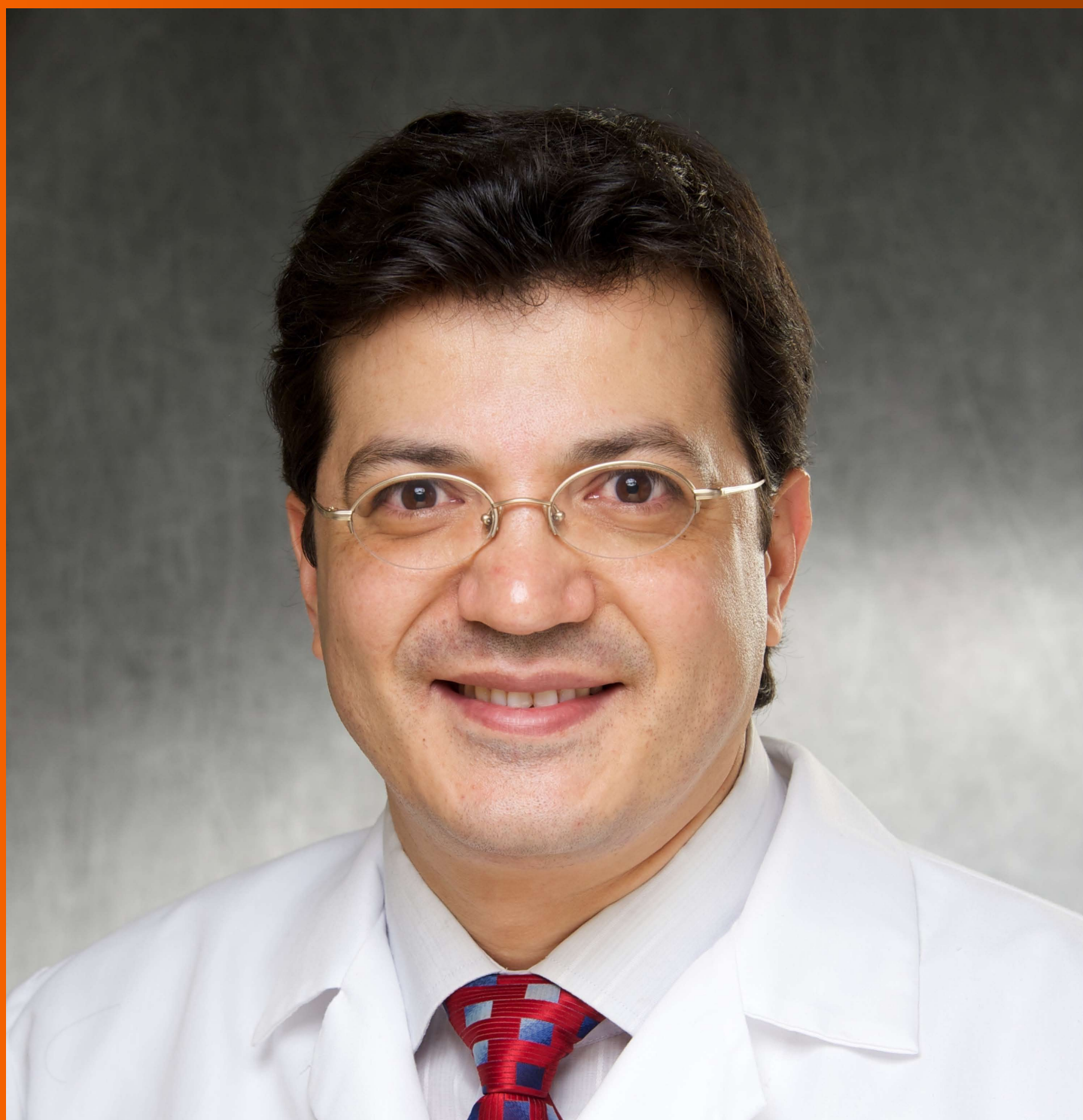


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## Strategies to tackle the challenges of external beam radiotherapy for liver tumors

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### Abstract

Primary and metastatic liver cancer is an increasingly common and difficult to control disease entity. Radiation offers a non-invasive treatment alternative for these patients who often have few options and a poor prognosis. However, the anatomy and aggressiveness of liver cancer poses significant challenges such as accurate localization at simulation and treatment, management of motion and appropriate selection of dose regimen. This article aims to review the options available and provide information for the practical implementation and/or improvement of liver cancer radiation programs within the context of stereotactic body radiotherapy and image-guided radiotherapy guidelines. Specific patient inclusion and exclusion criteria are presented given the significant toxicity found in certain sub-populations treated with radiation. Indeed, certain sub-populations, such as those with tumor thrombosis or those with larger lesions treated with transarterial chemoembolization, have been shown to have significant improvements in outcome with the addition of radiation and merit special consideration. Implementing a liver radiation program

requires three primary challenges to be addressed: (1) immobilization and motion management; (2) localization; and (3) dose regimen and constraint selection. Strategies to deal with motion include simple internal target volume (ITV) expansions, non-gated ITV reduction strategies, breath hold methods, and surrogate marker methods to enable gating or tracking. Localization of the tumor and organs-at-risk are addressed using contrast infusion techniques to take advantage of different normal liver and cancer vascular anatomy, imaging modalities, and margin management. Finally, a dose response has been demonstrated and dose regimens appear to be converging. A more uniform approach to treatment in terms of technique, dose selection and patient selection will allow us to study liver radiation in larger and, hopefully, multicenter randomized studies.

**Key words:** Hepatocellular carcinoma; Liver metastases; 4DCT; Image-guided radiotherapy; Stereotactic body radiation therapy

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**Core tip:** Primary and metastatic liver cancer patients are a growing population seen in cancer centers. This population often has few options and a poor prognosis. Radiation offers a safe non-invasive treatment option, but those implementing a liver radiotherapy program must address specific challenges not always seen in other disease sites. A growing and large number of papers have investigated a wide range of strategies. Our objective is to consolidate this literature to provide a concise review of options to allow a pragmatic selection of management strategies.

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## INTRODUCTION

Liver cancer is a major area of investigation as it is increasingly common and remains one of the deadliest diseases where clinicians have few options. According to Surveillance, Epidemiology, and End Results (SEER) statistics, the estimated numbers of cases of liver cancer (including intrahepatic bile duct cancers) will be 35660 in 2015 representing the second largest annual increase in incidence amongst all cancers in the United States<sup>[1]</sup>. Liver remains the most frequent site of metastatic disease for patients with colorectal cancer. Approximately 50%-60% of patients with colorectal cancer (CRC) will develop liver metastases and one third will die from liver failure from progressive disease<sup>[2]</sup>. In patients with only limited liver metastases, aggressive local treatment

with surgical extirpation could result in 5-year overall survival rates of 25%-40%<sup>[2]</sup>. Likewise, the mainstay of treatment for primary liver cancer is surgical resection or liver transplantation. Unfortunately, only 15%-25% of patients are eligible for curative resection or transplant at the time of diagnosis.

Traditionally, radiotherapy has not been routinely given to patients with liver tumors primarily due to the relatively low liver tolerance to radiation. With the advent of advanced radiation technology, it is now possible to deliver potentially curative radiation doses to liver tumors safely. Investigators from Sweden and Japan pioneered the use of stereotactic body radiotherapy (SBRT), a spin-off of intracranial stereotactic radiosurgery (SRS) for extracranial targets<sup>[3]</sup>. SBRT has also been applied for the treatment of liver tumors and the early results are promising. Following these results, advanced technologies such as protons have also been used to deliver radiotherapy to liver tumors with good results<sup>[4,5]</sup>.

Despite the availability of advanced radiotherapy technologies and evidence of efficacy, the use of radiotherapy for liver has not become standard<sup>[6]</sup>. This may be due to the fact that there are several difficult challenges for radiotherapy of liver lesions and a myriad of approaches to deal with these challenges. Clinicians must select an appropriate patient population, a safe and effective dose regimen, image guidance methods for tumor localization, methods to deal with respiratory motion, and methods to avoid radiation-induced complications. This overview will provide a practical review of the challenges and options for the treatment of primary and secondary liver tumors. This will assist the practical selection and implementation of options for a high-quality program that follows the guidelines on SBRT<sup>[7]</sup>.

## APPROPRIATE PATIENT SELECTION AND ACHIEVABLE CLINICAL OUTCOMES

### Hepatocellular carcinoma

There have been significant advances in the options available for hepatocellular carcinoma beyond surgery with level 1 evidence of an overall survival benefit for sorafenib, radiofrequency ablation and transarterial chemoembolization (TACE)<sup>[2]</sup>. Patient selection for sorafenib is limited to patients with hepatocellular carcinoma (HCC), that earlier treatments options are not suitable, or patients who have progressed on other treatments. This tends to be patients with extensive disease within or outside the liver, including patients with portal vein invasion based on two randomized controlled trials<sup>[8,9]</sup>. TACE has been shown in two randomized controlled trials and one metaanalysis to improve survival at two years<sup>[10-12]</sup>. Subsequent metaanalysis has added to the controversy indicating no improvement<sup>[13]</sup>. However, there are no prospective randomized studies to inform clinical practice beyond radiofrequency ablation, sorafenib, TACE and surgery. This makes selection of appropriate patients subject to interpretation of the evidence. For radiotherapy

there is no prospective randomized trial, and we must rely on interpretation of multiple studies reporting case series data with variable patient inclusion, treatment and length of follow-up (Table 1). However, the literature suggests that one subgroup can be identified: Unresectable, locally advanced disease without extrahepatic metastasis, Child-Pugh class A or B, and occupying less than 2/3 of the liver. Several guidelines already include radiation for this subgroup<sup>[14,15]</sup>. This is based on a growing body of level II evidence (retrospective and prospective case series data); this data indicates a 1-year overall survival of 48%-100%, a 1-year local control rate of 64%-100%, and a grade 3 or greater toxicity of 0%-36% (Table 1). Yet, the role of radiation for hepatocellular carcinoma can span early curative presentations to palliative treatment also based on retrospective data<sup>[16]</sup>. For tumors near luminal structures, conventional radiotherapy may be recommended over SBRT depending on the dose selection method and planning constraints applied. For the subgroup of recurrences or incomplete responses post chemotherapy or chemoembolizations, the data also suggest that radiation is a strong option. In the landmark trial by Shim, the 2-year overall survival for those receiving radiation was 37% compared to 14% for those who did not receive radiation<sup>[17]</sup>. This trial also highlighted the importance of tumor size in predicting success of TACE and the possible value of adding for certain patients. For tumors greater than 8 and 10 cm, no patients lived beyond 2-years with TACE. However, if external beam radiation was added, the survival was 50% and 17% for the 8 and 10 cm groups, respectively. Combination therapy, particularly in those with larger lesions where TACE is indicated, the addition of radiation may play a significant role.

Lastly, another group with a very poor outcome are those with portal vein thrombosis. Radiotherapy may be particularly useful for tumor thrombosis where current median survivals remain at 2-4 mo without radiation. To date there have been twelve retrospective<sup>[18-29]</sup> and 1 prospective case<sup>[30]</sup> series demonstrating a median survival improvement of two to five times historical cohorts. The larger studies used older radiotherapy techniques, including the largest from Yoon *et al.*<sup>[22]</sup>. Despite a relatively low median dose of 40 Gy in 2-5 fractions, the study achieved an impressive 43% one year overall survival and an acceptable 10% grade 3 or greater toxicity rate<sup>[22]</sup>. Randomized trials to address the value of radiation for patients with thrombosis are warranted given the possible survival benefit.

### Liver metastases

There is growing interest in radiation for oligometastatic disease and palliation. Høyer *et al.*<sup>[31]</sup> reviewed five retrospective and seven prospective trials to determine which patients should be considered for liver SBRT. This review of the literature by a subcommittee of the American Society of Radiation Oncology (ASTRO) including members from the European Society for Therapeutic Radiology and Oncology (ESTRO), the Canadian Asso-

ciation of Radiation Oncology (CARO) and the Trans-Tasman Radiation Oncology Group (TROG), concluded that the ideal radiotherapy candidate would have an ECOG 0-1, possess adequate hepatic function, have no extrahepatic disease, and have an uninvolved liver volume of 700 mL or greater. This would result in local control rates ranging from 56%-100% at 2 years. Table 2 summarizes prospective trials of SBRT for liver metastases. For a large proportion, extra-hepatic progression develops after local treatment. Though no threshold dose has been found, this group recommended liver metastases receive 48 Gy in three fractions based on the available evidence.

## TECHNICAL IMPLEMENTATION CHALLENGES

### Immobilization and motion management

Surveys demonstrate that there is no universal standard for liver SBRT<sup>[32]</sup>, but there are recommendations from large SBRT groups. This includes the CARO Scope of Practice guidelines that were published to ensure safe practice in the major SBRT sites<sup>[31,33]</sup>. In conventional treatments, a larger margin for internal target volume (ITV) may be acceptable as multiple fractions averaged the dose errors caused by inaccurate organ localization, motion or set up error. SBRT relies on the delivery of accurate high doses to the target and errors in localization could result in increased toxicity, geometric tumor miss and cannot be easily "corrected" in later fractions. Therefore, the use of techniques or devices to localize the radiation to the tumor, minimize margins and optimize on-treatment quality assurance is critical. Furthermore, with the use of IMRT, improved motion management results in fewer unplanned hot and cold spots due to the interplay of the motion of anatomical structures and MLC leaf motion<sup>[34]</sup>.

The primary motion with liver SBRT is respiratory motion which can be controlled with fixed immobilization, breath hold and/or tracking. For immobilization, vacuum-bag systems or fixed body immobilizers are used where arms are kept up and out of field. A simple margin expansion to account for ITV is then applied based on a 4DCT scan, fluoroscopy and/or slow CT scanning to capture the full range of motion. These are categorized as ITV methods or motion encompassing methods. An additional margin for set-up motion is added for planning target volume (PTV) with recommendations ranging between 2 and 5 mm<sup>[35]</sup>. These methods in isolation necessitate larger treated volumes, greater normal tissue inclusion and a lower chance for dose escalation. Shallow breathing may be sufficient in many patients especially if patients are compliant and can maintain regular breathing motions. The American Association of Physicists in Medicine (AAPM) Task Group 76 emphasizes that some method of respiratory assessment be applied and a step-wise algorithm be applied to determine the amount of respiratory management required<sup>[36]</sup>. However, in a large proportion of patients, additional motion management techniques are necessary to achieve greater dose escalation and



**Table 1** Summary of hepatocellular carcinoma radiotherapy studies

Ref.	No. of patients	Percent of Child-Pugh B patients	Median tumor diameter (range), cm	Dose (range)/No. of fractions	Median follow-up interval, mo	1-yr OS	2-yr LC	Toxicity
Scorsetti <i>et al</i> <sup>[68]</sup> , 2015	43	36%	≤ 6 cm	48-75 Gy/3 (51%) and 36-60 Gy/6 (49%)	8	77.9%	64.4%	≥ gr3: 0
Yamashita <i>et al</i> <sup>[69]</sup> , 2015	79	11%	2.7 cm	48 Gy (40-60)/4-10	21	53% at 2 yr	40%	gr3-4: 4.6% gr2: 2.3%
Huertas <i>et al</i> <sup>[70]</sup> , 2015	77	14.3%	2.4 cm	45 Gy/3	12	81.8	99%	14.9%
Zhong <i>et al</i> <sup>[71]</sup> , 2014	72	26%	13.1 cm	35.6 Gy/12	18	56%	NR	gr1-2: 5.6% liver gr1-2: 9.8% gastrointestinal RILD 9.4%
Lo <i>et al</i> <sup>[72]</sup> , 2014	53	NR	4.3 cm	40 Gy/4-5	13.1	70.1%		0
Van de Voorde <i>et al</i> <sup>[73]</sup> , 2014	5	NR	NR	93.6 Gy (62.5-150)/3-10	21	85.4%	NR	0
Sanuki <i>et al</i> <sup>[74]</sup> , 2014	63	16%	2.6 cm	35-40 Gy/5	31.1	100%	95%	gr3: early: 16% late: 21% gr4-5: 0% gr3: 4% gr4-5: 0%
Park <i>et al</i> <sup>[75]</sup> , 2013	26	27%	2.8 cm	40-50 Gy; 4-5 Gy per fraction	20.2	88.5%	87.6%	gr3: 21% gr4: 2.9% gr5: 6.9%
Bujold <i>et al</i> <sup>[30]</sup> , 2013	102	0%	9.9 cm	24-54 Gy (36)/6	31.4	75%	74%	gr3: 4.3% gr4: 1.0% gr5: 1.0%
Yoon <i>et al</i> <sup>[76]</sup> , 2013	93	26%	2 cm	45 Gy (30-60)/3-4	25.6	86.0%	94.8% <sup>1</sup> (2 yr)	gr3: 6.5% gr4: 1.9% gr5: 0%
Jang <i>et al</i> <sup>[65]</sup> , 2013	108	10%	3.0 cm	51 Gy (33-60)/3	30	83% <sup>1</sup>	87%	gr ≥ 3: 7% gr3: 4% gr4: 1.3% gr5: 0%
Jung <i>et al</i> <sup>[77]</sup> , 2013	92	26%	Vol: 8.6 cc	45 Gy (30-60)/3-4	25.7	86.9%	92.1% (3 yr)	gr3: 10% gr4-5: 0% gr2: 31.8% gr5: 1.1%
Bibault <i>et al</i> <sup>[78]</sup> , 2013	75	11%	3.7 cm	40-45 Gy/3	10	78.5%	89.8%	gr3: 2.4% gr4-5: 0% gr2: 3%
Honda <i>et al</i> <sup>[79]</sup> , 2013	30	23%	16 cm	48 Gy/4	12.3	100%	95% <sup>1</sup>	gr3: 6.4% gr4: 4.3% gr5: 0%
Yuan <i>et al</i> <sup>[80]</sup> , 2013	22	45%	4.3 cm	45 Gy (39-54)/3-8	53.4	73%	92.9%	gr3: 4.8% gr4: 4.8% gr5: 0%
Sanuki <i>et al</i> <sup>[81]</sup> , 2013	185	15%	CP-A: 27 cm CP-B: 24 cm	CP-A: 40 Gy/5 CP-B: 35 Gy/5	24	95%	93% (2 yr)	NR
Xi <i>et al</i> <sup>[18]</sup> , 2013	41	0%	Mean GTV vol: 65.4 cc (SD: 47.9)	30-48 Gy (36)	10	50.3%	NR	gr3: 35% gr4: 1.7% gr5: 0%
Huang <i>et al</i> <sup>[82]</sup> , 2012	36	NR	1.1-12.3 cm	37 Gy (25-48)/4-5	14	64% at 2 yr	98%	gr ≥ 3 22% gr3: early 8% late 4%
Kang <i>et al</i> <sup>[83]</sup> , 2012	47	13%	2.9 cm	42-60 Gy/3	17	83% <sup>1</sup>	94.6%	gr3: 0% gr4: 2% gr5: 0%
Ibarra <i>et al</i> <sup>[84]</sup> , 2012	21	NR	GTV vol: 334.2 cc	30 Gy (18-50)/1-10	12.9	87%	57% <sup>1</sup> (2 yr)	gr3: 13 instances gr4: 11.8% gr5: 0%
Price <i>et al</i> <sup>[85]</sup> , 2012	26	46%	Median GTV vol: 33.9 cc	42 Gy (24-48)/3-5	13	77%	NR	gr2: 33% 0
Andolino <i>et al</i> <sup>[86]</sup> , 2011	60	40%	3.1 cm	CP-A: 30-48 Gy/3 CP-B: 24-48 Gy/5	27	82% <sup>1</sup>	90% (2 yr)	gr3: 3% gr4-5: 0% PFS)
Chan <i>et al</i> <sup>[87]</sup> , 2011	11	25%	3 cm	45 Gy/10	24	62%	NR	NR
Louis <i>et al</i> <sup>[88]</sup> , 2010	25	12%	4.5 cm	45 Gy/3	12.7	79%	95%	gr3: 4.5% gr4-5: 0%
Kwon <i>et al</i> <sup>[89]</sup> , 2010	42	10%	Vol: 15.4 cc	30-39 Gy/3	28.7	92.9%	67.5%	
Cárdenes <i>et al</i> <sup>[66]</sup> , 2010	17	65%	≤ 6 cm	CP-A: 48 Gy/3 CP-B: 42 Gy/3 then 40/5	24	75%	100%	
Son <i>et al</i> <sup>[90]</sup> , 2010	47	8%	18.3 cm	36 Gy (30-39)/3	NR	NR	NR	
Goyal <i>et al</i> <sup>[91]</sup> , 2010	6	NR	9.3 cm	34 (24-45 Gy)/1-3	10	83%	100% at 9 mo	
Seo <i>et al</i> <sup>[92]</sup> , 2010	38	11%	Vol: 40.5 cc	33-57 Gy/3-4	15	68.4%	66.4% (local PFS)	
Choi <i>et al</i> <sup>[21]</sup> , 2008	22	14%	Vol: 23.5 cc	36 Gy (30-39)/3	11.5	88.1%	NR	

Tse <i>et al</i> <sup>[61]</sup> , 2008	31	0%	173 cc	36 Gy (24-54)/6	17.6	48%	NR	gr3: 29% gr4-5: 0%
Méndez Romero <i>et al</i> <sup>[93]</sup> , 2006	8	25%	3.2 cm	< 4 cm: 37.5 Gy/3 ≥ 4 cm: 25 Gy/5 or 30 Gy/3	12.9	75%	75% (22 mo)	gr5: 12.5% RILD

<sup>1</sup>Estimated from survival curve. CP-A: Child-Pugh class A; CP-B: Child-Pugh class B; gr: Grade; GTV: Gross tumor volume; LC: Local control; OS: Overall survival; NR: Not reported; PFS: Progression-free survival; RILD: Radiation induced liver disease; vol: Volume.

**Table 2 Prospective metastatic liver stereotactic body radiotherapy studies**

Ref.	No. of patients	Dose (Gy/fraction)	Median follow-up (mo)	2-yr local control (%)
Herfarth <i>et al</i> <sup>[94]</sup> , 2001	37	14-26 Gy/1	5.7	81 <sup>2</sup>
Hoyer <i>et al</i> <sup>[95]</sup> , 2006	44	45 Gy/3	51.6	79
Kavanagh <i>et al</i> <sup>[96]</sup> , 2006	36	60 Gy/3	19	93
Ambrosino <i>et al</i> <sup>[97]</sup> , 2009	27	Median 36 (25-60) Gy/3	13	74 crude <sup>2</sup>
Rusthoven <i>et al</i> <sup>[62]</sup> , 2009	47	36-60, 60 Gy/3	16	92
Lee <i>et al</i> <sup>[98]</sup> , 2009	68	Median 41.8 Gy/6	10.8	71 <sup>2</sup>
Méndez Romero <i>et al</i> <sup>[93]</sup> , 2006	17 <sup>1</sup>	30-37.5 Gy/2		86
Stintzing <i>et al</i> <sup>[99]</sup> , 2010	36 <sup>1</sup>	24 Gy/1	21.3	87 <sup>2</sup>
Goodman <i>et al</i> <sup>[100]</sup> , 2010	26 <sup>1</sup>	18-30 Gy/1	17	77 <sup>2</sup>
Rule <i>et al</i> <sup>[63]</sup> , 2011	27	30 Gy/5 50 Gy/5 60 Gy/5	20	56 89 100
Janoray <i>et al</i> <sup>[101]</sup> , 2014	56	45 Gy/3-60 Gy/3	12.5	64 <sup>2</sup>

<sup>1</sup>Included hepatocellular patients; <sup>2</sup>12-18 mo local control percentage.

safety. The AAPM suggests a cut-off of 5 mm after which respiratory management is recommended. The options can be categorized into three types: (1) non-gated ITV reduction strategies; (2) active or passive breath hold techniques; and/or (3) surrogate markers. These are applied uniformly based on institutional practice or after a trial assessment of patients who are then assigned to one or more additional motion control methods.

**Non-gated ITV reduction strategies:** Abdominal compression was one of the earliest motion management strategies and was first used in Karolinska Hospital for lung and liver lesions in the 1990s<sup>[37]</sup>. A compression plate was applied to the abdomen to reduce abdominal motion caused by respiration. Early data, primarily from lung cancer patients, has shown accuracy and reproducibility with median reductions of 7 mm<sup>[36,38]</sup>. Recent papers using fiducial markers to track motions have provided direct data on reproducibility and extent of motion reduction in liver patients using abdominal compression. Essentially, a motion minimization method, Wunderink and Méndez Romero<sup>[39]</sup> demonstrated reduced median excursion by 62% and essentially all residual excursions were reduced to less than 5 mm. Reproducibility was excellent between planning and treatment. Predating much of the 4D respiratory strategies, the appeal of this method includes better localization; this is due to more projection data from the entire breath cycle being available leading to better image quality than only a portion of the 4DCT data set. Another advantage of abdominal compression is the minimal technology requirements compared to more complicated strategies such as gating.

However, the magnitude of improvement may be

smaller than initially reported. Updated data from Eccles reported in 2011 that the decrease in motion averaged 2.3 mm and 0.6 mm in the CC and AP direction; 28% saw an increase in motion with abdominal compression so this option does come with caveats<sup>[40]</sup>. Motion of other important structures such as the kidney do not appear to be improved with the use of this type of device<sup>[41]</sup>. Furthermore, not all patients require or can tolerate abdominal compression. Patients with abdominal aortic aneurysm also may not be suitable for abdominal compression. Therefore, other motion correction methods have been tested such as using the mean respiratory position for planning<sup>[42]</sup>. This strategy determines the diaphragm's mean cranio-caudal position in the respiratory cycle or selects a mid-ventilation CT data set. Velec was able to show that this simple method resulted in a 34% lower irradiated volume due to the significantly smaller PTV compared to standard full-motion ITV-based and dose probability PTVs. However, this group demonstrated that rigid motion correction still results in an 8% and 7% change in dose accumulation for the tumor and normal tissues, respectively<sup>[43]</sup>. These changes were found in a majority of patients and suggest the need for some additional form of respiratory control and further investigation of adaptive SBRT to deal with organ deformation.

**Breath hold methods:** The second category of motion management techniques are the breath hold methods. The simplest application is deep inspiration breath hold (DIBH). Initially pioneered at the Memorial Sloan-Kettering Cancer Center<sup>[44]</sup>, DIBH has shown reproducibility within a margin of  $2.2 \pm 2.0$  mm<sup>[45]</sup>. Voluntary DIBH can reduce internal

motion from 12.9 to 2.8 mm<sup>[46]</sup>. Additional margins for set-up error (typically 2-5 mm) and assessment of intra- and inter-fraction motion is required<sup>[26]</sup>. The addition of assisted or active breath hold, such as Active Breathing Control (ABC), reduces variability further. ABC was commercialized by Elekta, Inc. and uses a spirometer that monitors the phase of breathing. Usually after two preparatory breaths, a valve is closed at expiration thereby “holding the patient’s breath”. Issues with this strategy include concerns regarding reproducibility, cost of non-reusable components, time required, maintenance and patient tolerance. Reproducibility assessments have demonstrated good intrafraction absolute offsets of 3 mm or less. However, interfraction errors > 3 mm are found in 46% of cases further emphasizing the need for image guidance<sup>[47]</sup>. Alternatively, shallow breathing and voluntary breath hold can be monitored using the spirometry system; planning margins, treatment activation, and reproducibility of set up can be determined using the same equipment. This is useful as not all patients can tolerate active breath hold. While it has its own limitations, voluntary breath hold has many advantages compared to gating including no marker/tumor motion lag issues, about half the treatment time required, less specialized equipment, less training and software, plus more efficient simulation<sup>[29]</sup>.

### Surrogate markers to enable gating or tracking:

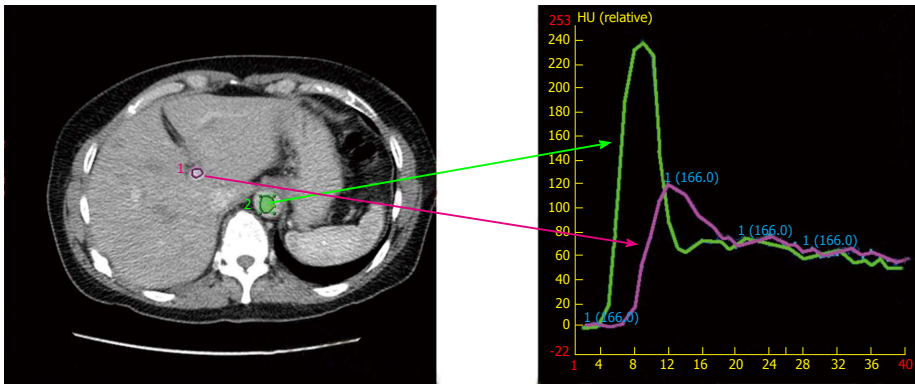
The third variation in motion management is the use of surrogate markers to enable gating. Depending on the surrogate, this overlaps with tumor localization strategies. Surrogates are used to assess the degree of motion which leads to individualization of motion management. Compared to breath hold techniques alone, this method is better tolerated and may represent a more accurate anatomical picture than deep inspiration or expiration. External respiratory surrogates include the Varian 3D infrared marker (Real-time Positioning Management or RPM) system, the Siemens respiratory strain gauge belt, and 3D laser surface respiratory assessment systems (C-RAD Sentinel). Internal surrogates include the diaphragm/lung interface, radiotransmitters (Calypso), and radio-opaque markers (including surgical clips, stents, lipiodol or anatomical calcifications). Internal markers provide the best surrogates, but usually are invasive to insert, risk complications and have a higher relative cost in time and money. Various gating options can be chosen based on the information from these surrogates. Respiratory gating can be grouped into three major categories: First, phase gating consists of treating the patient during a particular phase such as end-expiration. The advantage is that this portion of the breathing cycle is often the most stable with the least motion. Second, amplitude gating selects a certain portion of the respiratory cycle defined by a percentage of the amplitude of each cycle. As phase gating may result in binning errors due to breath to breath variations in slope, length of cycles and amplitudes, detractors suggest better sorting with fixed amplitude gating<sup>[48]</sup>. Third, gating may be based on the

surrogate marker at breath hold. In addition to motion assessment, these markers may be used during treatment for synchronizing treatment delivery. For example, simply gating the treatment beam when the surrogate indicates the tumor is in a certain position, or synchronizing the aperture *via* dynamic multileaf collimator (DMLC), or moving beam to the location of the lesion (Cyberknife) are valuable strategies<sup>[49]</sup>. A method to select an appropriate clinical target volume (CTV) to PTV margin has been developed by Keall and Vedam based on three challenges: (1) selection of amplitude vs phase gating; (2) accounting for phase shifts between markers and the lesion; and (3) the management of intrafraction motion vs increased delivery time<sup>[50]</sup>. Typical GTV to PTV margins are 5 mm axially and 10 mm craniocaudally<sup>[35]</sup>. Periodic monitoring during treatment is still necessary to confirm reproducibility of the motion compared to planning. Patient training plus visual or verbal prompting may allow better reproducibility and margins<sup>[50]</sup>.

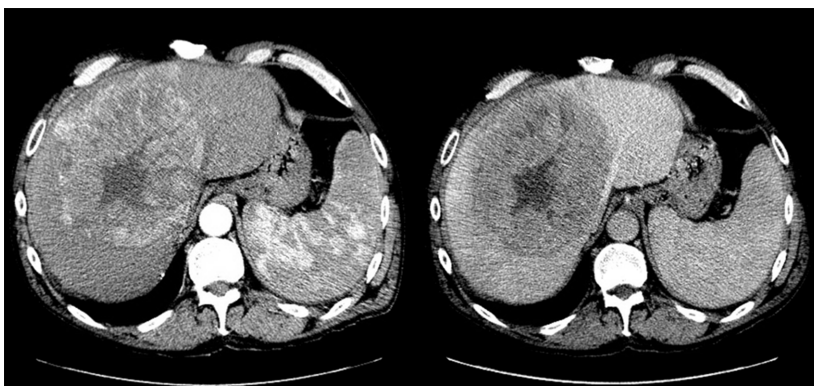
### Localization

At simulation, IV contrast is considered standard particularly for hepatocellular carcinoma. However, this does introduce fusion errors as the contrast infusion must be captured over several respiratory cycles. Various protocols are in place such as the MD Anderson standardized protocol<sup>[51]</sup> or those that individualize<sup>[52]</sup> binning by visualizing contrast in specific vessels. The later method accounts for patient differences in the time contrast reaches and leaves the lesion, anatomical location of the tumor relative to the start of the scan, body weight, time-density curves and cirrhosis (Figure 1). Figure 1 demonstrates the time-density intravenous contrast enhancement called Dynamic Contrast Enhanced CT (DCECT). Images are binned by location in respiratory cycle and when the contrast density within a vessel (such as the aorta or portal vein) signifies the arterial, portal venous, and delayed phase. Images are specific to each patient and individualized contrast enhanced images (Figure 2) offer the possibility of improved delineation without additional equipment or technology, but methods to eliminate motion during the long acquisitions are required<sup>[53,54]</sup>.

MRI and PET are becoming a standard part of management for liver lesions due to improved sensitivity and specificity<sup>[55]</sup>. MRI is particularly useful for small tumors, cirrhotic patients or those who are unable to tolerate IV contrast. MRI may play a greater role as experience with 4DCT, gated MRI and cine MRI accumulates<sup>[56,57]</sup>. Functional imaging assessments are useful for follow-up, and to determine the necessity to add additional treatments post radiation<sup>[58]</sup>. However, both MRI and PET have long acquisition times and require strategies to account for motion. Strategies such as multiple breath holds, parallel imaging for rapid acquisition and respiratory correlated PET are being investigated. Even if accurate localization is possible with the elimination of respiratory motion, strategies to register the MRI and/or PET to the CT image are then required. Additional margins will be required after deformable or rigid image registration in



**Figure 1** Image of a time-density intravenous contrast enhancement called Dynamic Contrast Enhanced computerized tomography. Images are binned by location in respiratory cycle and when the contrast density within a vessel (such as the aorta and portal vein) signifies the non-contrast, arterial and wash-out phase. Time is measured in seconds and density is measured in Hounsfield units (HU).



**Figure 2** Arterial and portal-venous phase images.

the range of 2.2 to 21.3 mm<sup>[59]</sup>. Therefore, the value of more accurate localization must be balanced against the additional margin, time, and cost.

Oral contrast is useful to localize luminal structures, which often represent the most critical organs at risk. The contrast is assigned a CT number for tissue equivalence prior to planning. Lastly, calcifications, vessels and other anatomical landmarks can be extremely useful. If possible, contouring these structures provides information to the therapists; communication with therapists to indicate which critical structures to localize, prioritize and/or avoid is a practical and valuable routine to incorporate.

At treatment, localization of the tumor in the liver is sometimes not possible in contrast to other sites such as lung cancer where the tumor location is often very clear. Internal and/or external surrogate markers or structures may be used as described earlier. At treatment the consistency of correlation with respiratory motion or breath hold ability at time of simulation must be verified. A commonly used structure is the diaphragm. Vedam has shown a strong linear correlation between the diaphragm and the external marker; a superior-inferior CTV-PTV margin of 0.8 cm provided sufficient coverage over multiple sessions with or without training<sup>[60]</sup>. Static images are acceptable, but real-time or near-real-time options exist. Some systems have the ability to acquire images in fluoroscopy or cine-mode and new systems now enable almost real-time dose accumulation to enable adaptive

treatment. However, with fewer fractions used in SBRT, the opportunity to correct dosing errors is limited and localization prior to and during each treatment remains the primary goal. Non-radiographic dependent internal tumor markers such as Calypso can track motion during treatment to provide a more accurate assessment of tumor motion. This real-time tracking has significant advantages over other motion control strategies including the ability to adjust beam delivery *via* synchronized aperture tracking methods or by directly following the lesion motion with the radiation beam.

#### **Dose selection and constraints**

An optimal dose for primary and secondary liver cancer has not been identified. Essentially there are two types of research approaches in the literature for dose finding: Radiobiologically-guided dose escalation and step-wise dose escalation. The first approach, such as the pioneering work of Tse *et al.*<sup>[61]</sup>, uses radiobiological calculation of risk to provide individualized dose recommendations. The second relies on maximally tolerated dose (MTD) techniques used successfully in drug trials. In many cases, the dose has been determined by normal tissue constraints. Furthermore, patient tolerance of radiation may vary due to underlying hepatic insufficiency, and previous or concurrent treatments (resections, chemotherapy). Despite the varied approaches and dose regimens, a convergence of dose recommendations may



be occurring (Table 1, summary of studies for HCC; and Table 2, summary of SBRT studies of metastatic cancer). For hepatic metastases, work published by Rusthoven demonstrated that 60 Gy in 3 fractions resulted in a local control rate of 92% at 2 years<sup>[62]</sup>. Similarly, Timmerman and Rule suggest that a 60 Gy in 5 fraction regimen is appropriate, particularly for tumors adjacent to critical structures<sup>[63]</sup>. Based on three dose escalation cohorts, the actuarial 24 mo local control was 100%. The authors state that a maximum tolerated dose was not reached; MTD was defined as the dose below which the dose limiting toxicity rate was  $\geq 33\%$ . Both groups used a critical volume model with at least 700 mL of normal liver receiving less than 15 Gy and 21 Gy for the 3 and 5 fraction regimen, respectively. However, in both studies tumors were highly selected with a median tumor size of less than 3 cm and few patients had centrally located lesions. Therefore, the excellent results may not be generalizable to a wider population especially those with larger lesions. However, for patients who can meet the trial constraints, the 100% local control rate is a strong argument that the optimal dose for hepatic metastases is 60 Gy in three or five fractions.

For primary liver cancer, a dose response relationship has been found<sup>[64]</sup>, but outcomes and regimens remain somewhat more varied than with metastatic disease (Table 1). HCC patient population is very heterogeneous with important parameters such as size of lesion, liver dysfunction, previous treatments received, presence of vascular invasion and number of lesions all influencing outcome. This heterogeneity increases the difficulty in generalizing data. Modeling suggests that a 90% probability of 6-mo control could be achieved with 84 Gy in 2 Gy equivalent doses<sup>[55]</sup>; much higher than the 53 Gy in 2 Gy equivalent required for metastatic disease. Review of trials reporting 2-year outcomes of greater than 90%, suggests that a dose of 45 Gy in 3-4 fractions or 35-40 Gy in 5 fractions need to be achieved (Table 1). A critical dose threshold likely exists for both local control and overall survival. In one of the larger SBRT studies, Jang *et al.*<sup>[65]</sup> demonstrated that above 54 Gy in 3 fractions, local control and survival was 100% and 71% at 2-years, respectively. However, if less than 45 Gy was achieved, the local control and overall survival dropped to 64% and 30%, respectively.

Unlike patients with liver metastases, a significant proportion of patients with HCC have underlying cirrhosis and/or other insufficiency. This factor has been a consistent parameter influencing dose selection, patient selection and outcome. Most commonly measured using the Child-Pugh score, groups have consistently found this issue to influence treatment and prognosis. Cárdenes *et al.*<sup>[66]</sup> from Indiana University conducted a phase I dose escalation trial of SBRT for HCC, where 17 Child-Pugh classes A or B patients with 25 tumors were included. The initial dose level was 36 Gy in 3 fractions and there was a 2-Gy per fraction increment. Patients received a maximum of two treatments per week. The protocol

required 700 cc of normal liver would receive  $< 15$  Gy. They were able to escalate the dose to 48 Gy in 3 fractions for Child-Pugh class A patients without causing dose-limiting toxicities. However, two Child-Pugh class B patients developed grade 3 liver toxicities when the dose was escalated to 42 Gy in 3 fractions. This observation has led these investigators to change the regimen for Child-Pugh class B patients, from 40 Gy in 3 fractions to 40 Gy in 5 fractions. This was considered the MTD and no further dose escalations are recommended. The most important factor associated with grade 3 or higher liver toxicities was a Child-Pugh score of  $\geq 8$ . Based on their experience, the group has recommended that the dose to one-third of the uninvolved liver should be restricted to  $\leq 10$  Gy (3.3 Gy/fraction) and  $\geq 500$  cc of uninvolved liver should receive  $< 7$  Gy (2.3 Gy/fraction) for Child-Pugh class A patients; for Child-Pugh class B patients, the dose to one-third of the uninvolved liver is restricted to  $\leq 18$  Gy, (3.6 Gy/fraction) and  $\geq 500$  cc of uninvolved liver should receive  $< 12$  Gy (2.4 Gy/fraction)<sup>[66]</sup>. A summary of suggested constraints based on recent randomized clinical trials with accepted fraction regimens is summarized in Table 3.

## FUTURE TRENDS

Patients with primary or secondary liver cancer are growing in incidence and have a rising mortality rate<sup>[1]</sup>. Current management with RFA, TACE, sorafenib and surgery often are not possible or result in moderate improvements<sup>[67]</sup>. Therefore, patients and their physicians must seek alternatives or combination of treatments. In addition to external beam radiation, work in the use of radionuclides, radiosensitizers (such as inhibitors of autophagy), epigenetic agents, liquid biopsies to better select patients, and immune modulation are exciting avenues of investigation. As for external beam radiotherapy, our review suggests that radiotherapy can be implemented safely and with high local control rates. In the future, we will continue to refine our technique and patient selection, but appropriate multidisciplinary randomized trials need to be completed before radiation can become a standard of care.

## CONCLUSION

Radiation plays an important role in the treatment of primary and metastatic liver cancer. Control rates can be high and toxicity is minimal in well-selected patients. Indeed it may play a primary role in subgroups such as large tumors and those with thrombosis. The wide ranging outcomes, differing techniques and varied dosing strategies make specific treatment recommendations difficult, but the literature is converging on a short list of important components of a high quality liver radiation program. This article aims to provide a practical review of options to provide the best care possible in this evolving field.

**Table 3** Summary of dose constraints by number of fractions

Organ at risk	3 fraction (RAS trial <sup>[102]</sup> )	5 fraction (RTOG 1112 <sup>[103]</sup> )	QUANTEC (1.8-2 Gy per fraction) <sup>[104]</sup>	Toxicity
Liver excluding CTV	700 mL < 15 Gy	V 10 < 70%	D mean < 30 Gy	Radiation induced liver dysfunction
Esophagus	D 1 mL < 21 Gy	D 0.5 mL < 32 Gy	V 35 < 50%	Esophagitis
Stomach	D 1 mL < 21 Gy	D 0.5 mL < 30 Gy	D 100 < 35 Gy	Ulceration
Kidney	D 35% < 15 Gy	D mean < 10 Gy	D mean < 28 Gy (1.8-2 Gy per fraction)	Renal dysfunction
Bowel and duodenum	D 1 mL < 21 Gy	D 0.5 mL < 30 Gy	D 45 < 195 cc	Enteritis/fistula
Spinal cord	Dmax < 18 Gy	D 0.5 mL < 25 Gy	Dmax = 45	Myelopathy
Heart	D 1 mL, 30 Gy	D 30 mL < 30 Gy	V 25 < 10%	Pericarditis

CTV: Clinical target volume.

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Retrospective Study

# Image quality and diagnostic performance of free-breathing diffusion-weighted imaging for hepatocellular carcinoma

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## Abstract

### AIM

To retrospectively evaluate the diagnostic performance of free-breathing diffusion-weighted imaging (FB-DWI) with modified imaging parameter settings for detecting hepatocellular carcinomas (HCCs).

### METHODS

Fifty-one patients at risk for HCC were scanned with both FB-DWI and respiratory-triggered DWI with the navigator echo respiratory-triggering technique (RT-DWI). Qualitatively, the sharpness of the liver contour, the image noise and the chemical shift artifacts on each DWI with  $b$ -values of 1000 s/mm<sup>2</sup> were independently evaluated by three radiologists using 4-point scoring. We

compared the image quality scores of each observer between the two DWI methods, using the Wilcoxon signed-rank test. Quantitatively, we compared the signal-to-noise ratios (SNRs) of the liver parenchyma and lesion-to-nonlesion contrast-to-noise ratios (CNRs) after measuring the signal intensity on each DWI with a b-factor of 1000 s/mm<sup>2</sup>. The average SNRs and CNRs between the two DWI methods were compared by the paired t-test. The detectability of HCC on each DWI was also analyzed by three radiologists. The detectability provided by the two DWI methods was compared using McNemar's test.

## RESULTS

For all observers, the averaged image quality scores of FB-DWI were: Sharpness of the liver contour [observer (Obs)-1, 3.08 ± 0.81; Obs-2, 2.98 ± 0.73; Obs-3, 3.54 ± 0.75], those of the distortion (Obs-1, 2.94 ± 0.50; Obs-2, 2.71 ± 0.70; Obs-3, 3.27 ± 0.53), and the chemical shift artifacts (Obs-1, 3.38 ± 0.60; Obs-2, 3.15 ± 1.07; Obs-3, 3.21 ± 0.85). The averaged image quality scores of RT-DWI were: Sharpness of the liver contour (Obs-1, 2.33 ± 0.65; Obs-2, 2.37 ± 0.74; Obs-3, 2.75 ± 0.81), distortion (Obs-1, 2.81 ± 0.56; Obs-2, 2.25 ± 0.74; Obs-3, 2.96 ± 0.71), and the chemical shift artifacts (Obs-1, 2.92 ± 0.59; Obs-2, 2.21 ± 0.85; Obs-3, 2.77 ± 1.08). All image quality scores of FB-DWI were significantly higher than those of RT-DWI ( $P < 0.05$ ). The average SNR of the normal liver parenchyma by FB-DWI (11.0 ± 4.8) was not significantly different from that shown by RT-DWI (11.0 ± 5.0); nor were the lesion-to-nonlesion CNRs significantly different (FB-DWI, 21.4 ± 17.7; RT-DWI, 20.1 ± 15.1). For all three observers, the detectability of FB-DWI (Obs-1, 43.6%; Obs-2, 53.6%; and Obs-3, 45.0%) was significantly higher than that of RT-DWI (Obs-1, 29.1%; Obs-2, 43.6%; and Obs-3, 34.5%) ( $P < 0.05$ ).

## CONCLUSION

FB-DWI showed better image quality and higher detectability of HCC compared to RT-DWI, without significantly reducing the SNRs of the liver parenchyma and lesion-to-nonlesion CNRs.

**Key words:** Diffusion weighted-imaging; Liver; Magnetic resonance imaging; Hepatocellular carcinoma; Free-breathing technique

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**Core tip:** This retrospective study evaluated the image quality of free-breathing diffusion-weighted imaging (FB-DWI) of the liver and its diagnostic performance for hepatocellular carcinoma compared with respiratory-triggered DWI. The free-breathing technique is widely believed to be inappropriate for body DWI because motion artifact causes decreased image quality. However, after a modification of imaging parameters, FB-DWI showed better image quality without significantly reducing the signal-to-noise ratio of the normal liver parenchyma

and the lesion-to-nonlesion contrast-to-noise ratio compared to respiratory-triggering-DWI. As a result, the improvement of the image quality of FB-DWI contributed to an increased rate of detection of hepatocellular carcinoma.

Takayama Y, Nishie A, Asayama Y, Ishigami K, Kakihara D, Ushijima Y, Fujita N, Shirabe K, Takemura A, Honda H. Image quality and diagnostic performance of free-breathing diffusion-weighted imaging for hepatocellular carcinoma. *World J Hepatol* 2017; 9(14): 657-666 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i14/657.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i14.657>

## INTRODUCTION

Diffusion-weighted imaging (DWI) has been widely adopted as a magnetic resonance imaging (MRI) method in clinical practice<sup>[1,2]</sup>. DWI can be used for the detection and characterization of malignant and nonmalignant lesions<sup>[3-5]</sup>. Liver DWI has been applied to quantify the degrees of chronic liver disease and fibrosis, and to detect and characterize liver lesions<sup>[2,6-9]</sup>. DWI can provide additional information that can be used to differentiate malignant liver lesions from benign liver lesions and to estimate the histological grade of hepatocellular carcinomas (HCCs)<sup>[6,7,10]</sup>. The combination of DWI and dynamic contrast-enhanced (DCE)-MRI has shown higher diagnostic performance compared to DWI or DCE-MRI alone<sup>[11-13]</sup>. Although DWI has an essential role to play in the assessment of HCCs on liver MRI, its sensitivity for detecting HCCs is thought to be low compared to that of DCE-MRI<sup>[2,14]</sup>.

DWI suffers from image distortion and/or chemical shift artifacts related to the echo planar imaging (EPI) technique and to motion and susceptibility artifacts<sup>[15-17]</sup>. With the goal of overcoming these issues, a previous study investigated MR parameter settings to obtain high spatial resolution and less artifacts without losing a significant level of the signal-to-noise ratio on liver MRI<sup>[18]</sup>. With this method, DWI with MR parameter settings provides improved image quality and detections of malignant liver tumors such as HCCs and metastatic liver tumors<sup>[18]</sup>. Here we hypothesized that DWI with modified MR parameter settings for the improvement of image quality might result in further improvement in the detectability of HCCs.

The purpose of this retrospective study was to evaluate the image quality and the detectability of HCCs in patients with chronic liver disease on DWI with modified MR parameter settings.

## MATERIALS AND METHODS

### Subjects

This study was approved by the Institutional Review Board of our institute. The requirement for written informed consent was waived due to the retrospective nature of the study. From November 2010 to September 2011, 468 consecutive patients who underwent liver MRI at

**Table 1** Details of magnetic resonance parameters

Imaging technique	FB-DWI	RT-DWI
	Spin echo single-shot EPI	Spin echo single-shot EPI
SENSE factor	2	2
TR/TE (ms)	6250/56	1877/55
Flip angle (degree)	90°	90°
Field of view (mm <sup>2</sup> )	380 × 299	380 × 299
Matrix (frequency × phase)	112 × 176	112 × 68
Slice thickness (mm)	7	7
Slice gap (mm)	1	1
No. of slice	25	25
No. of excitations	2	2
<i>b</i> -value (s/mm <sup>2</sup> )	0.500 and 1.000	0.500 and 1.000
Respiratory compensation	Free-breathing without navigator echo	Respiratory-triggered with navigator echo
Fat-suppression	SPAIR	SPIR
SPAIR delay (ms)	100	
SPAIR TR (ms)	250	
Frequency offset (Hz)	250	180
EPI factor	75	25
Band width (Hz/pixel)	4050.4	4438.5
Scan time (min:s)	3:32	3:20 <sup>1</sup>

<sup>1</sup>The mean scan time of RT-DWI, because these values vary depending on the subjects' respiration condition. FB-DWI: Diffusion-weighted imaging with modified MR parameter settings for image improvement by referring to the literature<sup>[18]</sup>; RT-DWI: Respiratory-triggered diffusion-weighted imaging without modified MR parameter settings for the image improvement; EPI: Echo planar imaging; TR: Repetition time; TE: Echo time; SPAIR: Spectral attenuation with inversion recovery; SPIR: Spectral presaturation with inversion recovery; SPAIR delay: Inversion time from exposure of SPAIR pulse; SPAIR TR: TR between SPAIR pulses during the scan; Frequency offset: Bandwidth from the frequency of fat tissue; EPI factor: The number of k-space profiles collected per excitation.

our institute were enrolled. The inclusion criteria were: (1) patients who were admitted to the Department of Surgery and were suspected to have HCCs due to chronic liver disease; (2) patients who underwent gadoteric acid-enhanced MRI (Gd-EOB-MRI) on the same MR scanners; and (3) patients who underwent treatments such as surgical resection, transcatheter arterial infusion chemotherapy (TAI) or transcatheter arterial chemoembolization (TACE). The exclusion criteria were: (1) patients with other malignant liver tumors, such as cholangiocellular carcinoma (ICC) and metastatic liver tumor; and (2) patients in whom follow-up computed tomography (CT) and/or MRI were not performed. Finally, 51 patients (age range: 26-82; mean: 63.8 years; male/female ratio: 33/18; Child-Pugh grades: A = 31, B = 16 and C = 4) were enrolled.

### Imaging protocol

**MR protocol:** MR examinations were performed on a clinical whole-body 3.0 Tesla MR system (Achieva 3.0 T TX; Philips Healthcare, Best, the Netherlands) using a 32-channel cardiac phased-array coil. For the comparison of imaging modalities, each patient was scanned with two different types of DWI. One type was DWI with modified MR parameter settings for the improvement of images referring to the literature<sup>[18]</sup>; we called this type of DWI "free-breathing (FB)-DWI" in this study because the FB technique was applied. The other type was DWI without modified MR parameter settings using a navigator-echo-based, real-time respiratory-gating and respiratory-triggering technique which we refer to as RT-DWI in this study. A navigator-echo-based technique was not applied

for respiratory-triggering (RT)-DWI.

The details of the MR parameters of the two DWI methods are summarized in Table 1. An apparent diffusion coefficient (ADC) map was developed for each DW image, by referring to the signal intensity decay on the DW image with *b*-values of 0, 500 and 1000 s/mm<sup>2</sup>.

Other imaging sequences included an axial T2-weighted single-shot turbo spin echo, axial dual-echo T1-weighted fast field echo, and a gadoteric acid-enhanced dynamic study. For the gadoteric acid-enhanced dynamic study, a multiphase dynamic study including arterial, portal, late and hepatobiliary phases was performed using axial enhanced T1 high-resolution isotropic volume excitation (eTHRIVE). First, pre-contrast images were scanned. Gadoteric acid (Primovist; Bayer, Osaka, Japan) at 0.1 mL/kg was injected through the antecubital vein for 5 s at a variable injection rate using a power injector, followed by a bolus administration of 20 mL of saline at the same injection rate. The timing of the arterial dominant phase was determined with a test injection of 0.5 mL of gadoteric acid. The scanning of the portal, late and hepatobiliary phases began at the arterial phase +30 s, 180 s and 20 min after the injection of the contrast agent, respectively.

**CT protocol:** On the follow-up CT examination, the scanning was performed before and after 100 mL of iodinated contrast medium (Iopamiron 370: Bayer Schering Pharma, Osaka, Japan; or Omnipaque 350: Daiichi-Sankyo, Tokyo) was administered, using a 64-MDCT scanner (Aquilion 64, Toshiba Medical, Tokyo). The contrast was intravenously administered at a rate of 3 mL/s. Contrast-enhanced



**Table 2** Image quality scores of sharpness of the liver contour, distortion and chemical shift artifacts

Score	Sharpness of the liver contour	Distortion and chemical shift artifacts
1	Unclear liver contour	Severe distortion or artifacts compromise the diagnostic capability of DWI in the whole liver
2	The liver contour is partially unclear	Distortion or artifacts are moderate, and they compromise the diagnostic capability of DWI in 50% or more of the liver
3	The liver contour is mostly clear	Distortion or artifacts are mild, and they compromise the diagnostic capability of DWI in less than 50% of the liver
4	The entire liver contour is clear	No distortion or artifacts; the diagnostic capability of DWI is not compromised

DWI: Diffusion-weighted imaging.

images were obtained during the arterial phase (43 s after the initiation of the injection), the portal venous phase (70 s), and the delayed phase (240 s). The imaging acquisition parameters were as follows: Voltage, 120 kV; electric current, automatic; collimation, 0.5 mm; image reconstruction thickness, 5 mm; and helical pitch, 53.

### Image quality assessment

Qualitatively, the sharpness of the liver contour, the image noise and the chemical shift artifacts on FB-DWI and RT-DWI with  $b$ -values of 1000 s/mm<sup>2</sup> were independently evaluated by three radiologists (Daisuke Kakihara, Yasuhiro Ushijima, and Nobuhiro Fujita, with 17, 17 and 12 years of experience in interpreting liver MRI, respectively) who were blinded to the imaging information and clinical data, using 4-point scoring. The details of the 4-point scoring are shown in Table 2. Quantitatively, the SNRs of the liver parenchyma and the lesion-to-nonlesion CNRs between the liver parenchyma and HCCs were calculated after drawing polygonal regions of interest (ROIs) on each DWI with a  $b$ -factor of 1000 s/mm<sup>2</sup>; this procedure was performed by one radiologist using a commercially available PACS workstation (SYNAPSE; Fujifilm Medical, Tokyo). The SNRs and CNRs were calculated using the following equations, as described in detail elsewhere<sup>[19,20]</sup>:

SNR of the liver parenchyma =  $S_{\text{liver}}/SD_{\text{liver}}$ , Lesion to nonlesion CNR =  $|S_{\text{liver}} - S_{\text{tumor}}|/SD_{\text{liver}}$

Where  $S_{\text{liver}}$  is the signal intensity of the liver parenchyma,  $S_{\text{tumor}}$  the signal intensity of the tumor, and  $SD_{\text{liver}}$  the standard deviation of the SI of the liver parenchyma. The SD of the liver parenchyma was taken as the estimated local noise for the calculation. In parallel imaging, noise is not distributed homogeneously throughout the image, and thus it is better to estimate noise in close proximity to the site of SI measurement<sup>[21]</sup>. The SNR cannot be calculated as a characteristic of the entire image but rather is calculated as a local property that characterizes the signal quality with respect to local noise levels<sup>[21]</sup>.

Three ROIs were made as large as possible on the normal liver parenchyma to avoid major vessels, tumors, and artifacts for each patient. The same ROIs were duplicated on each DW image. The range and averaged areas of ROIs of the normal liver parenchyma were 102.1–1414.0 mm<sup>2</sup> and 385.4 mm<sup>2</sup>, and the corresponding values for the hepatic lesions were 146.7–1237.4 mm<sup>2</sup> and 502.7 mm<sup>2</sup>. The measurements of the SNR of normal liver parenchyma and a lesion-to-nonlesion CNR were repeated

three times for each subject. The same ROIs were duplicated at the same slice and position for the two DWI methods.

### Confirmation of HCC

A final total of 105 HCCs (size range: 5–140 mm; mean: 17.1 mm; location, left lobe/right lobe: 52/53) was used for the assessment of the detectability of HCCs by the two different types of DWI. The number and location of HCCs were determined by one coordinator (Yukihisa Takayama, with 15 years of experience in interpreting liver MRI) who was a coordinator of this study and had knowledge of the clinical data of each patient. In the 38 patients who underwent surgery, 38 HCCs were identified using pathological reports after surgical resection. Of the other 67 HCCs in the 13 patients who underwent TAI or TACE, the HCCs were clinically defined using the enhancement in the early phase and hypointensity in the hepatobiliary phase of Gd-EOB-MRI and the nodular accumulation of emulsion of iodized oil (Lipiodol Ultrafluid; Terumo, Tokyo) on follow-up CT performed 2 wk after the TAI or TACE<sup>[22–24]</sup>.

The 3-mo follow-up Gd-EOB-MRI or DCE-CT confirmed the absence of HCCs in other liver parenchyma. The treated HCCs and hepatic hemangiomas were diagnosed based on the lack of early enhancement on Gd-EOB-MRI and DCE-CT and high signal intensity on T2-weighted imaging at an least 6-mo follow-up Gd-EOB-MRI or DCE-CT<sup>[24–26]</sup>. Liver tumors other than hemangiomas were not identified in 51 patients.

### Detectability of HCC

The detectability of HCC by each type of DWI was analyzed by three radiologists (Daisuke Kakihara, Yasuhiro Ushijima, and Nobuhiro Fujita, with 17, 17 and 12 years of experience in interpreting liver MRI, respectively) who independently interpreted the sets of DWI with  $b$ -values of 0, 500 and 1000 s/mm<sup>2</sup> and the ADC map at a 1-mo interval in random order. They were blinded to the clinical data and other imaging results such as those obtained by T1- and T2-weighted imaging, Gd-EOB-MRI, and DCE-CT.

On each type of DWI, HCC was diagnosed as a lesion showing mild-to-moderate hyperintensity compared to the liver parenchyma on DW images at a  $b$ -value of 0 s/mm<sup>2</sup> and restricted diffusion (*i.e.*, the lesion remained hyperintense) at a  $b$ -value of 500 and/or 1000 s/mm<sup>2</sup>, with an ADC value visually lower or equal to that of the surrounding liver parenchyma. Liver hemangiomas were

**Table 3** Results of qualitative assessment of the free-breathing diffusion-weighted imaging and respiratory-triggering-diffusion-weighted imaging results

	Sharpness of the liver contour		Distortion		Chemical shift artifacts	
	FB-DWI	RT-DWI	FB-DWI	RT-DWI	FB-DWI	RT-DWI
Observer 1	3.08 ± 0.81	2.33 ± 0.65 <sup>a</sup>	2.94 ± 0.50	2.81 ± 0.56 <sup>a</sup>	3.38 ± 0.60	2.92 ± 0.59 <sup>a</sup>
Observer 2	2.98 ± 0.73	2.37 ± 0.74 <sup>a</sup>	2.71 ± 0.70	2.25 ± 0.74 <sup>a</sup>	3.15 ± 1.07	2.21 ± 0.85 <sup>a</sup>
Observer 3	3.54 ± 0.75	2.75 ± 0.81 <sup>a</sup>	3.27 ± 0.53	2.96 ± 0.71 <sup>a</sup>	3.21 ± 0.85	2.77 ± 1.08 <sup>a</sup>

Data are the average ± SD. <sup>a</sup> $P < 0.05$  by the Wilcoxon signed-rank test. FB-DWI: Diffusion-weighted imaging with modified MR parameter settings for image improvement by referring to the literature<sup>[18]</sup>; RT-DWI: Navigator-echo-based, real-time respiratory-gating and respiratory-triggered diffusion-weighted imaging without modified MR parameter settings.

diagnosed by referring to the hyperintensity on DWI with a  $b$ -value of 0 s/mm<sup>2</sup> and an ADC value visually higher than that of the surrounding liver parenchyma. Each observer recorded the location of each HCC by placing an arrow on the image. The detectability of HCC was calculated on a tumor-by-tumor basis, and the detectability provided by the two types of DWI were compared. If liver hemangiomas were interpreted by each observer as HCC, those lesions were considered false-positives and were excluded from the assessment of detectability by the coordinator. In addition, the relationship between detectability and the sizes of the HCCs was analyzed.

### Statistical analysis

Image quality scores of the sharpness of the liver contour, the distortion, and the chemical shift artifacts between FB-DWI and RT-DWI were compared with the Wilcoxon signed-rank test. The SNRs of normal liver parenchyma and lesion-to-nonlesion CNRs between the two types of DWI were compared with a paired  $t$ -test. The detectability provided by the two DWI methods was compared using McNemar's test. A  $P$ -value  $< 0.05$  was considered to indicate a significant difference for each analysis. Statistical analyses were performed using IBM SPSS statistics 18.0 software (IBM Japan, Tokyo). The statistical methods of this study were reviewed by Akihiro Nishie from the Department of Clinical Radiology, Graduate School of Medical Sciences, Kyushu University and Junji Kishimoto from the Center for Clinical and Translational Research, Kyushu University.

## RESULTS

### Image quality assessments

The results of the qualitative assessment by the three observers are shown in Table 3. For all three observers, the average image quality scores of the sharpness of the liver contour, the distortion, and the chemical shift artifacts of FB-DWI were significantly higher than those of RT-DWI ( $P < 0.05$ ). There were no significant differences between FB-DWI and RT-DWI in the average or SDs of the SNR of the normal liver parenchyma (FB-DWI,  $11.0 \pm 4.8$ ; RT-DWI,  $11.0 \pm 5.0$ ) or the lesion-to-nonlesion CNR (FB-DWI,  $21.4 \pm 17.7$ ; RT-DWI,  $20.1 \pm 15.1$ ). Three representative cases are shown in Figures 1-3.

In Figure 1, the HCC is more clearly described as hyperintensity on FB-DWI than on RT-DWI. In Figure

2, the HCC was detected by FB-DWI, whereas it was concealed by a chemical shift artifact on RT-DWI. A pseudolesion caused by a chemical shift artifact from fat tissue between the liver parenchyma and diaphragm is shown in Figure 3.

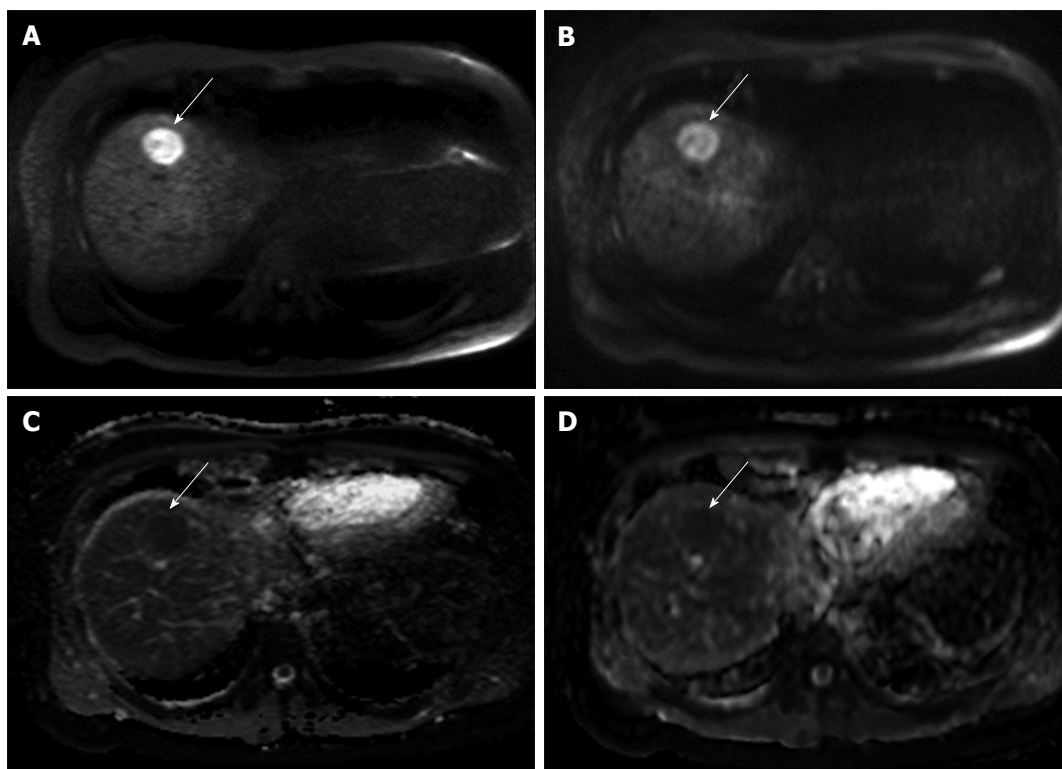
### Detectability of HCC

For all three observers, the sensitivity of FB-DWI [observer (Obs)-1, 43.6%; Obs-2, 53.6%; and Obs-3, 45.0%] was significantly higher than that of RT-DWI (Obs-1, 29.1%; Obs-2, 43.6%; and Obs-3, 34.5%) ( $P < 0.05$ ). Regarding the relationship between detectability and the size of the HCCs, the detectability of the two types of DWI was significantly different when the tumor size was 5-22 mm. FB-DWI showed significantly higher detectability of these HCCs (Obs-1, 36.0%; Obs-2, 48.3%; and Obs-3, 33.7%) compared to RT-DWI (Obs-1, 18.0%; Obs-2, 36.0%; and Obs-3, 24.7%) ( $P < 0.05$ ). There was no significant difference in the detectability of HCCs between the two types of DWI when the tumor size was  $> 22$  mm.

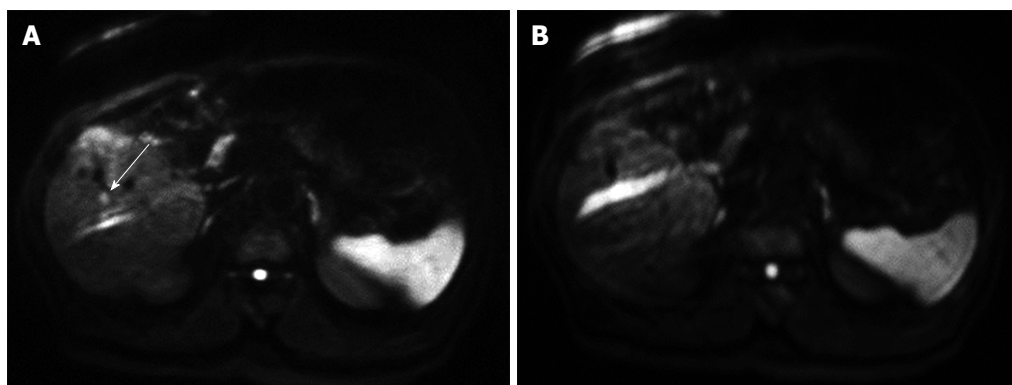
## DISCUSSION

The results of the present study revealed that, compared to RT-DWI, FB-DWI showed better image quality without significantly reducing the SNR of the normal liver parenchyma and the lesion-to-nonlesion CNR. The improvement of the image quality of FB-DWI might contribute to an increased detection rate of HCCs. The free-breathing technique was applied to FB-DWI of liver MRI even though it is widely believed that this technique is inappropriate due to its susceptibility to sensitive motion artifacts, which results in decreased SNRs and CNRs and image blurring<sup>[3,27]</sup>. However, our present findings demonstrated that FB-DWI had better image quality and equivalent SNRs of the liver parenchyma and lesion-to-nonlesion CNRs compared to RT-DWI.

Other studies have obtained similar results using DWI with a free-breathing technique, such as good image quality, good reproducibility of ADC values of liver tumors, and good diagnostic performance for liver lesions<sup>[28-31]</sup>. There are several possible reasons for the good image quality provided by DWI with a free-breathing technique; one is that an increased number of excitations contributes to a reduction in motion artifacts. However, FB-DWI used



**Figure 1** A 36-year-old male. The HCC in segment 8 of the liver showing hyperintensity on FB-DWI (A) is more clearly described than on RT-DWI (B). The ADC values of HCC were  $1.02 \times 10^{-3} \text{ mm}^2/\text{s}$  on the ADC map of FB-DWI (C) and  $1.16 \times 10^{-3} \text{ mm}^2/\text{s}$  on the ADC map of RT-DWI (D). HCC: Hepatocellular carcinoma; FB-DWI: Free-breathing diffusion-weighted imaging; RT-DWI: Respiratory-triggering diffusion-weighted imaging; ADC: Apparent diffusion coefficient.



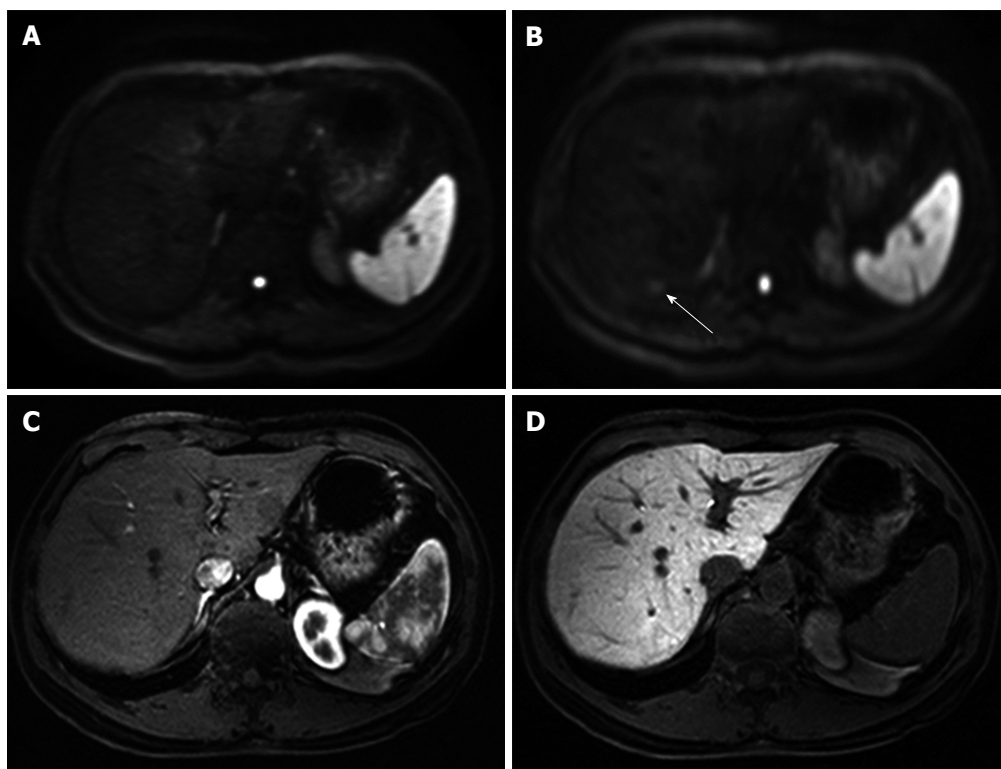
**Figure 2** A 78-year-old female. The HCC in segment 5 of the liver was detected by FB-DWI (A), whereas on RT-DWI, it was concealed by a chemical shift artifact (B). HCC: Hepatocellular carcinoma; FB-DWI: Free-breathing diffusion-weighted imaging; RT-DWI: Respiratory-triggering diffusion-weighted imaging.

only two excitations, and thus the results showing good image quality, SNR and CNR cannot be attributed to a multiplicity of excitations.

Another possible reason is the advances in MR technology in recent years (such as statistic and gradient magnetic fields, a 32-channel torso-cardiac phased-array coil, and dual-source parallel radiofrequency excitation and transmission technology) that address problems related to the EPI technique<sup>[9,18,32]</sup>. In particular, the rapid image acquisition of the EPI sequence allows minimization of the blurring from T2\* signal intensity decay during the gradient-echo train<sup>[9,18,32]</sup>, and it is insensitive to the effects of macroscopic patient motion because of the very fast

readout of the complete image data (within approximately 100 ms)<sup>[9]</sup>. This may account for the good image quality of FB-DWI in the present study.

Homogeneous fat suppression is essential for DWI, to avoid the image degradation caused by chemical shifts when using EPI<sup>[27]</sup>. For the FB-DWI in the present series, the SPAIR technique was applied because this technique is minimally affected by B1 inhomogeneity and is effective for obtaining homogeneous fat-suppression<sup>[33]</sup>. Chemical shift artifacts on DWI can be reduced by providing homogeneous fat-suppression as well. Fewer chemical artifacts on DWI are advantageous for the depiction of liver lesions. The present study's findings related to the



**Figure 3** A 53-year-old female. There was no detectable lesion on FB-DWI (A) but the nodular hyperintensity in segment 7 of the liver was seen on RT-DWI (B). It was diagnosed as a pseudolesion caused by a chemical shift artifact from fat tissue between the liver parenchyma and diaphragm, by referring to precontrast-enhanced imaging (C) and hepatobiliary phase imaging (D) of Gd-EOB-MRI. A follow-up MR examination also showed no progressive lesion (not shown). FB-DWI: Free-breathing diffusion-weighted imaging; RT-DWI: Respiratory-triggering diffusion-weighted imaging; Gd-EOB-MRI: Gadoteric acid-enhanced magnetic resonance imaging.

reduction of chemical shift artifacts occasionally mimicked a hepatic tumor. We thus conclude that FB-DWI will be advantageous for the depiction of HCCs.

In this study, we found that the detectability of HCC by FB-DWI was superior to that by RT-DWI. In fact, the detection of HCC by FB-DWI was equivalent to the reported results using breath-hold or respiratory-triggered techniques (45%–55%)<sup>[2,34,35]</sup>. That is, FB-DWI provides better spatial resolution, fewer chemical shift artifacts, and a comparable SNR and lesion-to-nonlesion CNR compared to RT-DWI<sup>[18]</sup>. This improvement in the detectability of HCCs, especially that of small-sized HCCs, is also probably due to the better spatial resolution and fewer chemical shift artifacts of FB-DWI.

The lower detectability of HCCs on DWI alone is a limitation because of the difficulty of differentiating a tumor from surrounding cirrhotic liver due to their similar diffusion properties and ADC values, and to the tumor grades of HCCs<sup>[2,7,14]</sup>. In contrast, two earlier meta-analyses showed that DWI had high sensitivity (81% and 93%) for detecting HCCs<sup>[13,36]</sup>. We speculate that the reasons for the difference in the rate of sensitivity between our results and those of the two prior meta-analyses might be related to patient selection bias, the background of liver parenchyma in patients with chronic liver disease (cirrhotic or not), and/or the tumor characteristics (*e.g.*, tumor size and malignant grade)<sup>[13,36]</sup>.

Generally, DCE-MRI is the first choice for the evalua-

tion of liver tumors, and DWI is not used alone. However, it is known that the combination of DCE-MRI and DWI improves the detectability and provides additional information to characterize liver lesions<sup>[10–12,37,38]</sup>. DWI thus plays an important role in the assessment of liver tumors for patients with contrast-agent contraindications, such as renal failure or a history of adverse reaction to a contrast agent<sup>[38,39]</sup>. DWI has also been applied for other evaluations of HCCs, such as for assessment of the treatment effects of TACE and molecular target therapy<sup>[40,41]</sup>. FB-DWI can also contribute to the detection or treatment assessment of HCCs in combination with DCE-MRI.

There are several limitations of this study. First, most of the HCCs examined in this study were confirmed by imaging findings, although some HCCs were confirmed by histological results after surgical resection. Therefore, we did not check the histological subtypes of the HCCs, such as whether they were well-, moderately or poorly differentiated. The more aggressive HCCs (*i.e.*, poorly differentiated HCCs) are known to show more water molecule restriction within the tumor compared to the well-differentiated HCCs. In other words, it was difficult to detect well-differentiated HCCs on DWI. In addition, there was a risk to include small ICCs which showed hypervascular tumor like HCCs. However, in this study, we did not focus on the differential diagnosis or characterization of liver tumors. The results of our study were not influenced even if a few ICCs were included in the subjects.



Second, several cases showed severe artifacts at the lateral segment of the liver because of cardiac and respiratory motions. In such cases, the artifacts hampered the visualization of the HCCs. The reduction of motion artifact at the lateral segment of the liver remains a problem to be solved.

In conclusion, FB-DWI provided better image quality and showed higher detectability of HCCs in patients with chronic liver disease compared to RT-DWI, without significantly reducing the SNR of the normal liver parenchyma or the lesion-to-nonlesion CNR. FB-DWI was better at detecting HCCs in patients with chronic liver disease compared to RT-DWI. Free-breathing diffusion-weighted imaging with modified MR parameter settings is advantageous in the diagnosis of HCCs.

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## COMMENTS

### Background

Diffusion-weighted imaging (DWI) is widely adopted as a magnetic resonance imaging (MRI) method in clinical practice because it is useful for the detection and characterization of benign and malignant lesions. DWI is also important for liver MRI to evaluate hepatocellular carcinomas (HCCs) in patients with chronic liver disease. Although dynamic contrast-enhanced MRI has shown higher diagnostic performance for HCCs, DWI can be used as a substitute for the detection and characterization of HCCs for patients who have a contraindication for contrast agents. However, liver DWI occasionally suffers from image distortion and/or chemical shift artifacts related to the echo planar imaging technique and to motion and susceptibility artifacts. To overcome these issues, a previous study investigated the ideal MR parameter settings for obtaining high spatial resolution and fewer artifacts without losing a significant portion of the signal-to-noise ratio or contrast-to-noise ratio on liver MRI. Although DWI with modified MR parameter settings for the improvement of image quality might result in further improvement in the detectability of HCCs, no previous study has investigated this, to our knowledge. In this study, the authors evaluated the image quality and the detectability of HCCs in patients with chronic liver disease on DWI with modified MR parameter settings.

### Research frontiers

DWI is an important diagnostic imaging tool for the detection and characterization of liver tumors, including HCC in patients with chronic liver disease. Especially in the case of patients who are contraindicated for a contrast agent, DWI plays an important role for the evaluation of HCCs. Nonetheless, few prior reports have analyzed free-breathing DWI for the liver. The results of the present study may help clarify the clinical utility of free-breathing DWI for the diagnosis of HCC in patients with chronic liver disease.

### Innovations and breakthroughs

In this study, the authors report the clinical utility of FB-DWI with modified MR parameter settings for the improvement of image quality for diagnosing HCC in patients with chronic liver disease. The free-breathing technique is generally avoided in liver DWI because it is hampered by image distortion and/or chemical shift artifacts related to the echo planar imaging technique and to motion and susceptibility artifacts. They evaluated the clinical impacts of previously reported modified MR parameter settings to overcome these issues with FB-DWI. FB-DWI with modified MR parameter settings provided better image quality without reducing the SNR of the normal liver parenchyma and the

lesion-to-nonlesion CNR. In addition, the improvement of the image quality of FB-DWI might help increase the detection of HCCs.

## Applications

These findings indicate that FB-DWI with modified MR parameter settings is especially useful for patients who are contraindicated for contrast agents and have difficulty holding their breath during the MRI scan. The improvement of image quality helps increase the detection of HCCs without reducing the SNR of the normal liver parenchyma and the lesion-to-nonlesion CNR.

## Terminology

FB-DWI: Diffusion-weighted imaging using free-breathing technique during the scan; RT-DWI: Diffusion-weighted imaging using a navigator-echo-based, real-time respiratory-gating and respiratory-triggering technique during the scan.

## Peer-review

This paper aims to show modified FB-DWI be to detect HCC than conventional MR sequence and patients who are contraindicated for contrast agents.

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## Protein tolerance to standard and high protein meals in patients with liver cirrhosis

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### Abstract

#### AIM

To investigate the plasma amino acid response and tolerance to normal or high protein meals in patients with cirrhosis.

#### METHODS

The plasma amino acid response to a 20 g mixed protein meal was compared in 8 biopsy-proven compensated cirrhotic patients and 6 healthy subjects. In addition the response to a high protein meal (1 g/kg body weight) was studied in 6 decompensated biopsy-proven cirrhotics in order to evaluate their protein tolerance and the likelihood of developing hepatic encephalopathy (HE) following a porto-caval shunt procedure. To test for covert HE, the "number connection test" (NCT) was done on all patients, and an electroencephalogram was recorded in patients considered to be at Child-Pugh C stage.

#### RESULTS

The changes in plasma amino acids after a 20 g protein meal were similar in healthy subjects and in cirrhotics except for a significantly greater increase ( $P < 0.05$ ) in isoleucine, leucine and tyrosine concentrations in the cirrhotics. The baseline branched chain amino acids/aromatic amino acids (BCAA/AAA) ratio was higher in the healthy persons and remained stable-but it decreased significantly after the meal in the cirrhotic group. After the high protein meal there was a marked increase in the levels of most amino acids, but only small changes occurred in the levels of taurine, citrulline, cysteine and



histidine. The BCAA/AAA ratio was significantly higher 180 and 240 min after the meal. Slightly elevated basal plasma ammonia levels showed no particular pattern. Overt HE was not observed in any patients.

### CONCLUSION

Patients with stable liver disease tolerate natural mixed meals with a standard protein content. The response to a high protein meal in decompensated cirrhotics suggests accumulation of some amino acids but it did not precipitate HE. These results support current nutritional guidelines that recommend a protein intake of 1.2-1.5 g/kg body weight/day for patients with cirrhosis.

**Key words:** Branched chain amino acids; Fischer's ratio; Liver; Protein; Cirrhosis; Tolerance; Nutrition; Amino acids; Diet

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**Core tip:** In this study we investigated the plasma amino acid response to standard and high protein meals in patients with liver cirrhosis and looked for evidence of protein intolerance by testing for the presence of either covert or overt hepatic encephalopathy. We sought to improve on previous methodology by selecting a more homogeneous group of patients with biopsy proven cirrhosis, and by using natural mixed protein meals at two protein levels: A standard (20 g) meal and a high (1 g/kg per body weight) protein meal. We found small differences in the plasma amino acid changes after the standard protein meal but there were marked increments in most amino acids after the high protein meal. Noteworthy no patient showed overt clinical signs of encephalopathy and minor electroencephalograph changes were seen in only one patient after the high protein meal. These results present experimental evidence to support current nutritional guidelines for patients with cirrhosis.

Campollo O, Sprengers D, Dam G, Vilstrup H, McIntyre N. Protein tolerance to standard and high protein meals in patients with liver cirrhosis. *World J Hepatol* 2017; 9(14): 667-676 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i14/667.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i14.667>

### INTRODUCTION

The liver plays a key role in the metabolism of amino acids and controls, to a great extent, their homeostasis in the plasma free amino acid pool; it removes them from the plasma, interconverts them and may incorporate them into new protein molecules. Consequently, patients with liver disease show abnormalities in their plasma amino-acid profile<sup>[1-4]</sup> and the fact that some patients with decompensated liver cirrhosis develop protein intolerance<sup>[5]</sup> has been a matter of major clinical concern

over the years<sup>[6]</sup>. The plasma amino acid increase after ingestion of amino acids or protein tends to be associated with an increase in plasma ammonia, which in turn has been implicated in the development of hepatic encephalopathy (HE)<sup>[7-9]</sup>. Under normal circumstances ammonia is detoxified in the liver.

Several studies have investigated the effect of protein ingestion on circulating amino acid levels in patients with liver cirrhosis. The findings have been used to plan therapeutic interventions involving the use of different mixtures of amino acids either to improve nutritional status or as an adjunct in the treatment of HE<sup>[10-13]</sup>.

However, both the type and dosage of protein feed or formula and/or the routes of administration have been varied<sup>[3,14-17]</sup>. Nevertheless, even though most nutritional guidelines recommend high protein diets for liver cirrhosis protein restriction is still considered appropriate in some clinics<sup>[18,19]</sup>. The aim of this study was therefore to investigate the plasma amino acid response to a natural meal with normal protein content in compensated cirrhotic patients compared to a group of healthy subjects in accordance with current guidelines<sup>[18,20-22]</sup>. Furthermore, a group of patients with decompensated cirrhosis were studied in a protocol where they received a meal with high protein content. All the patients were tested for both covert and overt HE to examine the concept of "protein tolerance".

### MATERIALS AND METHODS

#### Study subjects

We administered a 20 g mixed protein meal to 8 male patients with biopsy-proven compensated cirrhosis who were Child-Pugh class A (*i.e.*, without complications of cirrhosis)<sup>[19]</sup>. Patients were recruited from the liver clinic at the Royal Free Hospital. A control group comprising 6 healthy age matched volunteers also received the 20 g protein meal. A group of 6 patients (5 male and 1 female) with biopsy-proven decompensated cirrhosis Child-Pugh class C (*i.e.*, with ascites and esophageal varices but not HE) were also studied. They were being assessed for a porto-caval shunt procedure for the treatment of portal hypertension and so were studied before and after ingestion of a high protein meal (1 g protein/kg body weight). Exclusion criteria in this group were present or former HE and variceal bleeding within one week before the study. The high protein meal was used to predict the likelihood of HE developing following a shunt procedure<sup>[23]</sup>. To test for covert HE, the "number connection test" (NCT)<sup>[24]</sup> was performed in all patients and an electroencephalogram<sup>[25]</sup> was recorded in Child-Pugh stage C patients. This protocol was approved by the Ethics Committee of the Royal Free Hospital and all patients agreed to participate in the study.

#### Test meals

**Twenty grams protein meal:** We recorded the self-selected meals of 10 in-patients with compensated

**Table 1** Amino acid content of the 20 g protein meal

Amino acid	Content (mg)
Isoleucine	934
Leucine	1500
Lysine	1588
Methionine	563
Cysteine	240
Phenylalanine	867
Tyrosine	723
Threonine	872
Tryptophan	255
Arginine	1161
Histidine	637
Alanine	1104
Asparagine	2109
Glutamic acid	3229
Glycine	935
Proline	999
Serine	901

cirrhosis (5 males and 5 females) in order to design a test meal. The lunch of the 10 patients contained on average  $16.2 \pm 1.6$  g of protein,  $44.6 \pm 6.4$  g of carbohydrates and  $18.2 \pm 3.2$  g of fat. Afterwards we created a test meal consisting of beef, green beans, peach slices, ice cream and butter providing 546 kcal (2312.34 kJ), 19.4 g protein, 20.5 g fat and 76.5 g carbohydrate. The amino acid composition of the meal is presented in Table 1. To test for covert HE NCT were performed before and during the study at the same time as blood sampling and patients were checked for clinical signs of overt HE.

#### One gram per kilogram of body weight protein meal:

The Child-Pugh class C cirrhotics were allowed to select the source of protein from a variety of foods. Meat and chicken were the main sources of animal protein in the 1 g/kg of body weight protein dose. The proportions of fat and carbohydrate varied widely. The composition of the individual meals is shown in Table 2. Patients were also checked for clinical signs of overt HE, NCT were performed as mentioned previously, and an EEG was recorded before and 3 h after the meal.

#### Blood samples

Samples were taken before the meal and at 30, 60, 120, 180 and 240 min and were processed as described previously<sup>[26]</sup>. In the healthy persons the 240 min sample was not taken.

#### Laboratory analysis

Plasma ammonia was measured by an enzymatic method (No. 170-UV Sigma Diagnostics, St Louis, MO, United States)<sup>[27]</sup> and amino acid levels on an LKB 4151 Alpha plus amino acid analyzer with a 200 mm  $\times$  4.6 mm high performance analytical column filled with Ultropac 8 cation-exchange resin<sup>[26]</sup>. Values for glutamine and glutamic acid were inaccurate as their apparent concentration depends on the time period between sampling and analysis. Tryptophan was not measured. Because of

**Table 2** Protein content of the 1 g/kg protein meals

Patient	Energy (kcal)	Protein (g)	%kcal
1	914	94	40
2	973	78	32
3	590	56	36
4	653	38	23
5	1545	78	20
6	1507	65	17

technical problems citrulline was not reported in the healthy control group. Total alpha-amino nitrogen was determined by the fluorodinitrobenzene (DNFB) method<sup>[28]</sup>.

#### Statistical analysis

Differences in amino acid concentration between groups were compared using the Student's "t" test and differences within a group by the paired "t" test.

## RESULTS

#### Response to a 20 g protein meal

The plasma baseline levels of asparagine, cysteine, tyrosine and ornithine were significantly higher in the patients with stable cirrhosis compared to the healthy subjects ( $P < 0.05$ ). The total alpha-amino-N response to the meal in the cirrhotic subjects did not differ from that of healthy subjects (Figure 1 and Table 3). However, as to individual amino acids, isoleucine, tyrosine and leucine increased significantly more in the cirrhotic patients (Figure 2). The baseline branched chain amino acids/aromatic amino acids (BCAA/AAA) ratio was higher in the healthy persons and remained stable after the meal while there was a further significant decrease after two hours in the cirrhotic group (Table 3). At 60 and 120 min cirrhotic patients showed a significant increase in plasma ammonia concentration after the meal than normal subjects ( $P < 0.01$ , Figure 3). The NCT remained normal and there were no clinical signs of HE.

#### Response to a 1 g/kg body weight protein meal

Decompensated cirrhotics had different basal concentration of some amino acids compared to those with stable cirrhosis (elevated: Alanine, tyrosine, decreased: isoleucine, leucine) (Figure 4 and Table 4). Hence, the BCAA/AAA ratio was significantly lower in the patients with unstable cirrhosis. After the meal, the concentration of most plasma amino acids (except for taurine, proline, citrulline, cysteine and histidine) had increased significantly at 120 min (Figure 4 and Table 4). Those increments were significantly larger than those observed in the 20 g protein group (Figure 1). The largest increases were observed in the cases of isoleucine (148%), leucine (119%) and methionine (88%) (Figures 1 and 3). The BCAA/AAA ratio was significantly higher 180 and 240 min after the meal (Table 4). Slightly elevated basal plasma ammonia levels increased in two patients, decreased in one and showed no change in two (Figure 3). After the protein meal only

**Table 3** Plasma amino acid response to a 20-g protein mixed meal in cirrhotic patients and controls

Amino acid	Group	Basal ( $\pm$ SE)	30 min ( $\pm$ SE)	60 min ( $\pm$ SE)	120 min ( $\pm$ SE)	180 min ( $\pm$ SE)	240 min ( $\pm$ SE)
Tau	Control	69 $\pm$ 17	65 $\pm$ 10	74 $\pm$ 10	74 $\pm$ 11	69 $\pm$ 8	
	Cirrhotic	48 $\pm$ 5	46 $\pm$ 7	42 $\pm$ 5 <sup>a</sup>	47 $\pm$ 6	44 $\pm$ 4	42 $\pm$ 7
Thr	Control	101 $\pm$ 5	117 $\pm$ 10 <sup>c</sup>	110 $\pm$ 3	115 $\pm$ 8	114 $\pm$ 12	
	Cirrhotic	113 $\pm$ 10	124 $\pm$ 8	125 $\pm$ 11	118 $\pm$ 8	101 $\pm$ 8	111 $\pm$ 15
Ser	Control	94 $\pm$ 8	105 $\pm$ 8 <sup>f</sup>	104 $\pm$ 9 <sup>d</sup>	99 $\pm$ 11	92 $\pm$ 13	
	Cirrhotic	105 $\pm$ 7	117 $\pm$ 8 <sup>c</sup>	114 $\pm$ 10	108 $\pm$ 9	96 $\pm$ 7	103 $\pm$ 12
Asn	Control	23 $\pm$ 2	40 $\pm$ 2 <sup>d</sup>	41 $\pm$ 4 <sup>d</sup>	40 $\pm$ 4 <sup>d</sup>	35 $\pm$ 5 <sup>c</sup>	
	Cirrhotic	35 $\pm$ 3 <sup>a</sup>	43 $\pm$ 3 <sup>f</sup>	44 $\pm$ 5 <sup>d</sup>	44 $\pm$ 4 <sup>d</sup>	37 $\pm$ 4	38 $\pm$ 5
Glu	Control	246 $\pm$ 33	299 $\pm$ 35	263 $\pm$ 17	276 $\pm$ 24	237 $\pm$ 26	
	Cirrhotic	375 $\pm$ 30	388 $\pm$ 20	380 $\pm$ 34	375 $\pm$ 23	354 $\pm$ 22	378 $\pm$ 45
Gln	Control	163 $\pm$ 11	163 $\pm$ 14	154 $\pm$ 13	142 $\pm$ 11	144 $\pm$ 26	
	Cirrhotic	226 $\pm$ 21 <sup>a</sup>	217 $\pm$ 18	213 $\pm$ 14 <sup>a</sup>	213 $\pm$ 20	190 $\pm$ 12	180 $\pm$ 29
Pro	Control	145 $\pm$ 11	152 $\pm$ 13	151 $\pm$ 5	155 $\pm$ 12	135 $\pm$ 7	
	Cirrhotic	152 $\pm$ 15	147 $\pm$ 17	173 $\pm$ 19	185 $\pm$ 17 <sup>c</sup>	155 $\pm$ 16	150 $\pm$ 15
Gly	Control	178 $\pm$ 8	187 $\pm$ 11	190 $\pm$ 14	193 $\pm$ 16	176 $\pm$ 21	
	Cirrhotic	174 $\pm$ 14	177 $\pm$ 14	166 $\pm$ 13	176 $\pm$ 11	170 $\pm$ 12	165 $\pm$ 16
Ala	Control	271 $\pm$ 18	323 $\pm$ 21 <sup>d</sup>	339 $\pm$ 29 <sup>d</sup>	354 $\pm$ 31 <sup>d</sup>	309 $\pm$ 34	
	Cirrhotic	262 $\pm$ 17	310 $\pm$ 24 <sup>c</sup>	334 $\pm$ 34	345 $\pm$ 25 <sup>d</sup>	307 $\pm$ 26	284 $\pm$ 38
Cit	Cirrhotic	27 $\pm$ 3	23 $\pm$ 2	20 $\pm$ 2	27 $\pm$ 2	28 $\pm$ 2	33 $\pm$ 4
Val	Control	167 $\pm$ 13	186 $\pm$ 14 <sup>d</sup>	182 $\pm$ 16 <sup>c</sup>	188 $\pm$ 11 <sup>d</sup>	173 $\pm$ 16	
	Cirrhotic	180 $\pm$ 4	198 $\pm$ 10 <sup>c</sup>	203 $\pm$ 9 <sup>c</sup>	193 $\pm$ 10	170 $\pm$ 8	175 $\pm$ 18
Cys	Control	42 $\pm$ 1	41 $\pm$ 2	40 $\pm$ 2	42 $\pm$ 3	39 $\pm$ 4	
	Cirrhotic	58 $\pm$ 3 <sup>b</sup>	59 $\pm$ 2 <sup>e</sup>	60 $\pm$ 3 <sup>e</sup>	62 $\pm$ 2	58 $\pm$ 4 <sup>b</sup>	56 $\pm$ 5
Met	Control	25 $\pm$ 2	24 $\pm$ 1	25 $\pm$ 1	29 $\pm$ 4	27 $\pm$ 4	
	Cirrhotic	32 $\pm$ 3	30 $\pm$ 2 <sup>a</sup>	33 $\pm$ 3	31 $\pm$ 2	25 $\pm$ 3	25 $\pm$ 3
Iso	Control	49 $\pm$ 3	55 $\pm$ 4	54 $\pm$ 5	62 $\pm$ 5	57 $\pm$ 7	
	Cirrhotic	55 $\pm$ 2	68 $\pm$ 3 <sup>a,d</sup>	71 $\pm$ 4 <sup>a,d</sup>	59 $\pm$ 2	50 $\pm$ 3	55 $\pm$ 7
Leu	Control	91 $\pm$ 6	101 $\pm$ 8 <sup>c</sup>	97 $\pm$ 10	103 $\pm$ 8	95 $\pm$ 11	
	Cirrhotic	101 $\pm$ 2	126 $\pm$ 7 <sup>a,d</sup>	128 $\pm$ 7 <sup>a,d</sup>	106 $\pm$ 5	87 $\pm$ 5	95 $\pm$ 12
Tyr	Control	53 $\pm$ 3	56 $\pm$ 2 <sup>c</sup>	55 $\pm$ 3	56 $\pm$ 4	54 $\pm$ 6	
	Cirrhotic	73 $\pm$ 7 <sup>a</sup>	80 $\pm$ 7	83 $\pm$ 8 <sup>b</sup>	82 $\pm$ 8 <sup>a</sup>	73 $\pm$ 6	76 $\pm$ 10
Phe	Control	37 $\pm$ 0.8	39 $\pm$ 1	40 $\pm$ 2	42 $\pm$ 4	40 $\pm$ 5	
	Cirrhotic	48 $\pm$ 5	58 $\pm$ 7	61 $\pm$ 9	62 $\pm$ 9	57 $\pm$ 7	56 $\pm$ 7
Orn	Control	41 $\pm$ 2	44 $\pm$ 2	44 $\pm$ 3	47 $\pm$ 3	45 $\pm$ 5	
	Cirrhotic	61 $\pm$ 5 <sup>b</sup>	67 $\pm$ 4	69 $\pm$ 6	71 $\pm$ 7 <sup>d</sup>	68 $\pm$ 6	71 $\pm$ 11
Lys	Control	145 $\pm$ 10	169 $\pm$ 13 <sup>d</sup>	175 $\pm$ 13 <sup>d</sup>	194 $\pm$ 13 <sup>f</sup>	169 $\pm$ 17 <sup>c</sup>	
	Cirrhotic	144 $\pm$ 6	167 $\pm$ 9 <sup>c</sup>	171 $\pm$ 11 <sup>c</sup>	164 $\pm$ 11	153 $\pm$ 9	150 $\pm$ 15
His	Control	64 $\pm$ 3	73 $\pm$ 3 <sup>d</sup>	72 $\pm$ 4 <sup>c</sup>	72 $\pm$ 4 <sup>c</sup>	67 $\pm$ 8	
	Cirrhotic	63 $\pm$ 4	70 $\pm$ 3	73 $\pm$ 4	72 $\pm$ 4	69 $\pm$ 4	65 $\pm$ 5
Arg	Control	70 $\pm$ 4	83 $\pm$ 5 <sup>d</sup>	85 $\pm$ 6 <sup>c</sup>	88 $\pm$ 6 <sup>d</sup>	77 $\pm$ 8	
	Cirrhotic	73 $\pm$ 5	81 $\pm$ 6	85 $\pm$ 7	86 $\pm$ 6	80 $\pm$ 7	71 $\pm$ 8
BCAA/AAA	Control	3.44 $\pm$ 0.2	3.62 $\pm$ 0.2	3.49 $\pm$ 0.2	3.65 $\pm$ 0.2	3.54 $\pm$ 0.2	
	Cirrhotic	2.89 $\pm$ 0.2	2.97 $\pm$ 0.2	2.92 $\pm$ 0.2	2.62 $\pm$ 0.2 <sup>c</sup>	2.45 $\pm$ 0.2 <sup>d</sup>	2.53 $\pm$ 0.2 <sup>f</sup>

Plasma amino acid response to a 20 g protein mixed meal in cirrhotic patients and controls. The results are expressed as means  $\pm$  SEM. Healthy subjects  $n = 6$ , cirrhotics  $n = 8$ . Plasma amino acids are given in nmol/mL; significantly different from the corresponding value of control subject: <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ ; Significantly different from basal value within the same group <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , <sup>f</sup> $P < 0.001$ .

one patient presented mild electroencephalographic features of covert encephalopathy but there were no clinical manifestations.

## DISCUSSION

A characteristic pattern of plasma amino acids has been described in cirrhotic subjects<sup>[3,4,29-31]</sup> and metabolic and biochemical differences have been shown between stable and unstable cirrhotics<sup>[3,4,32]</sup>. In advanced liver disease there is usually an increased concentration of the AAA tyrosine, phenylalanine and tryptophan, and decreased concentration of the BCAA leucine, isoleucine and valine<sup>[3,4,6,9,14]</sup>. We have previously reported differences

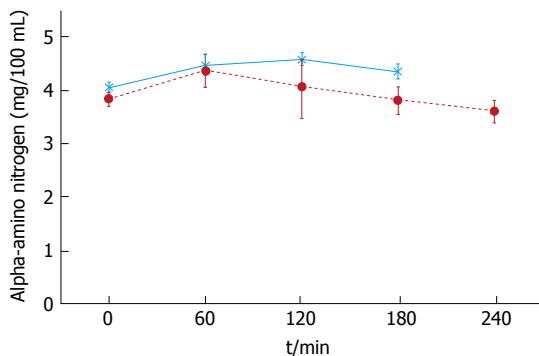
between different stages of liver disease and small or no significant differences between patients with stable liver disease and normal subjects<sup>[26]</sup>. Plasma amino acid concentrations change in the postabsorptive state reflects the balance between uptake by the liver and release by extrahepatic tissue, primarily muscle<sup>[2,3,29,33,34]</sup>. Following a mixed meal, the BCAA are transferred from the gut through the liver to peripheral tissues<sup>[35]</sup>. All other amino acids, including the AAA and methionine, are retained to a greater extent by splanchnic tissues and particularly by the liver<sup>[35,36]</sup>. The BCAA are then primarily metabolized in extrahepatic tissues i.e. skeletal muscle<sup>[28,36]</sup>.

In most previous studies the methods for patient selection (*i.e.*, disease severity) and nutritional intervention

**Table 4** Plasma amino acid response to a high (1 g protein/kg body weight) meal in cirrhotic patients

Amino acid	Group	Basal ( $\pm$ SE)	30 min ( $\pm$ SE)	60 min ( $\pm$ SE)	120 min ( $\pm$ SE)	180 min ( $\pm$ SE)	240 min ( $\pm$ SE)
Taurine	Cirrhotic	58 $\pm$ 1	54 $\pm$ 4	59 $\pm$ 5	58 $\pm$ 3	57 $\pm$ 4	54 $\pm$ 3
Threonine	Cirrhotic	150 $\pm$ 25	152 $\pm$ 22	169 $\pm$ 23	195 $\pm$ 29 <sup>c</sup>	216 $\pm$ 43	216 $\pm$ 31
Serine	Cirrhotic	114 $\pm$ 11	131 $\pm$ 17	151 $\pm$ 20	153 $\pm$ 16 <sup>f</sup>	156 $\pm$ 21	158 $\pm$ 15
Asparagine	Cirrhotic	46 $\pm$ 3	60 $\pm$ 5	67 $\pm$ 6 <sup>f</sup>	76 $\pm$ 3	76 $\pm$ 7	73 $\pm$ 8
Glutamic ac.	Cirrhotic	110 $\pm$ 30	94 $\pm$ 25	88 $\pm$ 36	109 $\pm$ 29	130 $\pm$ 26	153 $\pm$ 79
Glutamine	Cirrhotic	449 $\pm$ 29	462 $\pm$ 64	514 $\pm$ 56	560 $\pm$ 43	578 $\pm$ 39	520 $\pm$ 82
Proline	Cirrhotic	182 $\pm$ 35	212 $\pm$ 30	220 $\pm$ 26	278 $\pm$ 30	266 $\pm$ 32	245 $\pm$ 34
Glycine	Cirrhotic	199 $\pm$ 13	225 $\pm$ 20	245 $\pm$ 25	272 $\pm$ 12 <sup>d</sup>	276 $\pm$ 25	273 $\pm$ 21
Alanine	Cirrhotic	326 $\pm$ 23	408 $\pm$ 41	465 $\pm$ 48 <sup>c</sup>	462 $\pm$ 12	456 $\pm$ 29	475 $\pm$ 47
Citruline	Cirrhotic	48 $\pm$ 7	44 $\pm$ 7	48 $\pm$ 7	58 $\pm$ 5	50 $\pm$ 7	59 $\pm$ 10
Valine	Cirrhotic	146 $\pm$ 23	160 $\pm$ 19	185 $\pm$ 19	209 $\pm$ 29 <sup>c</sup>	224 $\pm$ 36	243 $\pm$ 29
Cysteine	Cirrhotic	53 $\pm$ 3	55 $\pm$ 4	61 $\pm$ 7	60 $\pm$ 4	57 $\pm$ 3	63 $\pm$ 4 <sup>d</sup>
Methionine	Cirrhotic	34 $\pm$ 4	41 $\pm$ 4	45 $\pm$ 4	54 $\pm$ 6 <sup>f</sup>	58 $\pm$ 6	64 $\pm$ 8
Isoleucine	Cirrhotic	43 $\pm$ 2	57 $\pm$ 6	63 $\pm$ 6 <sup>d</sup>	86 $\pm$ 12	97 $\pm$ 16	107 $\pm$ 11
Leucine	Cirrhotic	73 $\pm$ 10	93 $\pm$ 12	107 $\pm$ 14	135 $\pm$ 25 <sup>c</sup>	149 $\pm$ 31	160 $\pm$ 20
Tyrosine	Cirrhotic	109 $\pm$ 13	115 $\pm$ 8	123 $\pm$ 11	139 $\pm$ 8 <sup>c</sup>	144 $\pm$ 8	159 $\pm$ 15
Phenylalanine	Cirrhotic	58 $\pm$ 7	66 $\pm$ 5	77 $\pm$ 10	84 $\pm$ 10 <sup>f</sup>	86 $\pm$ 9	98 $\pm$ 12
Ornithine	Cirrhotic	62 $\pm$ 7	69 $\pm$ 6	73 $\pm$ 7	81 $\pm$ 4 <sup>f</sup>	90 $\pm$ 8	94 $\pm$ 9
Lysine	Cirrhotic	148 $\pm$ 10	179 $\pm$ 22	211 $\pm$ 27 <sup>c</sup>	240 $\pm$ 32	248 $\pm$ 40	253 $\pm$ 32
Histidine	Cirrhotic	68 $\pm$ 8	80 $\pm$ 8	89 $\pm$ 7	90 $\pm$ 8	91 $\pm$ 10	93 $\pm$ 7
Arginine	Cirrhotic	91 $\pm$ 9	99 $\pm$ 10	109 $\pm$ 14	134 $\pm$ 11 <sup>c</sup>	139 $\pm$ 18	152 $\pm$ 19
BCAA/AAA	Cirrhotic	1.69 $\pm$ 0.3	1.74 $\pm$ 0.2	1.81 $\pm$ 0.2	1.93 $\pm$ 0.3	2.03 $\pm$ 0.3 <sup>c</sup>	2.08 $\pm$ 0.4 <sup>c</sup>

The results are expressed as means  $\pm$  SEM. Plasma amino acids are given in nmol/mL. First value significantly different from basal value: <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , <sup>f</sup> $P < 0.02$ .



**Figure 1** Plasma alpha-amino nitrogen concentration in response to a 20 g protein meal. Cirrhotic patients ( $n = 6$ ), (closed circles); healthy subjects ( $n = 6$ ), (asterisk) (mean  $\pm$  SEM).

(type and dose of protein or amino acid formula) have varied widely, with very few controlled studies involving a “natural” meal<sup>[14,31,35,37]</sup>. This precludes the opportunity to make firm interpretations of the metabolic alterations in cirrhotic patients. In the present study we therefore investigated the plasma amino acid response to a *natural* meal administered to biopsy proven cirrhotic patients (Child-Pugh class A and C).

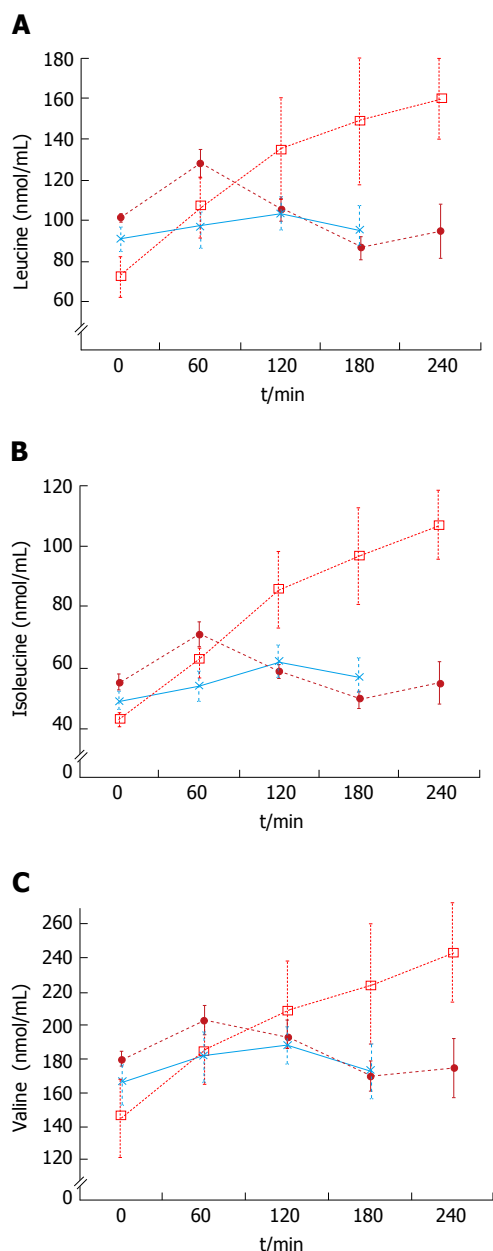
On the other hand ammonia is a toxic nitrogenous product of protein and amino acid metabolism<sup>[38]</sup> which under normal circumstances is mainly detoxified by the

liver. In patients with cirrhosis there is an increase in circulating ammonia caused by impaired hepatic detoxification and the presence (as in the decompensated cirrhotics group) of porto-systemic shunting<sup>[19,39]</sup>. Thus the rationale for a *protein tolerance test* is that if the patient develops HE after the test the risk of developing it after the shunt procedure is likely to be relatively high-information which helps surgeons decide which particular type of porto-systemic shunt or device to perform or use respectively.

### Twenty grams protein natural meal

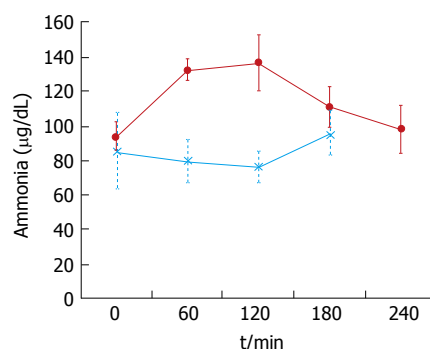
After intake of a mixed meal, there were only small differences for most plasma amino acids between cirrhotic patients and controls (Table 3). Only isoleucine, tyrosine and particularly leucine showed modest, but significantly higher increases in cirrhotic patients after the meal (Table 3 and Figure 2). The mean AAA concentration was also higher, but not significantly so. The higher BCAA and AAA increases observed in cirrhotics may be explained by their peripheral insulin resistance<sup>[14]</sup> which results in reduced muscle uptake of BCAA and a decreased inhibition of muscle catabolism after food intake. In previous studies, patients at different stages of liver disease were given either a protein load (ranging from 27 to 48 g)<sup>[3,14-16,31]</sup> or BCAA-enriched formulae<sup>[3,9]</sup> and showed amino acid “intolerance” to that load of protein or





**Figure 2** Plasma leucine (A), isoleucine (B) and valine (C) concentrations in response to protein meals. Twenty grams protein meal, cirrhotics ( $n = 8$ ), (closed circles); 20 g protein meal, healthy subjects ( $n = 6$ ), (asterix) (mean  $\pm$  SEM); 1 g/kg body weight protein meal, cirrhotics ( $n = 6$ ), (open squares).

amino acids. The term “intolerance” here being based on a persistent increase of amino acids in plasma<sup>[40,41]</sup>. It is known, however, that patient selection and factors such as protein type and dosage influence the plasma amino acid response<sup>[35,42-44]</sup>. Additionally the description “protein intolerant” is better reserved for patients who develop HE during protein intake. The BCAA/AAA ratio, showed a slight but significant decrease 120 min after the meal (Table 3). This is in agreement with previous reports suggesting that this ratio may be useful for detecting differences in amino acid metabolism in different groups of cirrhotics<sup>[26,36]</sup>. The differences found in our study suggest subtle alterations in the metabolism of BCAA and AAA evident 2 h after a protein meal, although the meal



**Figure 3** Plasma ammonia concentrations in cirrhotic patients. Blood ammonia after a 20-g protein meal. Cirrhotic patients ( $n = 6$ ), (closed circles); healthy subjects ( $n = 6$ ), (asterix) (mean  $\pm$  SEM).

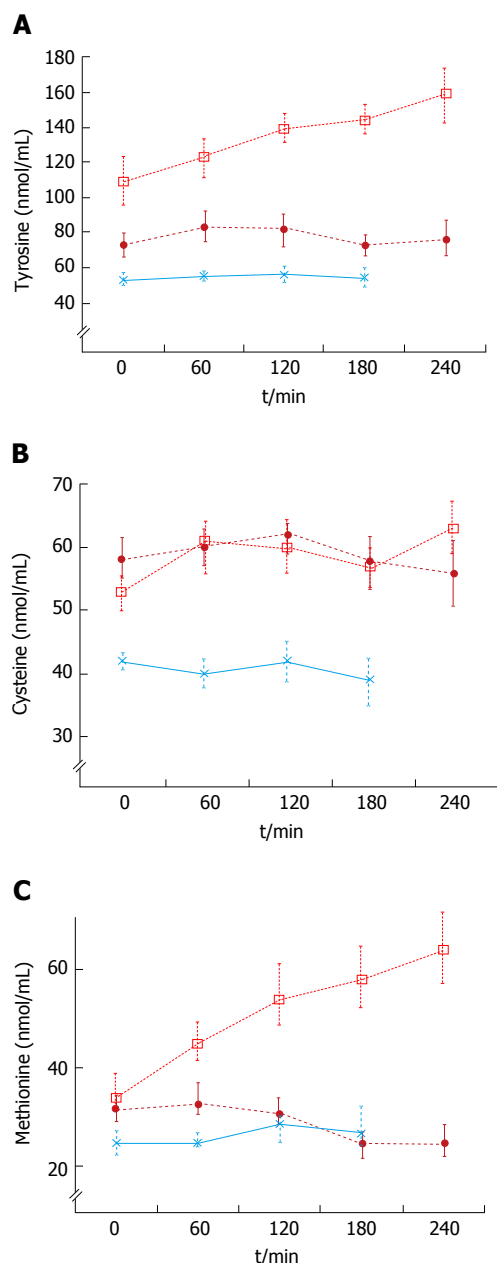
seemed otherwise well tolerated.

In the stable cirrhotic patients we observed a significant increase in the venous plasma ammonia concentration 60 min after food intake (Figure 3) although this protein meal had little effect on alpha amino nitrogen levels (Figure 1). This may be explained by the considerably larger amino nitrogen pool (13.8 mmol N) compared with that of ammonia (0.16 mmol N)<sup>[45]</sup> which might be more sensitive to cyclic changes in absorptive periods and by protein breakdown in the small intestine<sup>[46]</sup>. Additionally, a healthy liver has a huge capacity for increasing urea synthesis after protein ingestion, when ammonia is released from the gut into the portal blood. In patients with cirrhosis, liver ammonia clearance is diminished by the decreased functional liver mass, portosystemic shunting and loss of normal perisinusoidal glutamine synthetase activity<sup>[47-49]</sup>. Nevertheless, the increases in ammonia were modest and most importantly, we did not observe any overt (clinically detectable) HE. The NCT was carried out to test the patients for covert HE which is not clinically detectable. No patients had covert HE after ingestion of the meal. These results support more the role of those factors affecting the clearance of blood ammonia rather than the effect of diet in the development of HE<sup>[39]</sup>.

In this study, the plasma amino acid response to a 20 g natural protein meal was almost the same in cirrhotic patients and controls and we suggest that cirrhotic patients, with a reasonably good liver function have a good tolerance to a natural protein meal. This concurs with current guidelines for protein intake in patients with liver disease<sup>[20-22]</sup>.

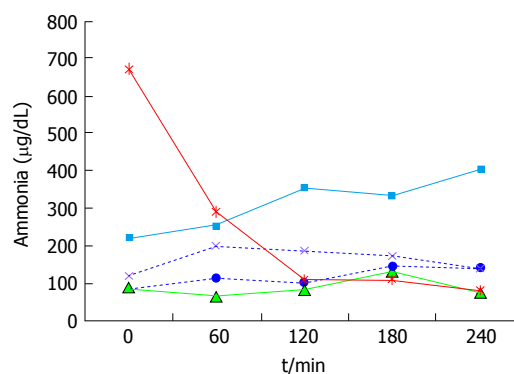
### High protein meal to decompensated patients with cirrhosis

The baseline results showed that the BCAA/AAA ratio was lower in decompensated cirrhotics than in patients with stable cirrhosis and healthy subjects (Table 4). This characteristic pattern of plasma amino acids has previously been described by us and others<sup>[14,26,29,31,34]</sup>. In this group administration of a high (1 g/kg body weight) protein meal led to significant increases in most plasma amino acid levels (Figures 2 and 4, Table 4).



**Figure 4** Plasma tyrosine (A), cysteine (B) and methionine (C) concentrations in response to protein meals. Twenty grams protein meal, cirrhotics ( $n = 8$ ), (closed circles); 20 g protein meal, healthy subjects ( $n = 6$ ), (asterisk) (mean  $\pm$  SEM); 1 g/kg body weight protein meal, cirrhotics ( $n = 6$ ), (open squares).

These results agree in general with those reported by Marchesini *et al*<sup>[14]</sup> and by Schulte-Frohlinde *et al*<sup>[31]</sup>. The fact that we found slightly lower increases of leucine, methionine, valine, arginine and glycine in our study might be explained by the type of meal administered (balanced and protein of mixed origin vs meat only in other reports)<sup>[31,35,43]</sup>, and by differences in the degree of liver disease in the study populations<sup>[14-16]</sup>. It is established that a balanced diet increases protein tolerance<sup>[43]</sup>. Apart from the expected increases in valine and methionine levels, our results showed that tyrosine, leucine, isoleucine, phenylalanine, arginine and glycine were also regularly increased after the meal (Figures 2 and 4, Table



**Figure 5** Plasma ammonia concentrations in cirrhotic patients. Individual results after a 1g/kg per body weight protein meal.

4). We also observed a significant increase in the BCAA/AAA ratio which remained elevated up to three hours. The increment in the BCAA/AAA ratio may have resulted from extreme elevations of the BCAAs included in this ratio, the more advanced degree of the disease and/or a paradoxical tendency to normalization of the BCAA/AAA ratio seen after a high protein dose. This should be further investigated.

Current nutrition guidelines recommend high protein diets (1.2-1.5 g/kg body weight/day) for liver cirrhosis<sup>[20-22]</sup> but this is mainly based on applied therapeutic interventions rather than on tolerance or challenge tests<sup>[20-22,40]</sup>. In this study we present experimental evidence supporting those recommendations.

In contrast to the patients with stable cirrhosis, no specific pattern in plasma ammonia concentration was observed in the high protein group (Figure 5), although the concentration in some of the patients reached higher levels than those seen after a standard (20 g) protein meal. The variability of the response in this group suggests an abnormal ammonia metabolism which would be in accordance with the Child-Pugh's grade of liver insufficiency (*i.e.*, C) and the presence of portal hypertension.

No patients experienced overt HE in spite of the amino acid elevations; only one of the six decompensated cirrhotic patients showed mild electroencephalographic changes compatible with covert HE. Previously, protein loads were thought to be a common precipitating factor for HE<sup>[23,27]</sup>. However, protein restriction worsens the nutritional status of cirrhotic patients<sup>[10,50]</sup> and a report by Córdoba *et al*<sup>[49]</sup> showed that diets with a normal-high protein content (1.2 g/kg per day) are metabolically more adequate than low-protein diets and can be administered safely to cirrhotic patients with episodic HE. Restriction of dietary protein did not have any beneficial effect<sup>[49]</sup>.

**Limitations:** We studied a small group of patients with decompensated cirrhosis. As they were following a protocol in preparation for a porto-caval shunt operation a protein tolerance test was done in order to predict the likelihood of the development of HE after the procedure. Those patients represented the high protein meal group

in this study. As that was not part of the protocol a control group for this part was not included, although we recognize that it would have given us more complete information and provided a better comparison group than just the standard protein group.

In conclusion, after a natural meal containing 20 g of protein, the overall plasma amino acid response in patients with cirrhosis was similar to that of healthy subjects. Plasma ammonia levels increased slightly but, importantly, no evidence of either covert or overt HE was observed. Patients with decompensated cirrhosis showed higher post-prandial concentrations of amino acids in response to a high protein meal. However, we did not observe any overt HE, hence the obvious benefits of a high protein regime should be considered in these patients<sup>[30,50,51]</sup>. In this patient group we therefore recommend following the current nutritional guidelines: protein intake of 1.2-1.5 g/kg body weight distributed daily in frequent small meals. If patients develop HE on a high-protein diet, consider supplementation with BCAA<sup>[12,13,20]</sup>.

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## COMMENTS

### Background

The plasma amino acid increase after ingestion of amino acids or protein tends to be associated with an increase in plasma ammonia, which in turn has been implicated in the development of hepatic encephalopathy. There has been long standing discussion over the adequate amount of protein to be administered to patients with liver cirrhosis in spite of generally accepted nutritional guidelines for these patients. Despite current nutrition guidelines recommend high protein diets, recommendations have not been completely adopted in some places where protein restriction is still considered as a general rule and proper dietary management is not readily followed.

### Research frontiers

While current nutrition guidelines are mainly based on applied therapeutic interventions there have been few reports investigating the tolerance to dietary protein nor they have studied protein tolerance or challenge tests. In this study the authors investigated the plasma amino acid response to standard and high protein natural meals in patients with liver cirrhosis and looked for evidence of protein intolerance by testing for the presence of either covert or overt hepatic encephalopathy.

### Innovations and breakthroughs

Several studies have investigated the effect of protein ingestion on circulating amino acid levels in patients with liver cirrhosis. However, both the type and dosage of protein feed or formula and/or the routes of administration have been varied and the selection of patients has been heterogeneous. The authors aimed to improve on previous methodology by selecting a more homogeneous group of patients with biopsy proven cirrhosis, and by using natural mixed protein meals at two protein levels: A standard (20 g) meal and a high (1 g/kg per body weight) protein meal. In this study they provide experimental evidence to support current nutritional guidelines.

## Applications

Current nutritional guidelines recommend normal to high protein diets (1.2-1.5 g/kg body weight/d) which the authors experimented in this study with good results. They did not observe any overt hepatic encephalopathy hence the obvious benefits of a high protein regime. If patients develop hepatic encephalopathy on a high-protein diet, temporary reduction of protein intake and supplementation with branched chain amino acids should be considered.

## Terminology

Protein tolerance test: A high protein meal (load) has been used to predict the likelihood of hepatic encephalopathy developing following a porto-caval shunt procedure for the treatment of portal hypertension. Therefore patients are studied before and after ingestion of a high protein meal (1 g protein/kg body weight) and clinical and psychological (*i.e.*, "number connection test") evaluations are performed to study overt and covert hepatic encephalopathy.

## Peer-review

The manuscript is well-structured, the rationale behind the study is clear, and the results are relevant for the field of nutrition in liver disease.

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## Inducible protein-10 as a predictive marker of antiviral hepatitis C treatment: A systematic review

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**Data sharing statement:** The technical appendix, and dataset are available from the corresponding author at [nina.weis@regionh.dk](mailto:nina.weis@regionh.dk).

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### Abstract

#### AIM

To investigate interferon- $\gamma$ -inducible protein-10's (IP-10) potential to anticipate rapid (RVR)- and sustained virological responses (SVR) to chronic hepatitis C (CHC) treatment.

#### METHODS

We included case series examining RVR or SVR in relation to 24 or 48 wk treatment for CHC, in patients treatment free for at least six months, with genotype 1 or 4, and in relation to 24 wk treatment for genotype 2 and 3, with pegylated interferon in combination with ribavirin. Patients had to have both a baseline IP-10 level as well as a hepatitis C virus (HCV)-RNA determination 4 wk after treatment initiation or 24 wk after end of treatment. Studies including patients with liver diseases other than CHC, human immunodeficiency virus-infection, treatment with immunosuppressants or cytostatics, alcohol dependency or active intravenous drug-use were excluded. We found 81 articles by searching the MEDLINE and EMBASE databases. Eight studies were eligible for inclusion. Their quality were assessed using an 18 point checklist for case series, developed using a modified Delphi technique. Information was extracted from the articles, and no raw data was requisitioned. The review protocol was

registered at the International Prospective Register of Systematic Reviews (reg. number: CRD42014008736).

## RESULTS

Three studies reported on baseline IP-10 level in association with RVR. A significant association was found for HCV genotype 1 infection by two studies. Only two studies reported on HCV genotype 4 infected and genotype 2 and 3 infected patients, respectively. A trend was seen for an association between RVR and baseline IP-10 for genotype 4, while no association was found for genotype 2 and 3. Seven studies provided information regarding baseline IP-10 and SVR. Following the pattern regarding rapid virological response all five studies examining SVR in relation to baseline IP-10 levels for HCV, genotype 1 infected patients showed a significant association. Likewise a significant association was seen for HCV, genotype 4 infected, while no association was found for HCV, genotype 2 and 3 infected. Though only two studies examined the association for HCV genotype 4 infected and HCV genotype 2 and 3 infected respectively.

## CONCLUSION

We found indications of a possible association between baseline IP-10 level and virological responses in patients with CHC genotype 1 and 4.

**Key words:** Chronic hepatitis C; Inducible protein-10's; Sustained virological response; Interferon- $\gamma$ -inducible protein-10; CXCL-10; Chemokine; Genotype; Pegylated interferon; Ribavirin; Rapid virological response

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**Core tip:** This is the first systematic review examining the association between baseline levels of interferon- $\gamma$ -inducible protein-10 (IP-10) and virological response to treatment with pegylated interferon and ribavirin among patients chronically infected with hepatitis C virus, genotype 1-4. We found a possible correlation for genotype 1 and 4 infected patients, indicating that baseline IP-10 levels could predict which patients, infected with genotype 1 or 4, would have the highest likelihood of benefitting from antiviral treatment with pegylated interferon and ribavirin. These findings can be especially relevant in countries, where treatments with direct acting antivirals are not readily applicable.

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## INTRODUCTION

Every year 3-4 million individuals are infected with hepatitis C virus (HCV) of whom only 20%-35% clear the

infection, meaning that 2.4-3.2 million individuals remain chronically infected, defined as detectable HCV-RNA in two consecutive measurements  $\geq$  six months apart. Globally, the prevalence of chronic hepatitis C (CHC) is estimated to 150 million people, with CHC being the leading cause of chronic liver disease<sup>[1]</sup>. CHC can lead to formation of connective tissue (fibrosis) in the liver. However, the rate and severity of the inflammation and fibrosis vary<sup>[2,3]</sup>. Though only 5%-20% of HCV infected patients develop cirrhosis, these patients have an increased risk of developing hepatocellular carcinoma, a condition responsible for more than 300000 deaths annually<sup>[1]</sup>.

Until recently, the standard of care for CHC was lengthy dual therapy with pegylated interferon plus ribavirin (peg-IFN/RBV), either as 180  $\mu$ g peg-IFN- $\alpha$ -2a weekly or peg-IFN- $\alpha$ -2b 1.5  $\mu$ g/kg per week in combination with ribavirin 15 mg/kg per day (minimum 1000 mg daily and maximum 1400 mg daily), fixed doses of 1000 mg for patients < 75 kg and 1200 mg for patients > 75 kg with genotype 1 or 4 or flat dosing of 800 mg daily for genotypes 2 and 3 - a treatment with modest success rates, severe adverse events and variation in treatment response between genotypes<sup>[4]</sup>. Therefore, a great effort has been put into identifying biomarkers to predict rapid virological response (RVR), defined as undetectable serum HCV-RNA at week four of antiviral treatment, and sustained virological response (SVR), defined as the undetectable HCV-RNA 24 wk after discontinuing antiviral treatment. One of the most promising chemokine biomarker candidate is interferon- $\gamma$  inducible protein-10 (IP-10). Both intrahepatic IP-10 mRNA and plasma levels of IP-10 are elevated in individuals with CHC<sup>[5,6]</sup>, strongly indicating that intrahepatic IP-10 is the source of plasma IP-10. Several studies have suggested that pretreatment levels of IP-10 have the capability to predict RVR and SVR<sup>[6-9]</sup>. In addition, hepatic inflammation and fibrosis have been shown to correlate with IP-10 levels<sup>[10-12]</sup>, and it has been proposed, that plasma levels of IP-10 can predict the risk of fibrosis progression<sup>[13]</sup>. Later years have seen the forthcoming of the new direct acting antivirals (DAA), and all current treatment recommendations for CHC patients from the European Association for the Study of the Liver contain at least one DAA<sup>[14]</sup>. The current DAAs are the NS5B polymerase inhibitor, sofosbuvir, the NS3/4A protease inhibitor simeprevir and the NS5A-replication-inhibitors daclatasvir and ledispavir or the so-called 3D regimen containing the dual NS3/4A protease inhibitors Paritaprevir/Ritonavir, the NS5A inhibitor Ombitasvir and the NS5B palm polymerase inhibitor Dasabuvir. This has yielded the possibility for treating CHC patients with interferon free, all-oral regimens, with high SVR-rates and fewer adverse events<sup>[14-18]</sup>. Despite of these great advantages, the cost of DAAs will without doubt substantially delay their introduction as standard treatment in low and middle-income countries by years to come. Moreover, even in high income countries, treatment with DAA therapy is reserved for patients with advanced liver disease, despite the fact that a majority of patients are expected to benefit from the treatment. Therefore,

peg-IFN/RBV treatment still has a role to play in treatment of patients with CHC, and the need for markers that can predict successful treatment outcomes to peg-INF- $\alpha$ /RBV are still needed.

Several studies have independently shown an association between virological response and baseline IP-10 concentrations for CHC patients infected with genotype 1 and 4<sup>[19-21]</sup>. However, the association seems to be lacking for CHC patients, infected with HCV genotype 2 and 3<sup>[21,22]</sup>. Despite this being the case, a systematic review to address and clarify the differences in IP-10 properties, in relation to the different HCV genotypes, is missing. The aim of this systematic review was therefore to examine IP-10's ability to predict RVR and SVR in patients with CHC genotypes 1-4 treated with peg-IFN/RBV. We succeeded in doing so, with data presented in the following.

## MATERIALS AND METHODS

On initiation of this review a protocol was made and registered at the International Prospective Register of Systematic Reviews (PROSPERO) - registration number: CRD42014008736. Protocol can be found at <https://www.crd.york.ac.uk/PROSPERO/>.

### Literature search

Using the search profiles listed in the Appendix I in the supporting information, suitable literature was identified in MEDLINE and EMBASE. The first article sorting was performed by rating the article headlines, while the second sorting was performed on abstract level. Papers passing both sorting rounds were considered for the review, and thoroughly scrutinized based on pre-defined inclusion and exclusion criteria as listed below. The initial search provided 81 articles; 34 in MEDLINE and 47 in EMBASE. After the first- and second-sorting, 14 articles remained from MEDLINE and 14 articles from EMBASE of which 10 were duplicates. One article was found by manual searching the references, bringing the total number of articles after the third sorting to 19. During the third sorting, 11 articles were excluded<sup>[6,8,22-30]</sup>. This left 8 studies for inclusion<sup>[7,9,19,21,31-34]</sup>. Overview of the entire sorting process is shown in Figure 1.

### Inclusion criteria

Case series examining RVR or SVR in relation to 24 or 48 wk treatment with either 180  $\mu$ g Peg-IFN- $\alpha$ -2a weekly or peg-INF- $\alpha$ -2b 1.5  $\mu$ g/kg per week in combination with ribavirin 15 mg/kg per day (minimum 1000 mg daily and maximum 1400 mg daily) or fixed doses of 1000 mg for patients < 75 kg and 1200 mg for patients > 75 kg or flat dosing of 800 mg daily, in CHC patients infected with HCV, genotypes 1 or 4, treatment free for at least six months prior to inclusion, with both a baseline IP-10 level- and HCV-RNA determination, as well as a HCV-RNA determination four weeks after treatment initiation to assess RVR and/or 24 wk after end of treatment to assess SVR.

Case series studies examining RVR or SVR, in rela-

tion to 24 wk treatment with either 180  $\mu$ g Peg-IFN- $\alpha$ -2a per week or peg-INF- $\alpha$ -2b 1.5  $\mu$ g/kg per week in combination with ribavirin 800 mg daily or fixed doses of 1000 mg for patients < 75 kg and 1200 mg for patients > 75 kg, in CHC patients infected with HCV, genotypes 2 or 3, treatment free for at least six months prior to inclusion, with both a baseline IP-10 level- and HCV-RNA determination 4 wk after treatment initiation to assess RVR and/or 24 wk after end of treatment to assess SVR.

### Exclusion criteria

Liver diseases other than CHC, Co-infection with human immunodeficiency virus (HIV), co-infection with hepatitis B virus (HBV), alcohol dependency (regular intake of  $\geq$  75 g/d), active intravenous drug-use, treatment with immunosuppressants or cytostatica and prior treatment for CHC within the last 6 mo.

### Quality assessment

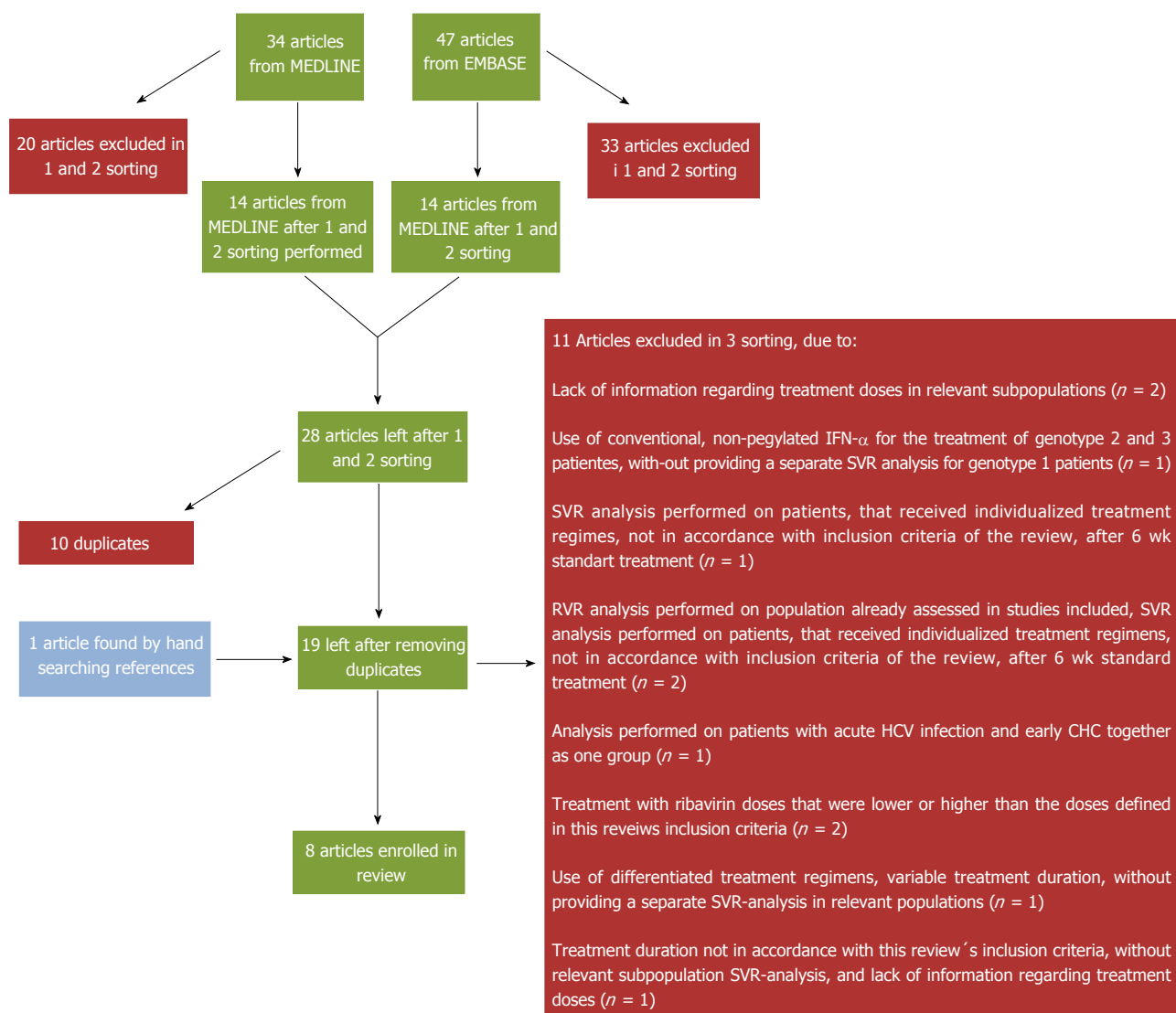
The quality of the 8 included articles were appraised using an 18 point checklist for case series, developed using a modified Delphi technique<sup>[35]</sup>. Each criterion can be answered with "yes", "no" or "partially reported/unclear", with the 18 criteria being weighted equally. In line with a pilot study conducted testing the assessment tool, we choose to rate studies with 14 or more "yes responses" as "high-quality studies", and studies with 13 or less "yes responses" as "low-quality studies". No studies were excluded on the basis of the criteria scores. The full checklist can be found in the Appendix II in the supporting information. Table 1 shows the sum score of the checklist. The baseline demographics regarded as important for the appraisal of the studies were: Number of patients included, patient ethnicity, patient age, male/female ratio, HCV RNA, liver enzyme level [alanine transaminase (ALT) or aspartate transaminase (AST)], body mass index (BMI), genotype, liver fibrosis stage and distribution on interleukin 28B (IL28B) single nucleotide polymorphism (SNPs).

## RESULTS

### Patient baseline demographic

All information was extracted from the articles, no raw data was requisitioned. Overall, presentation of baseline demographic data was missing in one study. Instead, this study provided baseline demographics in the following subpopulations: IL28 *rs12979860* (CC, CT, TT), *rs12980275* (AA, AG, GG), *rs8099917* (TT, TG, GG)<sup>[34]</sup>. Only baseline characteristics for *rs12979860* are reported in the review, as these were representative for the study population. All studies provided baseline information on total number of patients included, gender and age. Four studies failed to provide BMI<sup>[7,9,19,31]</sup>, and four studies did not supply exact information regarding patient ethnicity<sup>[7,9,19,32]</sup>. Information regarding number of patients included, ethnicity, age, male/female ratio, and BMI is reported in Table 2. ALT or AST values were not reported by two studies<sup>[33,34]</sup>, one of these however stated that all patients included had two





**Figure 1 Flow chart depicting the sorting of articles.** The chart depicts the number of articles found by searching the MEDLINE and EMBASE databases 04.15.2014, the number of articles excluded during the first and second sorting, the number of duplicates, the number of articles found by manual searching references, and the number of articles excluded in the third sorting, with indication of the reason for exclusion. Articles progressing down the chart from the original search to final inclusion are marked with green boxes, articles found by manual search are marked with blue boxes, and articles excluded are marked with red boxes. SVR: Sustained virological responses; RVR: Rapid virological responses; CHC: Chronic hepatitis C; HCV: Hepatitis C virus.

serum ALT values above the upper limit of normal within 6 mo of treatment initiation<sup>[34]</sup>. Effect on liver parenchyma and HCV-RNA load are shown in Table 3. Regarding fibrosis stage, four studies used the Ishak score<sup>[7,31,33,34]</sup>, two studies used the Scheuer score<sup>[9,32]</sup>, and two studies used the Metavir staging system<sup>[19,21]</sup>. Overview of genotype and fibrosis stage is presented in Table 4. Information regarding treatment regimens can be found in Table 5. Four studies provided information on IL28B SNP distribution. An overview of SNPs can be seen in Table 6.

### Rapid virological response

An overview is presented in Table 7. Lagging *et al.*<sup>[34]</sup> (2011) examined IP-10's ability to predict virological response and treatment outcome in 170 patients with genotype 1, from the DITTO-HCV study group. After six weeks, patients were randomized to individualized treatment,

or continued on the standard combination therapy as no sub analysis on SVR for patient receiving therapy, corresponding with the review's inclusion criteria for the course of 24-48 wk, was provided. Only results regarding RVR are featured in the review. The study found that patients obtaining RVR had significantly lower median baseline IP-10 levels than patients without a RVR. These findings were similar to results reported by Fattovich *et al.*<sup>[21]</sup> that patients infected with HCV, genotype 1, who achieved RVR, had a significant lower mean baseline IP-10, than those who did not. However, this association was not seen for patients infected with HCV genotype 2 or 3. The study also enrolled genotype 4 infected patients, but due to insufficient numbers ( $n = 15$ ), these were excluded. Al-Ashgar *et al.*<sup>[19]</sup>, 2013 studied the relationship between IP-10 and virological response in patients infected with genotype 4, and showed a trend

**Table 1 Overview of the modified Delphi, 18-point quality assessment checklist for studies included in the review**

Ref.	Yes response	No response	Partiel reported/unclear	Assessment
Fattovich <i>et al</i> <sup>[21]</sup>	16	2	0	High-quality
Diago <i>et al</i> <sup>[7]</sup>	15	2	1	High-quality
Apolinario <i>et al</i> <sup>[9]</sup>	14	2	2	High-quality
Darling <i>et al</i> <sup>[31]</sup>	14	1	3	High-quality
Lagging <i>et al</i> <sup>[34]</sup>	14	3	1	High-quality
Al-Ashgar <sup>[19]</sup>	13	3	2	Low-quality
Derbala <i>et al</i> <sup>[32]</sup>	13	2	3	Low-quality
Kurelac <i>et al</i> <sup>[33]</sup>	12	3	3	Low-quality

Studies was ranked as high quality if they provided  $\geq 14$  "yes" answers, or low quality if they provided  $\leq 13$  "yes" answers.

towards lower mean baseline IP-10 in patients with RVR, than in those without, though the association was not significant.

### SVR

An overview is presented in Table 8. Following the pattern regarding RVR, all five studies examining SVR in relation to baseline IP-10 levels for HCV genotype 1 infected patients, showed a significant association.

Apolinario *et al*<sup>[9]</sup> enrolled 63 Spanish patients from clinical trials and out patient clinics. Forty-three patients had genotype 1, while 20 had a non-1 genotype. Among the 43 HCV genotype 1 infected patients, mean baseline IP-10 levels were significantly lower in patients who reached a SVR compared to those who did not. Because some of the genotype non-1 infected patients received 48 wk of therapy, the results for these are not provided in Table 7. Diago *et al*<sup>[7]</sup> also found a significant association between mean baseline IP-10 and SVR for their overall population of Spanish patients. An association that remained significant, when the analysis was restricted to HCV genotype 1 infected patients. The same significant association between lower baseline IP-10 and SVR for HCV genotype 1 infected, were reported for Italian, Croatian and American patients<sup>[21,31,33]</sup>. An interesting aspect of the study by Kurelac *et al*<sup>[33]</sup>, 2012 was, that the greatest difference in IP-10, between patients with a SVR vs non-SVR, was seen at treatment week 4, where median IP-10 levels were 185 pg/mL (63-518) and 424 pg/mL (90-815) ( $P < 0.0001$ ), respectively. Darling *et al*<sup>[31]</sup> noted, that the significant association between baseline IP-10 levels and SVR remained when patients were grouped as Caucasian Americans (CA) or African Americans (AA) ( $447 \pm 44$  pg/mL vs  $677 \pm 69$  pg/mL,  $P < 0.001$  and  $418$  pg/mL  $\pm 35$  vs  $716$  pg/mL  $\pm 55$ ,  $P < 0.001$ , respectively). Fattovich *et al*<sup>[21]</sup> were the only ones that reported on HCV genotype 2 and 3 patients. As with the results regarding RVR, no association was found between SVR and baseline IP-10. Derbala *et al*<sup>[32]</sup> and Al-Ashgar *et al*<sup>[19]</sup> studied HCV genotype 4 infected Egyptian and Saudi patients, respectively, and showed significantly higher values of baseline IP-10 in non-SVRs

than in SVRs. Interestingly, a sub analysis, performed by Al-Ashgar *et al*<sup>[19]</sup> on genotype 4a and 4d, showed that this correlation was present for genotype 4d ( $465.9$  pg/mL  $\pm 349.1$  vs  $904.9$  pg/mL  $\pm 532.1$ ,  $P < 0.001$ ), but not for genotype 4a ( $564.7$  pg/mL  $\pm 288.9$  vs  $568$  pg/mL  $\pm 384.9$ ,  $P = 0.300$ ). Derbala *et al*<sup>[32]</sup> failed to provide information on the exact levels of IP-10, and instead provided a graphic depiction, which could not be interpreted to adequate results.

### DISCUSSION

Several studies have independently shown levels of IP-10 to be associated with both RVR and SVR to peg-IFN/RBV treatment for CHC patients infected with HCV, genotype 1 and 4, but not for genotype 2 and 3. We conducted this systematic review to assess variation in IP-10's predictive ability for RVR and SVR to peg-INF/RBV treatment in patients chronically infected with HCV genotypes 1-4.

Our main findings indicate that a correlation exist between baseline IP-10 and SVR- and in part for RVR - for genotype 1 and possibly for genotype 4, however not for genotype 2 or 3.

Three studies provided information on baseline IP-10 in relation to RVR<sup>[19,21,34]</sup>. Studies reporting on HCV, genotype 1 infected patients, found significant lower baseline IP-10 values in patients achieving RVR compared to those who did not<sup>[21,34]</sup>. Only a trend, failing to reach significance, was described between baseline IP-10 and RVR in genotype 4 infected patients<sup>[19]</sup> and no significant relation was found in relation to genotype 2 or -3<sup>[21]</sup>. Seven studies provided information on baseline IP-10 in relation to SVR<sup>[7,9,19,21,31-33]</sup>. All five studies reporting on HCV genotype 1 infected patients<sup>[7,9,21,31,33]</sup> found significantly lower IP-10 levels of SVR than non-SVR. Diago *et al*<sup>[7]</sup> did not provide separate results for the group of genotype non-1 patients included, which was surprising, as three quarters of the patients were infected with HCV, genotype 1, and could very well be the reason for finding a significant association in the overall population, when all genotypes were analyzed together. In line with this, Apolinario *et al*<sup>[9]</sup> stated that no associations was found for their genotype non-1 group. However lacking differentiation into sub genotypes, compromise the value of information, especially as no association were found for HCV, genotype 2 or 3 infected<sup>[21]</sup>, and both studies reporting on genotype 4 infected patients<sup>[19,32]</sup> found significant lower baseline IP-10 level in their populations, when comparing patients achieving SVR vs non-SVR. It should be noted, that while Fattovich *et al*<sup>[21]</sup> considered two-sided  $P$ -values  $< 0.05$  as statistical significant, only results of statistical tests with a  $P$ -value  $< 0.01$  were considered of interest, because of the multiple comparisons between subjects with and without SVR. Therefore IP-10 was not considered to be associated with SVR, for HCV genotype 3 infected individuals, even though the  $p$ -value was found to be 0.02.

One study observed that the greatest difference in IP-10 levels was found at week 4. Patients, who at this

**Table 2** Baseline total patient number, number of male patients, mean age, body mass index and ethnicity for the 8 included studies

Ref.	Patients		Mean age (yr)	BMI (kg/m <sup>2</sup> )	Ethnicity		
	Males	Total (n)			Caucasian	African American	Asian
Apolinario <i>et al</i> <sup>[9]</sup>	40	63	41 (± 9.3) <sup>5</sup>	Information not provided	Information not provided <sup>6</sup>		
<sup>1</sup> Lagging <i>et al</i> <sup>[34]</sup>	169	252 <sup>2</sup>			252 <sup>9</sup>		
IL28B rs12979860 CC	64	93	41.6 (± 10.1) <sup>5</sup>	25.1 (± 3.6) <sup>5</sup>			
IL28B rs12979860 CT	77	123	41.9 (± 9.5) <sup>5</sup>	25.0 (± 3.5) <sup>5</sup>			
IL28B rs12979860 TT	28	36	41.9 (± 11.4) <sup>5</sup>	25.0 (± 3.5) <sup>5</sup>			
Diago <i>et al</i> <sup>[7]</sup>	77	137	42 (± 9.7) <sup>5</sup>	Information not provided	Information not provided		
Fattovich <i>et al</i> <sup>[21]</sup>	133	226 <sup>3</sup>	46 (± 11) <sup>5</sup>	24.7 (± 3.8) <sup>5</sup>	226		
Kurelac <i>et al</i> <sup>[33]</sup>	17	46	41.5 (± 12.4) <sup>5</sup>	23.7 (21.9-25) <sup>4</sup>	46		
Darling <i>et al</i> <sup>[31]</sup>	176	272	48.4 (± 7.4) <sup>5</sup>	Information not provided	138	134	
Darbala <i>et al</i> <sup>[32]</sup>	144	159	46.47 (± 8.83) <sup>5</sup>	30.18 (± 5.05) <sup>5</sup>	Information not provided <sup>7</sup>		
Al-Ashgar <i>et al</i> <sup>[19]</sup>	41	64	38.7 (± 11.5) <sup>5</sup>	Information not provided	Information not provided <sup>8</sup>		

<sup>1</sup>Study did not provide baseline characteristics for their entire population, but instead provided baseline demographics in accordance with IL-28 genotype (only baseline characteristics for rs12979860 are reported in the review, as these were representative for the study population); <sup>2</sup>253 was reported to be enrolled, however when adding the males and female patients, it sums to 252; <sup>3</sup>Only 226 out of 280 patients had serum available for iP-10 testing; <sup>4</sup>Median (25-75 percentiles); <sup>5</sup>Mean (SD); <sup>6</sup>51 patients from clinical trials had Spanish nationality; <sup>7</sup>Egyptian nationality; <sup>8</sup>Saudi nationality; <sup>9</sup>95% of the original DITTO patient population were Caucasian. BMI: Body mass index.

**Table 3** Hepatitis C virus-RNA and patient liver enzyme status for the 8 included studies

Ref.	HCV-RNA				Liver enzyme level	
	High viral load		Low viral load		AST	ALT
	<i>n</i>	Limit	<i>n</i>	Limit		
Apolinario <i>et al</i> <sup>[9]</sup>	28	≥ 6.3 log IU/mL <sup>5</sup>	35	< 6.3 log IU/mL <sup>5</sup>		118 IU/L (± 64) \$
<sup>1</sup> Lagging <i>et al</i> <sup>[34]</sup>						
IL28B <i>rs12979860 CC</i>					6.3 log IU/mL (± 0.8) <sup>3</sup>	Information not provided
IL28B <i>rs12979860 CT</i>					6.1 log IU/mL (± 0.7) <sup>3</sup>	
IL28B <i>rs12979860 TT</i>					5.9 log IU/mL (± 0.8) <sup>3</sup>	
Diago <i>et al</i> <sup>[7]</sup>	85	≥ 5.7 log IU/mL <sup>5</sup>	52	< 5.7 log IU/mL <sup>5</sup>		117.2 IU/L (± 81.6) <sup>3</sup>
Fattovich <i>et al</i> <sup>[21]</sup>	147	≥ 5.6 log IU/mL <sup>5</sup>			5.74 log IU/mL (± 0.9) <sup>3,5</sup>	92 IU/L (± 78) <sup>3</sup>
Kurelac <i>et al</i> <sup>[33]</sup>					5.55 log IU/mL (5.52-6.1) <sup>2,5</sup>	Information not provided
Darling <i>et al</i> <sup>[31]</sup>					6.66 log IU/mL (± 6.76) <sup>3,5</sup>	
Darbala <i>et al</i> <sup>[32]</sup>					4.95 log IU/mL (3.6-5.63) <sup>4,5</sup>	38 IU/L (27-51) <sup>4</sup>
Al-Ashgar <i>et al</i> <sup>[19]</sup>	45	≥ 5.78 log IU/mL	19	< 5.78 log IU/mL <sup>5</sup>	67.5 IU/L (43.5-106.8) <sup>2</sup>	51 IU/L (34-87) <sup>4</sup>
						56.0 IU/L (32.0-86.0) <sup>2</sup>

<sup>1</sup>Lagging *et al* provided baseline demographics in accordance with IL-28 genotype (only baseline characteristics for rs12979860 are reported in the review, as these were representative for the study population); <sup>2</sup>Median (25-75 percentiles); <sup>3</sup>Mean (SD); <sup>4</sup>Median (IQR); <sup>5</sup>Recalculated into log IU/mL. HCV-RNA is shown as number of patients with high or low viral load or as the mean or median for the entire population. Depending of the presentation in the original article, levels of ALT, AST or both are shown. HCV: Hepatitis C virus; ALT: Alanine transaminase; AST: Aspartate transaminase.

point had IP-10 levels higher than 250 pg/mL, had a 40-fold risk of not reaching SVR compared to patients with IP-10 levels lower than 250 pg/mL<sup>[33]</sup>. This might indicate, that IP-10 levels at treatment week 4, could be used to assess if peg-INF/RBV treatment should be discontinued or not in genotype 1 patients - and perhaps could also be used to evaluate the need for adjacent DAA treatment (*i.e.*, using a 4 wk lead in phase with peg-INF/RBV treatment before apprising the need for DAAs). However, the small number of patients participating calls for caution when interpreting these results, and further studies of IP-10 levels at treatment week 4 should be encouraged.

One study<sup>[31]</sup> showed that the correlation between baseline IP-10 and SVR remained significant even when the population was grouped according to ethnicity ( $P < 0.001$ ). The latter is interesting as AA ethnicity is otherwise considered an unfavorable prognostic factor for obtaining SVR<sup>[36-38]</sup>, and might imply that IP-10 could help aid the

decision as to whom would have the greatest potential benefit from peg-INF/RBV treatment regardless of ethnicity. In this context it is interesting that it has previously been shown that HCV infected AA had higher IP-10 levels than corresponding CA patients, while uninfected AA had IP-10 levels similar to uninfected CA<sup>[28]</sup>. The effect of race on Interferon Stimulated Genes, once at the stage of CHC, should therefore be examined further.

Findings, regarding SVR for HCV genotype 2 and 3, followed the same pattern as the results for RVR with no association between baseline IP-10 and SVR present for genotype 2 or 3<sup>[21]</sup>. Supporting our findings, this lacking correlation in patients with HCV genotype 2 and 3, has also been shown when treating patients with standard and low (90 µg once weekly) peg-INF/RBV regimens<sup>[22]</sup>.

As mentioned, a significant correlation between IP-10 and SVR was reported by both studies, including HCV genotype 4 infected patients<sup>[19,32]</sup>. One of these<sup>[19]</sup> also performed differentiated analyses on HCV genotype 4

**Table 4 Genotype and liver fibrosis stage for the 8 included studies**

Ref.	Genotype (n)				Method	Liver fibrosis stage						
	1	2	3	4		0	1	2	3	4	5	6
Apolinario <i>et al</i> <sup>[9]</sup>	43	20			Scheuer score	28		35				
Lagging <i>et al</i> <sup>[34]</sup>	170 <sup>1</sup>	23 <sup>1</sup>	49 <sup>1</sup>	11 <sup>1</sup>	Ishak score	11	61	65	30	15	20	14
IL28B <i>rs12979860</i> CC	44	13	33	3		3	18	27	11	5	12	5
IL28B <i>rs12979860</i> CT	96	7	15	5		7	35	27	17	7	6	6
IL28B <i>rs12979860</i> TT	30	3	1	3		1	8	11	2	3	2	3
Diago <i>et al</i> <sup>[7]</sup>	103	9	25		Ishak score	106				31		
Fattovich <i>et al</i> <sup>[21]</sup>	92	87	47		Metavir <sup>2</sup>	121			21			
Kurelac <i>et al</i> <sup>[33]</sup>	46				Ishak		34			12		
Darling <i>et al</i> <sup>[31]</sup>	272				Ishak	220				52		
Darbala <i>et al</i> <sup>[32]</sup>				159	Scheuer score		109		50			
Ashgar <i>et al</i> <sup>[19]</sup>				64	Metavir	34 <sup>3</sup>			10 <sup>3</sup>			

<sup>1</sup>Baseline information for the sub analysis of IL28 12979860. Two hundred and fifty-two patients are reported to be enrolled, however adding the genotypes yields 253 patients. Likewise biopsies from 228 patients are described, however when adding the Ishak scores only yields 216 patients; <sup>2</sup>Biopsies only available for 142 patients; <sup>3</sup>Histology available for 44 patients; <sup>4</sup>Lagging *et al* provided baseline demographics in accordance with IL-28 genotype (only baseline characteristics for rs12979860 are reported in the review, as these were representative for the study population). A box stretching over two - or more genotypes or fibrosis stage, indicates that the number refers to the combined group.

**Table 5 Overview of the treatment regimens for pegylated interferon in combination with ribavirin, for the 8 studies included, in relation to dose and duration**

Ref.	Genotype		Duration	Interferon treatment	Ribavirin treatment
Apolinario <i>et al</i> <sup>[9]</sup>	Multi-centerpatients	1	48 wk	180 µg peg-INF-α-2a once weekly	800 mg per day or 1000 mg < 75 kg, 1200 mg > 75 kg
		Non-1	24-48 wk		
	Out patiens	1	48 wk	peg-INF-α2b 1.5 µg/kg per week	1000-1200 mg per day
		Non-1	24 wk		
Lagging <i>et al</i> <sup>[34]</sup>	1		6 wk <sup>1</sup>	180 µg peg-INF-α-2a once weekly	1000 mg < 75 kg, 1200 mg > 75 kg per day
Diago <i>et al</i> <sup>[7]</sup>	1		48 wk	peg-INF-α-2b 1.5 µg/kg per week or 180 µg peg-INF-α-2a/week	1000 mg < 75 kg, 1200 mg > 75 kg per day
	Non-1		24 wk		
Fattovich <i>et al</i> <sup>[21]</sup>	1 and 4		48 wk	peg-INF-α-2b 1.5 µg/kg per week or 180 µg peg-INF-α-2a/week	800-1200 mg per day
	2 and 3		24 wk		
Kurelac <i>et al</i> <sup>[33]</sup>	1		48 wk	peg-INF-α-2b 1.5 µg/kg per week	Weight based ribavirin treatment <sup>2</sup>
Darling <i>et al</i> <sup>[31]</sup>	1		48 wk	180 µg peg-INF-α-2a once weekly	1000-1200 mg per day
Derbala <i>et al</i> <sup>[32]</sup>	4		48 wk	Peg-IFN once weekly <sup>3</sup>	1000 mg < 75 kg, 1200 mg > 75 kg per day
Al-Ashgar <sup>[19]</sup>	4		48 wk	180 µg peg-INF-α-2a once weekly	1000 mg < 75 kg, 1200 mg > 75 kg

<sup>1</sup>After 6 wk, patients were randomized to differentiated treatment regimes; <sup>2</sup>No further information on ribavirin treatment was provided; <sup>3</sup>No further information on the subtype of peg-IFN was provided. Apolinario *et al*<sup>[9]</sup> feature patients from both an outpatient clinic as well as patients from two multicenter trials receiving different treatment regimens, illustrated by the segregation in the genotype column. peg-IFN: Pegylated interferon.

subtypes, 4a and 4d, showing a significant association only for the latter ( $P = 0.330$  and  $P < 0.001$ , respectively). It would have been interesting to examine if this was also the case for RVR, as it could be speculated that the association between baseline IP-10 and RVR in HCV genotype 4 infected patients failed to show significance, because both subtype 4a and 4d were analyzed as a whole. Therefore, subsequent studies making RVR and SVR assessments should be encouraged to perform differential analysis on individual viral subtypes, in order to uncover more specific associations. The setup for this study, did not allow us to investigate, what specific mechanisms account for the differences in correlation between baseline IP-10 and HCV genotype 1 and 4 compared with HCV genotype 2 and 3. However it is of great interest that these differences occur, and should be investigated further. Inversely patients infected with HCV genotype 2 or 3 generally has a more favorable response to treatment with PEG-IFN and RBV. Therefore, in a clinical setting the underlying mechanism

might not be relevant, as genotype 2 and 3 patients would readily be treated, whereas clinicians might be more reluctant to initiate peg-INF treatment to genotype 1 and 4 - infected individuals and here IP-10 levels might help to show which patients should undergo treatment.

This review focused on the association between pre-treatment IP-10 levels and virological responses. However, IL28B SNPs should be addressed when considering IP-10, as they are strongly linked with treatment response to Peg-INF/ RBV<sup>[39-45]</sup>. Especially are homozygote genotypes at markers *rs8099917* (TT), *rs12979860* (CC) and *rs12980275* (AA) associated with a favorable outcome to treatment. While IL28B polymorphisms were not found to be predictive for treatment response in HCV genotype 2 and 3 infected individuals by Fattovich *et al*<sup>[21]</sup>, pretreatment IL28B polymorphisms, HCV-RNA- and IP-10 levels independently predict RVR in HCV genotype 1 infected individuals, with RVR in turn being the strongest predictor of SVR. Combining the IL28B polymorphisms and HCV-



**Table 6** Overview of the marker distribution, in the four studied that supplied information on interleukin 28B single nucleotide polymorphism

Ref.	Genotype (n)	rs12979860			rs12980275			rs8099917			rs11881222		
		CC	CT	TT	AA	AG	GG	TT	TG	GG	AA	AG	GG
Lagging <i>et al</i> <sup>[30]</sup>	1 (253)	93	123	37	101	115	37	153	90	10			
Fattovich <i>et al</i> <sup>[21]</sup>	1 (92)	33	44	15	33	45	14	49	38	5			
	2 (87)	34	43	10	34	42	11	47	34	6			
	3 (47)	25	21	1	25	20	2	34	13	0			
Darling <i>et al</i> <sup>[31]</sup>	1 (201)	63	103	44									
Derbala <i>et al</i> <sup>[32]</sup>	4 (159)	57	77	25				96	55	8	64	75	20

Genotype column indicates specific genotype, and total number of patients with the specific genotype. Each marker column is divided into allelic distribution for the IL28B SNP genotype. SNP: Single nucleotide polymorphism.

**Table 7** Overview of rapid virological response in the 3 studies providing information on baseline inducible protein-10's, and hepatitis C virus RNA levels at week 4

Ref.	Patients (n)	IP-10 measurement method	Genotype (n)	Baseline IP-10 concentration, grouped by rapid virological response (pg/mL)			Overall RVR (n)	
				RVR	Non-RVR	P-value	RVR	Non-RVR
Lagging <i>et al</i> <sup>[34]</sup>	170	ELISA (Quantikine, R and D systems, Minneapolis, MN, United States)	1 (170)	222	401	$P < 0.01$ (median)	33	137
<sup>1</sup> Fattovich <i>et al</i> <sup>[21]</sup>	226	ELISA (Quantikine, R and D systems, Minneapolis, MN, United States)	1 (92)	2.4 ( $\pm 0.28$ )	2.6 ( $\pm 0.25$ )	$P < 0.01$ (log mean $\pm$ SD)	172	108
			2 (87)	2.38 ( $\pm 0.31$ )	2.3 ( $\pm 0.30$ )	$P > 0.05$ (log mean $\pm$ SD)		
			3 (47)	2.45 ( $\pm 0.23$ )	2.48 ( $\pm 0.39$ )	$P > 0.05$ (log mean $\pm$ SD)		
Al-Ashgar <i>et al</i> <sup>[19]</sup>	64	ELISA (Quantikine, R and D systems, Minneapolis, MN, United States)	4 (64)	483.9 ( $\pm 261.6$ )	609.9 ( $\pm 424.3$ )	$P > 0.05$ (mean $\pm$ SD)	12	52

<sup>1</sup>Entire patient population was 280, genotype 4 infected were removed from the analyses, and IP-10 results was available for 226 patients. IP-10: Inducible protein-10; RVR: Rapid virological response.

RNA yielded a specificity of 98% but a low sensitivity of 39%. By including IP-10 values in the equation, the sensitivity and the negative predictive value was raised from 81% to 94%, however lowering the positive predictive value from 87% to 76%. This is consistent with other findings in HCV genotype 1 infected, homozygous carriers of the favorable IL28B SNPs, with low IP-10 level, which also significantly predicted a first phase decline of HCV RNA, which translated into increased rates of RVR and SVR<sup>[30]</sup>. While the two latter studies was carried out solely on Caucasian patients infected with HCV genotype 1, the additive predictive effect has also been shown for both HCV genotype 1 infected AA and CA patients<sup>[31]</sup>, and HCV genotype 4 infected patients<sup>[32]</sup>, respectively. Although low in numbers, these results could indicate, that both variables should be considered in a clinical context, before initiating treatment with Peg-INF/RBV in patients infected with HCV genotype 1 or 4. Further studies examining the association in HCV genotype 2 and 3 infected patients should be encouraged.

Conducting a systematic review with clear and stringent in- and exclusions criteria, is an obvious strength of this study, ensuring homogeneity between the studies included, hereby allowing an unbiased assessment of the current evidence. Another strength of our study was that we assessed the quality of the studies included, and provided a detailed declaration of the studies aim,

method - including treatment regimens and duration, as well as baseline patient demographics for the individual studies - supplying a solid ground for interpreting the results put forth. Although some authors recommend the use of quality assessments, other consider them misleading<sup>[46]</sup>, and there remains uncertainties about the relationship between methodology, validity and the use of sum scores to judge the quality of studies<sup>[47]</sup>. Therefore we chose not to exclude any articles based on their quality score (e.g., high quality or low quality), but instead presented the ratings of the studies in the review to serve as an objective guide to interpret the review's results, rather than a tool for selecting studies for the review. As seen by the exclusion criteria, we wished to eliminate the possible uncertainties that could arise by including studies treating HIV/HCV - or HBV/HCV co-infected patients. Therefore, it should be mentioned that even though there was no indication towards inclusion of co-infected patients, three of the included studies, based in the United States, Croatia and Egypt, contained no clear exclusion criteria for HIV- or HBV- infection<sup>[31-33]</sup>.

Only a limited number of articles fulfilled the in- and exclusion criteria to be assessed in this review. Hence, more work is needed to establish a sufficient ground for final conclusions to be made. Further, there was an overweight of studies that addressed the association between SVR and baseline IP-10 in CHC patients infected

**Table 8 Overview of sustained viral response in the 8 studies providing information on baseline inducible protein-10's, and hepatitis C virus-RNA levels 24 wk after end-of- treatment**

Ref.	Patients (n)	IP-10 measurement method	Genotype (n)	Baseline IP-10 concentration, grouped by sustained virological response (pg/mL)			Overall SVR	
				SVR	Non-SVR	P-value	SVR	Non-SVR
Apolinario <i>et al</i> <sup>[9]</sup>	63	ELISA (OptEIA, Pharmingen, San Diego, CA, United States)	1 (43)	245 (± 154)	381 (± 138)	$P < 0.05$ (mean ± SD)	36	27
Diago <i>et al</i> <sup>[7]</sup>	137	ELISA (Human immunoassay kit; BioSource Europe SA, Nivelles, Belgium)	1 (103)	347 (± 197.4)	500.6 (± 311.2)	$P < 0.01$ (mean ± SD)	79 <sup>2</sup>	58 <sup>2</sup>
			1 (103)	332.4 (± 222.1)	476.8 (± 305.3)	$P < 0.01$ (mean ± SD)		
			2 (9)					
<sup>1</sup> Fattovich <i>et al</i> <sup>[21]</sup>	226	ELISA (Quantikine, R and D systems, Minneapolis, MN, United States )	1 (92)	2.47 ± 0.23	2.65 ± 0.28	$P < 0.001$ (log mean ± SD)	209 <sup>2</sup>	71 <sup>2</sup>
			2 (87)	2.37 ± 0.31	2.33 ± 0.35	$P > 0.05$ (log mean ± SD)		
			3 (47)	2.42 ± 0.21	2.67 ± 0.46	$P < 0.05^a$ (log mean ± SD)		
Kurelac <i>et al</i> <sup>[33]</sup>	46	ELISA (Quantikine, R and D systems, Minneapolis, MN, United States )	1 (46)	185 (63-518)	395.5 (111-926)	$P < 0.0001$ (median, range)	26	20
Darling <i>et al</i> <sup>[31]</sup>	272	ELISA (Quantikine, R and D systems, Minneapolis, MN, United States )	1 (272)	437 (± 31)	704 (± 44)	$P < 0.001$ (mean ± SD)	157	115
Derbala <i>et al</i> <sup>[32]</sup>	159	Luminex, Cytokine multiplex immunoassay kit (Merck Millipore, Billerica, MA, United States)	4 (159)	Exact data not provided, only graphic presentation		$P < 0.001$ (median, IQR)	98	61
Al-Ashgar <i>et al</i> <sup>[19]</sup>	64	ELISA (Quantikine, R and D systems, Minneapolis, MN, United States )	4 (64)	462 (± 282.6)	840.4 (± 490.6)	$P < 0.01$ (mean ± SD)	41	23

<sup>1</sup>Entire patina population was 280, genotype 4 removed from the analyses, and IP-10 results was available for 226 patients Note that M. Derbala *et al* did not provide written specification on IP-10 levels for SVR compared to non-SVR, and only supplied a graphic depiction, which could not be interpreted to adequate results; <sup>2</sup>SVR for the entire population. <sup>a</sup> $P = 0.02$ . Only the results of statistical tests with a  $P$  value  $< 0.01$  were considered of interest, because of the multiple comparisons between subjects with and without SVR. SVR: Sustained viral response; IP-10: Inducible protein-10.

with HCV genotype 1, whereas there was only a small fraction addressing the association between SVR and baseline IP-10 for genotype 2, 3 and 4, as well as studies examining the relationship between RVR and baseline IP-10 constituting an insufficient base for assessing baseline IP-10's predictive ability in these regards.

In this systematic review, we found correlations between baseline IP-10 levels and SVR in patients chronically infected with HCV genotype 1 and 4, while no such association was found for patients infected with HCV genotype 2 or 3. Likewise, we found indications of a possible correlation between baseline IP-10 and RVR for HCV genotype 1 infected patients, while no such association were found for HCV genotype 2 or 3 patients, and only a trend was found for HCV genotype 4 infected patients. However, the amount of information regarding baseline RVR for genotypes 1-4, and SVR's relation with baseline IP-10 for genotypes 2, 3 and 4 were insufficient for final conclusions.

## COMMENTS

### Background

Until recently, the standard of care for chronic hepatitis C (CHC) patients was lengthy dual therapy with pegylated interferon plus ribavirin (peg-IFN/RBV), a treatment with modest success rates, severe adverse events and variation in treatment response between hepatitis C virus (HCV) genotypes. Therefore, efforts to identifying biomarkers that can predict virological responses to treatment have been made. Interferon- $\gamma$  inducible protein-10 (IP-10) is one such promising

marker, with several studies independently showing an association between virological response and baseline IP-10 concentrations for CHC patients infected with HCV genotype 1 and 4. However, the association seems to be lacking for CHC patients, infected with HCV genotype 2 and 3.

### Research frontiers

IP-10 has been shown to be expressed at higher levels in HCV genotype 1 infected CHC patients with moderate to severe fibrosis compared to patients with mild or non fibrosis. Therefore, studies are being made to examine if this correlation is also found in HCV genotype 2 and 3 infected CHC patients. In addition to this, examinations of baseline IP-10 ability to predict fibrosis progression in CHC patients are pending. IP-10 research in relation to CHC is therefore expanding from the possible correlation between virological response to treatment with peg-IFN/RBV at baseline, to also include fibrosis score at baseline and fibrosis progress over time.

### Innovations and breakthroughs

Despite the work done so far to correlate IP-10 levels to treatment response, this is to our knowledge, the first systematic review to address and clarify the differences in IP-10 properties, in relation to the different HCV genotypes and virological response. The authors found indications of correlations between baseline IP-10 levels and SVR in CHC patients infected with HCV genotype 1 and 4, but not in patients infected with HCV genotype 2 or 3. Likewise, the authors found indications of a possible correlation between baseline IP-10 and RVR for HCV genotype 1 infected patients, while no such association were found for HCV genotype 2 or 3 patients, and only a trend was found for HCV genotype 4 infected patients.

### Applications

Despite of the great advantages with the new treatment options with direct acting antivirals (DAA), the cost of DAAs will without doubt substantially delay their introduction as standard treatment in low and middle-income countries by

years to come. In addition, DAA in high-income countries is still reserved for patients with advanced liver disease. Therefore, peg-IFN/RBV treatment still has a role to play in treatment of patients with CHC. Their findings of a possible correlation between baseline IP-10 levels and SVR in CHC patients infected with HCV genotype 1 and 4 but not for genotypes 2 and 3 could be beneficial in a clinical setting. Genotype 2 and 3 patients would readily be treated, as these patients generally have a favorable outcome to peg-IFN/RBV compared to genotype 1 and 4 infected individuals. In such patients, IP-10 levels might help to show which patients would have the best prognosis for a positive outcome to treatment.

### Terminology

Interferon- $\gamma$  inducible protein-10, more commonly denoted IP-10 or CXCL10, is a non-ELR-CXC chemokine, binding to the CXC-receptor-3. It functions as a chemotactic, attracting T lymphocytes and NK cells to the site of inflammation. Within the liver, IP-10 mRNA is produced by hepatocytes in inflammatory areas, and both intrahepatic IP-10 mRNA - and plasma levels of IP-10 are elevated in individuals with CHC, indicating that intrahepatic IP-10 is the source of plasma IP-10. The hypothesis therefore is that IP-10 can function as proxy for the level of liver inflammation, which in turns lead to fibrosis formation.

### Peer-review

This is a well written and comprehensive systemic review to explore the association between baseline levels of interferon- $\gamma$ -inducible protein-10 and virological response to treatment with pegylated interferon and ribavirin among patients chronically infected with hepatitis C virus, genotype 1-4.

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## Coffee: The magical bean for liver diseases

Ryan D Heath, Mihir Brahmbhatt, Asli C Tahan, Jamal A Ibdah, Veysel Tahan

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risk of hepatocellular carcinoma, reduce advancement of fibrotic disease in a variety of chronic liver diseases, and perhaps reduce ability of hepatitis C virus to replicate. This review aims to catalog the evidence for coffee as universally beneficial across a spectrum of chronic liver diseases, as well as spotlight opportunities for future investigation into coffee and liver disease.

**Key words:** Coffee; Hepatocellular carcinoma; Liver; Hepatitis; Fatty liver

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**Core tip:** Coffee is one of the most popular beverages consumed in the United States, with about 75% of the population reporting consuming it. Coffee has also long been associated with hepatoprotective effects, the extent of which there appears to be an ever growing body of benefits as well as a wide variety of etiologies of chronic liver disease it may positively affect. This article reviews recent available literature and summarizes the potential positive or preventive effects of coffee on liver malignancy as well as chronic liver disease secondary to alcohol, viral hepatitis, and fatty infiltration. These studies collectively suggest a simple lifestyle modification patients may be able to incorporate to enhance their own health.

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### Abstract

Coffee has long been recognized as having hepatoprotective properties, however, the extent of any beneficial effect is still being elucidated. Coffee appears to reduce

### INTRODUCTION

With 1.4 billion kilograms of coffee consumed yearly

in the United States alone, coupled with 74.7% of the population being coffee drinkers some may call drinking coffee the national pastime<sup>[1,2]</sup>. Beyond the taste and stimulating effects, coffee has been associated with improved outcomes with chronic liver disease, hepatocellular cancer (HCC), cirrhosis, colorectal cancer, esophageal cancer, breast cancer, prostate cancer, pancreatic cancer, ovarian cancer, kidney cancer, hepatitis B virus (HBV), hepatitis C virus (HCV), and non-alcoholic fatty liver disease (NAFLD). A recent 2015 meta-analysis of 16 case-control and cohort studies of Western populations demonstrated significantly reduced incidence of cirrhosis amongst coffee drinkers when compared to those who did not drink the beverage<sup>[3]</sup>. As coffee continues to grow in popularity, with daily consumption of coffee-based beverages increasing from 19% to 41% in the 25-39 years old age group from 2008, the documented benefits of increased coffee intake have also grown<sup>[4,5]</sup>. Furthermore, coffee is generally considered to have a wide safety profile, with the American Food and Drug Administration noting caffeine as a substance generally recognized as safe, not known to be a health hazard<sup>[6]</sup>. Many countries' health agencies set no upper limit for daily caffeine intake; in 2006 Health Canada did set an upper limit of 450 mg per day as safe<sup>[6]</sup>. Over 30 million Americans have chronic liver disease and about 31000 deaths have been attributed to it yearly<sup>[7]</sup>. Studies evaluating coffee's potential hepatoprotective effect on liver disease are important as they may represent a simple lifestyle modification patients can incorporate to enhance their own health.

## COFFEE AND AN ASSOCIATION WITH DECREASED LIVER ENZYMES

In numerous studies, it has been noted that coffee consumption has been associated with decreased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), and alkaline phosphatase (ALP). One of the first studies to document consumption of coffee with relatively decreased GGT was in 1985 in the Tromsø Heart Study<sup>[8]</sup>. That same year, another study noted an inverse relationship between coffee consumption and AST and ALT levels amongst both Korean and Japanese immigrants<sup>[9]</sup>. These studies began an investigation into elucidating a more direct relationship between coffee and possible hepatoprotective properties. The Tromsø study looked at multiple beverages, notably including green tea. Since 1985 multiple other studies have been performed with similar findings when testing specifically for the possible effect of coffee consumption on liver disease.

One such study, performed in 1993, tested an Italian population of 2240 with findings indicating not only a

decrease in GGT but also ALT and ALP in drinkers of three or more cups of coffee daily when compared to groups that drinking less than this amount<sup>[10]</sup>. Another Japanese study in 1998 of 12687 participants with no history of liver disease or abnormal serum aminotransferases indicated significantly decreased levels of GGT, ALT, and AST in men; however, this finding was unable to replicate in women greater than 50 years of age in the study. Another noteworthy aspect of this study was the lack of similar effect on green tea, suggesting a specific role for coffee in liver disease.

A later 2000 Japanese study of 1353 men demonstrated lower GGT levels in coffee drinkers<sup>[11]</sup>. A follow-up study by this same group noted lower AST and ALT in Japanese men aged 35-56 years of age<sup>[12]</sup>, noting a decrease in these liver enzymes over a 4 year period with increased coffee consumption. A 1998 amongst Japanese men and women, excluding those with a history of chronically elevated liver enzymes or chronic liver disease, evaluated GGT levels amongst subgroups of alcohol drinkers, body mass index (BMI), and cigarette smoking, and green tea consumption<sup>[13]</sup>. While coffee consumption was not associated with significantly decreased GGT activity in male non-alcohol drinkers, the response was noted to be significant in male alcohol consumers. Results published in 2014 from the National Health and Nutrition Examination Survey utilized self-reported dietary logs, demonstrating individuals drinking > 3 cups of coffee daily demonstrated significantly lower levels of AST, ALT, ALP and GGT<sup>[14]</sup>. A 2001 Japanese study evaluated AST and ALT levels amongst 7313 men, excluding former alcohol drinkers or a history of a chronic liver disease, examining for a dose related response by subgrouping men amongst self-reported drinking of < 1, 1-2, 3-4, or > 5 cups of coffee daily<sup>[15]</sup>. Transaminases were significantly lower in groups reporting increased coffee usage. Of note, men reporting ongoing alcohol use with concurrent coffee consumption exhibited a relatively reduced rise in AST compared to non-coffee drinking alcohol users. A 2010 study in Japan evaluated levels of AST, ALT, and GGT amongst various subgroups of men and women with high BMI, low BMI, and high and low alcohol consumption. Transaminases were noted to be lower amongst men and women with higher coffee consumption, the relationship appearing to be stronger in those with higher alcohol consumption and lower BMI<sup>[16]</sup>.

While studies had been performed previously testing for coffee consumption and its association with liver enzyme levels, one study evaluated effect of coffee in patients with risk factors for chronic liver disease: consumption of greater than two alcoholic beverages daily, positive serum HBV antigen, positive serum HCV antibody, transferrin saturation > 50%, elevated BMI, and uncontrolled diabetics<sup>[17]</sup>. This study demonstrated relatively reduced levels of ALT amongst these higher

risk groups.

## COFFEE AND LIKELY PROTECTIVE EFFECTS AGAINST DEVELOPMENT OF FIBROSIS

Given the above information the association between coffee and relative reduction of liver enzymes appears clear, however, the benefits of coffee appear to extend further. In a 2015 population-based prospective cohort study demonstrated coffee intake with reduced mortality from chronic liver disease<sup>[18]</sup>. In fact, as little as 1 cup of coffee consumed daily resulted in 15% reduction in risk of death from chronic liver disease; 4 cups daily was associated with 71% reduction, suggesting a dose-dependent response. This study appears to reaffirm findings of an earlier 2005 study noting that consumers of coffee and tea exhibited significantly decreased risk of chronic liver diseases<sup>[19]</sup>. The study followed 9849 participants for a median of 19 years and a decreased risk of hospitalization or death with a chronic liver disease; a dose-dependent response was seen again in this group, with consumption of 2 or more cups of coffee doubling the relatively reduced risk of complications than those drinking 1 cup. A 2003 Norwegian study found similar findings, noting progressively improved mortality with increasing coffee consumption, though the effect appears to negligible beyond drinking 4 cups of coffee daily<sup>[20]</sup>. In addition to less frequent complications of liver disease, there is evidence demonstrating coffee has an association with reduced fibrosis. A 2010 study evaluated effect of coffee intake over a six month period in a group of 177 patients with variable degrees of liver fibrosis<sup>[12]</sup>. In this study, intake of at least 2 cups of coffee daily was associated with the less advanced observed fibrotic disease. A 2011 study echoes these findings, noting that advanced fibrosis in a population of chronic HCV patients was not only seen significantly less frequently in coffee drinkers but that the frequency decreased with increasing reported coffee intake, again suggesting a dose-dependent response<sup>[21]</sup>. A 2014 Brazilian study reinforces this impression, evaluating 136 patients with biopsy, ultrasound, or endoscopic evidence of fibrotic disease, finding that individuals drinking higher amounts of coffee demonstrated a significantly lower frequency of advanced fibrosis on liver biopsy<sup>[22]</sup>. A 2015 study of 910 chronic HCV male patients evaluated the association of daily intake of various caffeinated beverages, including coffee, finding a higher percentage of coffee drinking amongst patients without advanced fibrosis than those with demonstrated fibrotic disease<sup>[23]</sup>. A recent meta-analysis of multiple cohort studies and case-control studies independently demonstrated a significantly reduced risk of cirrhosis with consumption of at least 2 cups of coffee daily<sup>[24]</sup>.

Coffee clearly correlates with reduced frequency

of fibrosis, but is coffee itself responsible for these effects, or can its probable protection against fibrosis be seen utilizing any caffeinated beverage? Other studies referenced above seem to suggest hepatoprotection is unique to coffee amongst caffeinated beverages, however, a 2001 study attempted to answer this question head-on<sup>[25]</sup>. This group noted that caffeine intake from other beverages did not show significant odds ratio along with no evidence of significant trends over the amount of intake whereas with coffee intake there was an inverse association with cirrhosis and coffee consumption with just one cup of coffee daily<sup>[15]</sup>. A 2012 study found a similar association of reduced observation of advanced in coffee drinkers but not in espresso<sup>[26]</sup>.

There is always a concern when findings of a beverage are correlated with health benefits that there may be confounding factors in play. In a case-control study performed in Italy, it was confirmed that the inverse relationship between coffee consumption and cirrhosis across strata of tobacco use, alcohol consumption, age, and sex. A consistent inverse relationship was still noted in moderate alcohol drinking indicating the relationship between coffee consumption and cirrhosis is not restricted to alcohol-related cirrhosis<sup>[27]</sup>.

The variety of different liver diseases, as well as a variety of ethnicities, involved in the aforementioned studies, suggests a possibly universal effect of coffee on this disease spectrum, however, further studies have been done in populations with more homogenous liver pathology. As one can glean from the above information, chronic viral hepatitis etiologies appear to be most heavily represented population in liver disease literature related to coffee.

## COFFEE AND EVIDENCE OF HEPATOPROTECTION IN PATIENTS WITH VIRAL HEPATITIS

In the United States, the most predisposing factors to hepatocellular cancer are alcohol abuse, HBV, and HCV. The aforementioned case-control study in Italy determined that the inverse relationship exists between coffee consumption and cirrhosis across varying degrees of alcohol consumption it is documented that hepatocellular carcinoma risk is also decreased with the intake of coffee<sup>[27]</sup>.

A similar Italian case-control study performed a few years later also demonstrated a substantial decrease in hepatocellular carcinoma risk in drinkers of coffee of 3 or more cups of coffee daily, going on to note a decreased risk of hepatocellular carcinoma regardless of etiology of chronic liver disease<sup>[28]</sup>. A large prospective study of 776 participants with advanced HCV-related liver disease was also exhibited lower rates of disease progression with regular coffee consumption. This prospective study noted that drinkers of 3 or more cups



of coffee per day had 53% lower risk of liver disease progression than non-coffee drinkers with advanced HCV-related liver disease<sup>[29]</sup>. A 2014 study evaluated levels of AST and ALT levels in HCV-HIV co-infected patients, with those self-reporting higher levels of coffee (> 3 cups/d) demonstrating lower levels of liver enzymes<sup>[30]</sup>. A 2013 cohort study amongst 229 HCV patients with normal baseline ALT levels found that 189 retained normal ALT levels one year after being followed; daily coffee drinks were three times more likely to maintain their baseline ALT level than non-coffee drinkers<sup>[31]</sup>. Another 2013 study evaluated 40 HCV patients, splitting them into two groups; one drank 4 cups of coffee/day, the other drank no coffee. HCV viral loads were significantly higher in the non coffee drinking group, as well as oxidative damage *via* telomere length and measured 8-hydroxydeoxyguanosine levels<sup>[32]</sup>. Studies demonstrating a dose-dependent response in patients specific for HCV mediated disease are lacking, however, the previously presented data suggests direction for future studies. A common thread one may note amongst these and aforementioned studies is a large number of studies of HCV infected population. One 2011 cross-sectional study of Asian populations with HBV did not demonstrate any correlation between caffeine drinking and liver fibrosis using elastography as a tool for evaluating severity of disease<sup>[33]</sup>. While this evidence does not demonstrate that coffee intake in this population may not be associated with decreased risk of HCC, it does suggest that coffee's protective mechanism may be unrelated to prevention of fibrosis.

Beyond demonstrating an association with decreased fibrotic disease, studies are beginning to emerge suggesting a more specific hepatoprotective role for coffee in patients with HCV. A 2015 study utilizing human hepatoma cell line infected with HCV demonstrated significantly decreased HCV viral load in lines introduced to caffeic acid, an organic acid found in coffee, compared to control lines infected with HCV<sup>[34]</sup>. Another study done in 2015 yielded similar results, with caffeine inhibiting HCV replication a hepatic cell line infected with the virus<sup>[35]</sup>.

## COFFEE, METABOLIC SYNDROME, AND NAFLD

While alcohol has been noted to be hepatotoxic, it has long been observed not all alcohol abusers develop cirrhosis. Development and progression of fibrosis appear to involve multiple factors at play in the disease process. Metabolic syndrome appears to be linked to increased risk of fibrosis, though the relationship has not been fully described at this juncture. Research involving coffee and liver disease appears to demonstrate a close relationship between these disease states. In a mortality follow-up study of 51036 individuals, it was noted that coffee drinking had an inverse association with cirrhosis risk

in the setting of four or more cups of coffee consumed daily<sup>[20]</sup>. A fair question, again, concerns whether coffee is a confounding variable; are individuals consuming this much coffee are generally avoiding other foods and beverages which would predispose one to liver disease? Two 2008 Japanese studies appear to reinforce this belief, noting that metabolic syndrome appears to be associated with increased risk of HCC, whilst coffee drinkers appear to be less likely to have metabolic syndrome<sup>[36,37]</sup>. Given that metabolic syndrome appears to be a risk factor HCC, perhaps due to steatosis, this would imply an indirect benefit of coffee. It is worth noting the second study was done in exclusively HCV patients, suggesting again that coffee is hepatoprotective against a large spectrum of liver disease<sup>[37]</sup>. Studies have also indicated an association between coffee consumption and NAFLD and liver fibrosis. An inverse relationship between NAFLD patients and fibrosis was noted in a 2011 cross-sectional study<sup>[38]</sup>. Another two studies was performed using bright liver score as a method to gauge the advancement of NAFLD, noting again an inverse relationship between progression of fibrosis and coffee consumption<sup>[39,40]</sup>. A 2003 study noted relatively decreased fibrosis in obese women drinking coffee compared to those that did not<sup>[25]</sup>. The mechanism of possible hepatoprotection in NAFLD is unclear. One 2015 cross-sectional study of a random German patients demonstrated expected correlations between NAFLD and obesity, however, saw no significant difference in either levels of ALT nor sonographic evidence of NAFLD when comparing coffee drinkers vs those who did not drink coffee, though is unable to comment on coffee's effect on rate of disease<sup>[41]</sup>. An earlier noted meta-analysis, it should be stated, did note its protective effect in coffee drinkers significant for HCV and alcoholic liver disease populations, though not in NAFLD<sup>[6]</sup>. While the NAFLD population is not heavily represented in this study, one must consider the possibility that coffee's potential protective effect on NAFLD may be due to disease modifying effects on metabolic syndrome. Taken together, however, these studies suggest evidence for a positive influence of coffee consumption on NAFLD.

## COFFEE AND DECREASED RISK OF HEPATOCELLULAR CARCINOMA

There have been numerous studies performed which have indicated the association between coffee consumption and risk of HCC. We have previously presented information suggesting protective effects of coffee in patients with viral hepatitis, a known risk factor for HCC. Further studies demonstrate broad support for the hypothesis that coffee protects again HCC in general. An earlier referenced population-based prospective cohort study performed involving > 215000 men and women found that when compared with non-coffee drinkers that consumption of 2-3 cups per day had 38% reduction in

**Table 1** Summary of findings from studies evaluating coffee consumption and reduced risk of hepatocellular cancer

Studies	Year	Study type	Summary
Setiawan <i>et al</i> <sup>[18]</sup>	2015	Prospective cohort	2-3 cups/d noted to have 38% HCC reduction risk 4 cups/d noted to have 41% risk reduction
Yu <i>et al</i> <sup>[45]</sup>	2013	Prospective cohort	Significantly decreased risk of HCC noted among coffee drinkers
Bravi <i>et al</i> <sup>[44]</sup>	2013	Meta-analysis (14 studies)	40% HCC risk reduction with 1-3 cups coffee/day
Bravi <i>et al</i> <sup>[43]</sup>	2007	Meta-analysis (10 studies)	Inverse association noted between coffee consumption and HCC
Larsson <i>et al</i> <sup>[42]</sup>	2007	Meta-analysis (9 studies)	43% HCC risk reduction
Gelatti <i>et al</i> <sup>[28]</sup>	2005	Case control	Inverse relationship noted between coffee and HCC

HCC: Hepatocellular cancer.

risk for HCC and with 4 cups per day found to have 41% reduction in HCC<sup>[18]</sup>. Yet another hospital-based case control study found that regardless of the etiology of HCC, there was an inverse relationship of observed HCC with coffee consumption<sup>[28]</sup>. According to a meta-analysis done involving relevant studies from 1966 to 2007 indicated a 43% reduced risk of liver cancer with the consumption of two cups of coffee<sup>[42]</sup>. Yet another meta-analysis performed involving ten studies with 2260 HCC cases and six case-control studies from southern Europe and Japan with 1551 cases and four cohort studies from Japan accounting for 709 cases also confirmed an association with decreased risk of liver cancer and coffee consumption<sup>[43]</sup>. A 2013 meta-analysis of studies through 1966 to 2012 found 14 studies demonstrating a pooled reduced risk of HCC by 40%, suggesting strong evidence that coffee consumption is associated with decreased risk of HCC, though the necessary minimum appears to be anywhere from 1 cups daily to 3 cups<sup>[44]</sup>. Another 2013 study of Western populations who recorded their consumption of coffee for 24 years, stratifying for age, BMI, as well as smoking and alcohol use with a decreased risk of HCC demonstrated amongst this group of people<sup>[45]</sup>. These studies together (Table 1) suggest a universally decreased risk of HCC amongst people of all ethnicities with potentially a variety of different risk factors to develop HCC.

## COFFEE AND EVIDENCE OF DECREASED RISK OF OTHER GI TRACT MALIGNANCIES

As though the already stated benefits of coffee consumption were not enough there has been emerging data of other malignancies that may also be affected by coffee consumption. In a hospital based case-control study conducted in Italy and Switzerland, it was noted that with greater than three cups of coffee consumed daily was associated with an odds ratio of 0.6 when compared to drinkers of one or less cups of coffee daily in relation to pharyngeal cancer. The same study also noted an odds ratio of 0.6 for esophageal cancer; indicating a decreased risk of pharyngeal and esophageal cancer with greater than three cups of coffee<sup>[46]</sup>. One case-control

study performed earlier indicated an inverse relationship with coffee consumption and colon cancer along with rectal cancer. However, the same study was unable to find a significant association with cancers of the mouth, stomach, or pancreas<sup>[47]</sup>. Ultimately; coffee consumption appears to have an association with decreased risk of colon, rectal, esophageal, and pharyngeal cancer.

## DISCUSSION

With coffee growing in popularity its documented health benefits are also growing. With the benefits of coffee consumption ranging from liver enzyme laboratory test improvement to improved mortality from cirrhosis, HCC, as well as other malignancies, and chronic liver diseases secondary to HBV, HCV and NAFLD.

In summary, the etiology of coffee's apparent beneficial effects have been greatly debated. One hypothesis involves the observation that coffee consumption is associated with better lifestyle choices, confounding the positive effects that had been associated with coffee consumption. One previously discussed cohort study argues against this hypothesis, demonstrating subjects that were prone to increased coffee consumption actually had higher median consumption of cigarettes, lower education levels, and higher median intake of alcohol than those with decreased coffee consumption<sup>[16]</sup>.

An additional question regarding coffee consumption's benefits relates to the attribution of the caffeine content than the coffee itself. In a study involving inpatient cirrhotics, it was noted that caffeine intake from beverages other than coffee did not show significant odds ratio at least in relation to liver cirrhosis<sup>[15]</sup>. Multiple studies referenced above demonstrate beneficial effects related to coffee that are generally not reproducible when testing against other caffeinated beverages. Regardless, as a biologic mechanism has not been proposed, the link is still unclear.

A few hypotheses exist to possibly demonstrate a physiologic basis of coffee's beneficial effects. One hypothesis is that coffee activates enzymes that detoxify the liver *via* activation of uridine 5'-diphospho-glucuronosyltransferases<sup>[48]</sup>. A 2002 study demonstrates increased expression of such enzymes in mice fed coffee specific compounds known as diterpenes, kahweol and cafestol,

conferring protection against toxins associated with colon cancer<sup>[48]</sup>. A 2007 study demonstrated that kahweol and cafestole administered to hepatocytes subsequently treated with carbon tetrachloride significantly prevented markers of liver injury as compared to control *via* measured ALT and AST levels, reduced glutathione content and lipid peroxidation<sup>[49]</sup>. Another hypothesis suggests the anti-oxidant properties of polyphenols present in coffee mediate its hepatoprotective effects<sup>[25,50]</sup>. As for the mechanism with which coffee prevents worsening of hepatic fibrosis, one thought involves caffeine decreasing transforming growth factor- $\beta$  (TGF- $\beta$ ), a mediator of fibrogenesis<sup>[51]</sup>. Hepatic stellate cells are induced by TGF- $\beta$  for differentiation to myofibroblasts, synthesizing connective tissue involved in fibrogenesis. A study in rat hepatocytes demonstrated caffeine inhibited TGF- $\beta$  signaling by upregulating peroxisome proliferator activated receptor  $\gamma$  (PPAR- $\gamma$ )<sup>[52]</sup>. As noted above, not all studies reviewed suggest a modifying effect of fibrogenesis as the protective etiology conferred in what appears to be a generally positive outcome effect on chronic liver disease, however, further studies appear warranted to evaluate for any possible delayed onset of fibrosis amongst coffee drinkers vs non coffee drinkers in comparable populations at risk for cirrhosis. In addition, the studies demonstrating the potential effects of caffeic acid on HCV replication suggest a possible mechanism for the apparent positive affects of coffee in this population. Further studies need to be done to verify these and others noted above, such as coffee potentially preventing HCV replication. Regardless, with the wealth of evidence suggesting a positive disease modifying effect of coffee on chronic liver diseases in a multitude of patient populations, there is clearly a strong basis with which to move forward with studies evaluating the potential causative agent. To conclude, while the reason why coffee is good for you is not yet completely clear, these studies should encourage the vast number of patients with chronic liver disease to enjoy the beverage as many others already do.

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## Cardiovascular assessment in liver transplant for non-alcoholic steatohepatitis patients: What we do, what we should do

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### Abstract

Non-alcoholic fatty liver disease (NAFLD) is increasing considerably due to the current lifestyle, which means that it is becoming one of the main indications for liver transplantation. On the other hand, there is a strong association between NAFLD and cardiovascular disease. This has been evidenced in many studies revealing a higher presence of carotid plaques or carotid intima-media thickness, leading to cardiovascular events and, ultimately, mortality. According to the liver transplant guidelines, screening for heart disease in transplant candidates should be performed by electrocardiogram and transthoracic echocardiography while a stress echocardiogram should be reserved for those with more than two cardiovascular risk factors or greater than 50 years old. However, there are no specific recommendations in NAFLD patients requiring a liver transplantation, despite its well-known cardiovascular risk association. Many studies have shown that these patients probably require a more exhaustive assessment and a global approach including other specialists such as cardiologists or nutritionists. Also, the incidence of cardiovascular disease is also increased in NAFLD patients in the post-transplantation period in comparison with other etiologies, because of the pre-existent risk factors together with the immunosuppressive therapy. Therefore, an early intervention on the lifestyle and the individualized selection of the immunosuppressive regimen could lead to a modification of the cardiovascular risk factors in NAFLD patients requiring a liver transplantation.

**Key words:** Cardiovascular risk; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Pre-transplant assessment; Liver transplantation

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**Core tip:** Non-alcoholic fatty liver disease is a growing condition due to the current lifestyle. It is considered the liver manifestation of the metabolic syndrome, so it is

strongly related to cardiovascular disease. Given that is one of the main indications of liver transplantation, it is essential to carry out an adequate assessment of the pre-transplant cardiovascular risk, as well as an individualized management of the patient in the post-transplantation period (due to the pre-existent cardiovascular risk factors and the immunosuppressive therapy).

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinical-pathological condition that encompasses a wide range of liver damage not caused by chronic alcohol consumption, including steatosis, non-alcoholic steatohepatitis (NASH) and cirrhosis<sup>[1]</sup>. NAFLD is considered a hepatic manifestation of metabolic syndrome. Its prevalence has increased considerably over last years, especially in Western countries, due to the current lifestyle (diet, sedentary lifestyle, obesity)<sup>[2,3]</sup>. It has been calculated that up to 30% of the population shows NAFLD, representing up to the 70% in patients with type 2 diabetes mellitus (DM)<sup>[4]</sup>. On the other hand, the prevalence of NASH (characterized by the presence of inflammation) is around 3%-5%. In NASH patients, cardiovascular (CV) risk represents one of the leading causes of mortality due to the frequent association with dyslipidemia, DM and other features of metabolic syndrome<sup>[5]</sup>. In fact, NASH patients suffer more subclinical atherosclerosis, heart disease, and CV clinical events than those without it<sup>[6]</sup>. This latter, together with NASH has become the second cause of liver transplantation (LT) in the United States and Europe<sup>[7]</sup>, makes especially relevant the adequate cardiovascular assessment in LT setting.

## CV RISK IN NAFLD PATIENTS

Several studies have clearly demonstrated the link between NAFLD and CV risk. It is not surprising, considering that they share many risk factors derived from metabolic syndrome (such as obesity, insulin resistance, DM, sedentary lifestyle, hypertension, dyslipidemia) and genetics (PNPLA3, TM6SF2)<sup>[8]</sup>. Gut microbiota also plays an important role. In both mice and humans, a high-fat diet results in an increase of lipopolysaccharides in plasma (a cellular component of Gram-negative bacteria) by modifying the microbiota and, therefore, the intestinal permeability. That is the reason to increase TLR4 receptor expression, stimulating liver cells to produce inflammatory cytokines and creating a systemic pro-inflammatory status, which favors atherosclerosis<sup>[9,10]</sup>. According to CV risk, we can classify it in three steps: Subclinical

atherosclerosis, clinical events, and mortality.

Firstly, a higher prevalence of subclinical atherosclerosis has been well-documented (Table 1). In 2005, Brea *et al*<sup>[11]</sup> published that NAFLD patients showed an increased carotid artery intima-media thickness (CIMT) and a higher prevalence of carotid plaques (50% vs 25%) compared to healthy controls. Regarding NAFLD subjects, NASH patients showed greater subclinical atherosclerosis in comparison with those with simple steatosis and the CV risk was progressively increased according to liver fibrosis<sup>[11]</sup>. Later, Kim *et al*<sup>[12]</sup> identified that patients with NAFLD had a higher percentage of coronary artery calcification (by computerized tomography) independently of other known factors. More recently, Puchner *et al*<sup>[13]</sup> again assessed the link between NAFLD and advanced coronary arterial disease. After performing a coronariography by computerized tomography, they found that the presence of significant coronary stenosis (16% vs 5%), global carotid plaques (78% vs 24%) and high-risk carotid plaques (59% vs 19%) were more prevalent in individuals with NAFLD. All of these findings have been confirmed in a recent meta-analysis, as NAFLD patients showed a greater link with subclinical atherosclerosis regarding CIMT [OR 2.04 (95%CI: 1.65-2.51)] and the presence of carotid plaques [OR 2.82 (95%CI: 1.87-4.27)]<sup>[14]</sup>. Secondly, NAFLD patients suffer more CV events than the overall population. In 2016, Fracanzani *et al*<sup>[15]</sup> aimed to evaluate the incidence of CV and cerebrovascular events in patients with NAFLD, who had been monitored for 10 years. Patients presented a higher number of CV events than the control group (19% vs 10%), being the presence of carotid plaques [OR 5.08 (95%CI: 2.56-10.95)] and liver steatosis [OR 1.99 (95CI: 1.01-3.94)] the main risk factors<sup>[15]</sup>. As a consequence of the higher prevalence of subclinical atherosclerosis and clinical events, CV mortality is ultimately increased as well. In fact, CV-related death appears to be one of the leading causes of death in most of the studies in NASH patients (Table 2). Ekstedt *et al*<sup>[16]</sup> followed-up 229 patients during more than 30 years, concluding that CV disease was the first cause of mortality for NASH patients without cirrhosis.

Taking all together, the European Association for the Study of the Liver recommend screening for CV disease in patients with NAFLD, irrespective of the presence of other traditional risk factors<sup>[17]</sup>.

## CV EVALUATION PRE-LT

CV disease is a major cause of death in post-LT knowing that this risk is bigger in patients showing pre-LT risk factors (irrespective of the etiology). For example, coronary artery disease has been observed in as many as 60% of potential LT candidates and, obviously, its presence increases the CV morbi-mortality pre and post-surgery<sup>[18]</sup>. Therefore, it is essential an adequate CV assessment to prevent these complications and increase post-LT survival rates.

To be included on the liver transplant list, comprehensive evaluation must be performed to evaluate the

**Table 1** Methods to detect subclinical atherosclerosis<sup>[8]</sup>

Carotid ultrasound	CIMT	> 0.9 mm
CT coronary angiography	No. of calcifications in coronary arteries	≥ 1
Endothelial function	Flow-mediated vasodilation brachial artery	
	Carotid-femoral pulse wave velocity	> 12 m/s
Morpho-structural alteration	Electrocardiogram (left ventricular hypertrophy)	Sokolov-Lyon > 38 mm; cornell > 2444 mm*ms
Renal function	Slight increase in plasmatic creatinine	M: 1.3-1.5 mg/dL
		F: 1.2-1.4 mg/dL
	Low glomerular filtration	Creatinine clearance < 60 mL/min
	Microalbuminuria	30-300 mg/24 h
		Alb/Cr ≥ 22 (M) or ≥ 31 (F) mg/g Cr
Inflammatory biomarkers	TNF, IL-6, C-reactive protein	
Thrombogenic biomarkers	PAI-1, fibrinogen, factor VII	

CIMT: Carotid intima-media thickness; CT: Computerized tomography; M: Male; F: Female; TNF: Tumor necrosis factor; IL: Interleukin; PAI-1: Plasminogen activator inhibitor type 1.

**Table 2** Cardiovascular mortality in non-alcoholic fatty liver disease patients

Ref.	Year	NAFLD diagnosis	Follow-up	CV mortality	Cause of mortality
Dam-Larsen <i>et al</i> <sup>[48]</sup>	2004	Histology	20 yr	38%	1 <sup>st</sup>
Adams <i>et al</i> <sup>[49]</sup>	2005	Histology	8 yr	25%	2 <sup>nd</sup>
Ong <i>et al</i> <sup>[50]</sup>	2008	Ultrasound	9 yr	25%	1 <sup>st</sup>
Rafiq <i>et al</i> <sup>[51]</sup>	2009	Histology	29 yr	13%	1 <sup>st</sup>
Söderberg <i>et al</i> <sup>[52]</sup>	2010	Histology	28 yr	35%	1 <sup>st</sup>
Angulo <i>et al</i> <sup>[53]</sup>	2013	Histology	9 yr	38%	1 <sup>st</sup>
Stepanova <i>et al</i> <sup>[54]</sup>	2013	Histology	12 yr	28%	1 <sup>st</sup>
Ekstedt <i>et al</i> <sup>[16]</sup>	2015	Histology	26 yr	43%	1 <sup>st</sup>

CV: Cardiovascular; NAFLD: Non-alcoholic fatty liver disease.

peri-surgery risk that could prevent from good long-term results. Regarding to CV assessment, current recommendations include<sup>[19]</sup>: (1) to carry out an electrocardiogram and a trans-thoracic echocardiography to rule out underlying heart disease; (2) in patients with > 2 CV risk factors or those older than 50 years old, an ergometry or a stress echocardiogram with dobutamine to detect subclinical ischemic cardiopathy; and (3) whether a significant coronary artery disease is detected during the usual evaluation, a coronariography must be performed (if this latter results effective, the survival rate after LT is similar to those who do not have previous CV disease<sup>[20,21]</sup>). Sometimes, non-invasive methods to screen for CV disease have low sensitivity and specificity compared to other tests (*i.e.*, angiography)<sup>[22]</sup>. However, there is no sufficient evidence to recommend invasive tests to evaluate CV risk before LT in asymptomatic patients. Therefore, the American Heart Association and the American College of Cardiology Foundation<sup>[23]</sup> propose to perform a coronariography in CV high-risk candidates, defined as those who have > 2 CV risk factors (DM, age > 60 years, smokers, AHT, and dyslipidemia). On the other hand, they recommend non-invasive stress tests in those patients with low risk of CV disease<sup>[24]</sup>.

Given that CV risk factors before LT have a great impact, it has been proposed that the Framingham Risk Score (an algorithm to predict CV risk at 10 years including age, sex, smoking, DM, arterial hypertension, and dyslipidemia) could be useful for predicting post-LT CV risk in candidate patients. This strategy could lead

to performing individualized diagnostic and therapeutic tests depending on the score<sup>[25]</sup>.

Clinical guidelines for NAFLD patients recommend that CV risk must be carefully evaluated in LT setting because theoretically these subjects have more risk factors to suffer CV-related clinical events and mortality. Even more, some of them probably would require invasive tests but the best method remains unclear. The British guideline<sup>[26]</sup> proposes the evaluation of the functional capacity of the patient measured by the MET unit (energy expenditure during physical activity), guiding the following tests according to the result. Consequently, patients able to climb at least two flights of stairs (equivalent to 4 METs) and those who do not present CV risk factors, may not require further tests. On the other hand, those with a MET < 4 or showing at least one CV risk factors (myocardial infarction, heart failure, stroke/transient ischaemic attack, renal dysfunction, DM requiring insulin therapy) will need a stress echocardiogram or cardiopulmonary exercise test. Likewise, they recommend the simultaneous evaluation with a cardiologist in CV high-risk patients, especially those who have suffered a CV disease before LT<sup>[23]</sup>. Despite all this, pre-LT CV assessment in NAFLD patients is not routinely different to those patients who have cirrhosis for other etiologies.

## CV RISK IN NAFLD POST-LT

Post-LT survival rates have been increasing over time,



**Table 3** Immunosuppressive drugs and metabolic side effects affecting post-liver transplantation cardiovascular risk<sup>[33]</sup>

Drug	Group	Side effects
Corticosteroids		Dyslipidemia ++ AHT +++ DM +++
Mycophenolate mofetil	<i>De novo</i> purine synthesis inhibitor	Renal impairment - Dyslipidemia - AHT - DM -
Cyclosporine Tacrolimus	Calcineurin inhibitors	Renal impairment - Dyslipidemia ++ AHT +++ DM ++
Sirolimus Everolimus	mTOR inhibitors	Renal impairment ++ Dyslipidemia +++ AHT - DM - Renal impairment -

(+): Positive association; (-): No association; AHT: Arterial Hypertension; DM: Diabetes mellitus.

due to the loss of the liver graft is less common and the short-term mortality is lower<sup>[27]</sup>. After the transplant, patients usually gain weight, and the incidence of metabolic syndrome is greater (as much as two-thirds of patients at 5 years) probably related to the lifestyle and the immunosuppressive treatment, respectively<sup>[28,29]</sup>. In this scenario, metabolic and CV complications are currently the main responsible for affecting the mid- and long-term survival.

Among non-liver-related 1-year mortality after the LT, CV disease is the second cause after tumors, followed by infections and kidney failure<sup>[30]</sup>. Madhwal *et al.*<sup>[31]</sup>, based on a meta-analysis including twelve observational studies, observed that CV events were present in 13.6% (95%CI: 9%-18%) of NAFLD patients within 10 years. Also, they noted that the incidence of CV disease was especially relevant in those who had additionally metabolic syndrome (four times higher of suffering a CV event)<sup>[31]</sup>. Precisely, NAFLD patients who required LT are older and have more prevalence of DM and obesity (as well as chronic kidney failure or previous CV disease) in opposition to the rest of etiologies<sup>[32]</sup>.

The prevalence of metabolic syndrome is around 50%-60% of the post-LT population<sup>[28,33]</sup>, influenced by the appearance of several risk factors. Obesity (BMI > 30 kg/m<sup>2</sup>) is approximately 24%-64% after LT<sup>[27]</sup>, due to the fact that the weight increases after the operation (reversion of cirrhosis and its hypercatabolic state, increase in appetite, absence of the chronic disease, effects of steroids) which means an increase in DM and dyslipidemia, as well as in vascular events and kidney disease<sup>[34]</sup>. On the other hand, DM (the most important risk factor of NAFLD) is diagnosed in 10%-64% of post-LT patients<sup>[28,35]</sup>, and is being considered more and more the main complication after LT. Its appearance is multifactorial, but the main modifiable factor (apart from lifestyle) is the choice and dose of the immuno-

suppressive therapy. Corticoids have diabetogenic effects producing resistance to insulin and increasing the gluconeogenesis, while the calcineurin inhibitors can directly damage the pancreatic cells (tacrolimus has a significantly higher risk than cyclosporine<sup>[36]</sup>). The immunosuppressive therapy is also responsible, at least in part, of the appearance of post-transplant AHT (40%-85%) and dyslipidemia (40%-66%)<sup>[37]</sup> (Table 3). All of this means that the liver disease can return after the LT (*de novo* NAFLD). Out of NASH patients who are transplanted, this entity reappears in 75%, being the post-LT hypertriglyceridemia, BMI and steroid treatment, the main risk factors<sup>[38]</sup> (causing a positive feedback for post-LT CV risk).

In this scenario, several studies have evaluated whether patients with NAFLD show a higher risk of post-LT CV disease in comparison with other etiologies. Yalamanchili *et al.*<sup>[39]</sup> evaluated 2152 patients with liver cirrhosis, of which 12% had NAFLD or cryptogenic cirrhosis. Survival rate at 10 years after the LT was similar regardless of the etiology, but a significant increase was observed in CV mortality in NAFLD patients (21% vs 14%)<sup>[39]</sup>. VanWagner *et al.*<sup>[40]</sup> compared the incidence of CV events between NAFLD and alcohol after the LT. Authors observed an increase in CV-related 1-year mortality after LT in NAFLD group (26% vs 8%) and, more interestingly, the most of the CV events occurred in the peri-surgery period (70%)<sup>[40]</sup>. The same research group has recently determined a group of risk factors clearly associated with post-LT CV mortality: Age > 55 years old, male sex, DM, and kidney failure<sup>[32]</sup>. Wang *et al.*<sup>[41]</sup> performed a meta-analysis in NAFLD patients to estimate post-LT results regarding overall survival, CV mortality, sepsis and liver graft failure. Authors concluded that survival rates were similar in patients with or without NAFLD, as far as 5 years after LT. However, it was found that NAFLD patients were more likely to die because of CV complications [OR 1.65 (95%CI: 1.01-2.70)]<sup>[41]</sup>.

## RECOMMENDATIONS IN NAFLD LIVER TRANSPLANT

### *Pre-liver-transplantation recommendations*

Taking into account the information exposed before, the pre-LT CV assessment in patients with NAFLD should be more exhaustive than in the rest of etiologies. However, there are no specific recommendations probably due to there is no an ideal procedure regarding cost, availability, and reliability.

NAFLD is not considered a CV risk criterion to influence the decision of the selection of the CV evaluation in the pre-LT assessment. Consequently, many NAFLD patients only undergo a trans-thoracic echocardiogram or a computerized coronary tomography with calico-score. Some authors have proposed the stress echocardiography with dobutamine as an initial test in NAFLD candidates for LT because it shows a high negative predictive value to detect low-risk patients<sup>[42]</sup>. In high CV

risk patients (age > 55 years, male gender, DM, kidney failure), it probably should be the initial test.

NAFLD is a condition that, more than a specific treatment, needs a multidisciplinary approach whose aim is a dramatic change in the lifestyle<sup>[43]</sup>. Thus, it is crucial to have a systematic intervention of a nutritionist during the LT evaluation in NAFLD patients (overweight, obesity, unhealthy diet) to reinforce and maintain a healthy lifestyle after the LT<sup>[44]</sup>.

### Post-liver-transplantation recommendations

Given that liver transplant recipients have an increased risk of CV disease, an early and effective treatment is required, as well as changing of the other risk factors (lifestyle, treatment of co-morbidities, immunosuppressive therapy). One example is the obligation of starting the treatment to control AHT, dyslipidemia or DM as soon as possible<sup>[44]</sup>.

Regarding the immunosuppressive drugs, most of them can cause and enhance various CV risk factors. Mycophenolate mofetil is associated with an increased risk of CV disease in post-LT patients<sup>[45]</sup>. More recently, the use of mTOR inhibitors (sirolimus, everolimus) was associated to lower CV risk than calcineurin inhibitors<sup>[46]</sup>. Therefore, mTOR inhibitors could be considered for patients with metabolic syndrome and multiple CV risk factors, such as NAFLD patients. Nevertheless, these findings must be confirmed and validated in prospective cohorts. On the other hand, we should use a steroid-free regimen (or an early steroid withdrawal) preferably considering an, for example, a basiliximab-based induction therapy<sup>[26]</sup>.

A healthy diet and regular exercise are effective and complementary therapies<sup>[47]</sup>. Exercise is effective to lower the CV risk in non-transplant patients, but the connection between the benefits and the possible damage of regular exercise after LT has not been established. Also, there are no data concerning the impact of these exercise programs on the prevalence of metabolic syndrome or its individual components after LT.

## CONCLUSION

The increased CV risk in patients with NAFLD, compared to other etiologies of liver disease, has important implications both in pre- and post-LT. An adequate stratification of CV risk and an early detection of the different features of metabolic syndrome is required to prevent or decrease CV-related morbi-mortality. In this scenario, an active intervention on lifestyle and an individualized management of immunosuppression could be the most suitable strategies to maintain an adequate balance between risks and benefits.

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## Observational Study

# Trend of hepatocellular carcinoma incidence after Bayesian correction for misclassified data in Iranian provinces

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## Abstract

### AIM

To study the trend of hepatocellular carcinoma incidence after correcting the misclassification in registering cancer incidence across Iranian provinces in cancer registry data.

### METHODS

Incidence data of hepatocellular carcinoma were extracted from Iranian annual of national cancer registration reports 2004 to 2008. A Bayesian method was implemented to estimate the rate of misclassification in registering cancer incidence in neighboring province. A

beta prior is considered for misclassification parameter. Each time two neighboring provinces were selected to be entered in the Bayesian model based on their expected coverage of cancer cases which is reported by medical university of the province. It is assumed that some cancer cases from a province that has an expected coverage of cancer cases lower than 100% are registered in their neighboring facilitate province with more than 100% expected coverage.

## RESULTS

There is an increase in the rate of hepatocellular carcinoma in Iran. Among total of 30 provinces of Iran, 21 provinces were selected to be entered to the Bayesian model for correcting the existed misclassification. Provinces with more medical facilities of Iran are Tehran (capital of the country), Razavi Khorasan in north-east of Iran, East Azerbaijan in north-west of the country, Isfahan in central part and near to Tehran, Khozestan and Fars in south and Mazandaran in north of the Iran, had an expected coverage more than their expectation. Those provinces had significantly higher rates of hepatocellular carcinoma than their neighboring provinces. In years 2004 to 2008, it was estimated to be on average 34% misclassification between North Khorasan province and Razavi Khorasan, 43% between South Khorasan province and Razavi Khorasan, 47% between Sistan and balochestan province and Razavi Khorasan, 23% between West Azerbaijan province and East Azerbaijan province, 25% between Ardebil province and East Azerbaijan province, 41% between Hormozgan province and Fars province, 22% between Chaharmahal and bakhtyari province and Isfahan province, 22% between Kogiloye and boyerahmad province and Isfahan, 22% between Golestan province and Mazandaran province, 43% between Bushehr province and Khozestan province, 41% between Ilam province and Khuzestan province, 42% between Qazvin province and Tehran province, 44% between Markazi province and Tehran, and 30% between Qom province and Tehran.

## CONCLUSION

Accounting and correcting the regional misclassification is necessary for identifying high risk areas and planning for reducing the cancer incidence.

**Key words:** Trend of hepatocellular carcinoma; Cancer incidence registry; Misclassification; Bayesian correction

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**Core tip:** In many developing countries and even in some developed countries some errors occur in disease registry system. Since registered data is used for planning at the national and sub-national level, correcting the existed errors has a great importance. One of these errors is misclassification in registering cancer incidence. It occurs because some patients from divested provinces prefer to get more qualified diagnostic and treatment services at their adjacent provinces with more medical facilities

without mentioning their permanent residence. The aim of this study is to investigate the trend of hepatocellular carcinoma after correcting for misclassification error in Iran's cancer registry using Bayesian method.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the 5<sup>th</sup> most common cancer worldwide<sup>[1]</sup>. It is the fifth most common cancer in men (7.5% of the total, 554000 cases) and the ninth most common cancer in women (3.4% of the total, 228000 cases). Eighty-three percent of the estimated new cancer cases worldwide occurred in less developed regions in 2012 that 50% of that belongs to China alone<sup>[2]</sup>. HCC is the second most common cause of cancer death in the world<sup>[1]</sup> and it is estimated to be responsible for nearly 746000 deaths based on Globocan report 2012<sup>[2]</sup>. The major risk factors for HCC, are infection with the hepatitis B virus (HBV) and hepatitis C virus<sup>[3]</sup>. The most common cause of HCC in Iran is HBV and 80% of HCC cases are positive for at least one of the markers of HBV<sup>[4-6]</sup>. It is estimated that approximately 1.5 million people in the country are infected with this virus and 15% to 40% of them are at risk of developing cirrhosis or HCC<sup>[7,8]</sup>. The other known risk factors are Gender (HCC is more common in males than in females), Race (Pacific Islanders and Asian Americans have the highest rates of HCC, followed by American Indians and Hispanics, African Americans, and whites), Cirrhosis, Non-alcoholic fatty liver disease, Heavy alcohol use, Obesity, Aflatoxins and Tobacco use<sup>[9]</sup>. Overall mortality to incidence ratio of HCC is 0.95, so the geographical patterns of incidence and mortality are similar<sup>[2,3]</sup>. The regions of high incidence are Eastern Asia and South-Eastern Asia, the regions of intermediate incidence are Southern Europe and Northern America (9.3) and the lowest rates are occur in South-Central Asia and Northern Europe<sup>[2]</sup>. Iran is located in Middle East, an area with low risk for HCC<sup>[1,10]</sup> with an annual incidence much less than 5 per 100000 populations<sup>[4,11]</sup> but, while prognosis for HCC is very poor, the true prevalence of HCC in Iran is unknown and up to 40% of its death statistics are underreported; so it is not considered as an uncommon malignancy<sup>[2-4]</sup>.

Nowadays having a thorough information of geographic distribution of cancers has become so important<sup>[12]</sup>. Cancer registries are known as the main resource of epidemiologic data by registering the mortality, incidence, prevalence and survival for different disease in a systematic manner that is used by health policy makers for cancer control planning and evaluation of cancer

screening programs, detecting the impact of treatments and interventions, and allocating of resources to various provinces based on their need to healthcare facilities<sup>[13]</sup>. In addition to poor diagnosis of HCC, some patients want to get healthcare in facilitate neighboring provinces outside their resident without reporting their permanent address. It causes misclassification error in cancer registry system. Misclassification error is the disagreement between the observed and the true value. The expected coverage of cancer incidence in different provinces is the evidence of existence of misclassification error; that the observed rate of incidence is more than expected rate in some of the provinces, but then, it is much less than expected rate in their neighboring provinces<sup>[14]</sup>, while it is expected that the rate of cancer incidence be about the same in neighboring provinces that are similar in lifestyle and environmental conditions. Misclassification error in registered data leads to erroneous estimates of the incidence rates of cancer in different provinces and consequently affects need assessments. There are two methods to correct for misclassification error. The first is using a valid data that usually is not available or it is so time consuming and costly to valid a sample data and generalizing the results to the population<sup>[15]</sup>. The second is implementing Bayesian method. This is a statistical method that can be used to import the researcher's prior knowledge about the rate of misclassification to the analysis and updating prior information with observed data to estimate the misclassification rate<sup>[16]</sup>.

The aim of this study is to assess the trend of HCC incidence after correcting for misclassification error in registering cancer incidence in neighboring provinces of Iran, using a Bayesian method.

## MATERIALS AND METHODS

Incidence data of HCC was extracted from Iranian annual of national cancer registration report from 2004 to 2008<sup>[14]</sup>. Annual of 2008 was the last available data to use. The Age Standardized Rate (ASR) for HCC [coded based on the 10<sup>th</sup> revision of the International Classification of Diseases (ICD-10; C22)] was calculated for all provinces of Iran in each year with direct standardization method and using the standard population reported by World Health Organization for both genders and four age groups (0-14 years, 15-49 years, 50-69 years and over than 70 years old). Age standardized rate was used to achieve comparative statistics on cancer in Iran with those for other countries<sup>[17]</sup>.

The expected coverage of cancer cases was calculated for medical universities of each province that is considered to be 113 per 100000 population. In the process of cancer incidence registry, all new diagnosed cancer cases by diagnostic centers are reported to the medical university. Reported data are entered to software which is made by ministry of health. Medical university of each province sends its temporary data bank to the ministry of health. Ministry of health after removing duplicates and coding the recorded cancers based on

10<sup>th</sup> revision of international coding of disease provides a permanent data bank of cancer cases and sends it back to medical university of each province. So medical universities have an observed number of cancer cases in addition to the expected rate. Percent of expected coverage for each province is calculated by dividing the observed number to the expected number of cancer cases.

The data were entered to the Bayesian model in the form of two vectors  $y_1$  and  $y_2$ . Vector  $y_1 = (y_{11}, y_{21}, \dots, y_{r1})'$  contained the data of the province that has an expected coverage less than 100% and vector  $y_2 = (y_{12}, y_{22}, \dots, y_{r2})'$  contained the data of a neighboring province with more than 100% expected coverage. Subscript  $r$  is the indicator of covariate patterns that is made by age-sex group combinations. A Poisson distribution was considered for  $y_1$  and  $y_2$  that are count data<sup>[18,19]</sup>. An informative beta prior distribution was assumed for the misclassified parameter  $\theta$  as the probability of registering a data in misclassified group; so  $\theta \sim \text{beta}(a, b)$ <sup>[20-22]</sup>. In order to the expectation of beta distribution which is  $a/(a + b)$  get converged to the misclassified rate, prior values for  $b$  were selected based on the calculated expected coverage of the medical university with lower than 100% expected coverage and  $a$  was calculated with subtracting  $b$  from 100. Since misclassified parameter is unknown, a latent variable approach was employed to correct the misclassification effect<sup>[18,19]</sup>. The latent variable  $U$  was considered as the number of events from the first group that are incorrectly registered in the misclassified group with binomial distribution, i.e.,  $U_i | \theta, y_1, y_2 \sim \text{Binomial}(y_{12}, \theta)$  that  $P_i = (\lambda_{1i}\theta) / (\lambda_{1i}\theta + \lambda_{2i})$ .

Finally by multiplying likelihood function in prior distribution, posterior distribution obtained in the following form;  $\theta | U_i, y_1, y_2 \sim \text{Beta}(\sum U_i + a, \sum y_{1i} + b)$ <sup>[18,23-25]</sup>. Misclassified parameter was estimated by using a Gibbs sampling algorithm and averaging the generated posteriors. After estimating the misclassification rate between each two neighboring provinces, the rates of HCC incidence for each province were re-estimated and the trend of HCC were checked out during 2004 to 2008. Analyses were carried out using R software version 3.2.0.

## RESULTS

All registered HCC cases from 2004 to 2008 in Iran were included in the study. The ASR of HCC for female increases from 0.43 per 100000 population (103 cases) in 2004, to 1.56 per 100000 (376 cases) in 2008. Also ASR of HCC for male increases from 0.66 per 100000 population (180 cases) in 2004, to 2.03 per 100000 (574 cases) in 2008. The trend of HCC from 2004 to 2008 for Iranian male and female is shown in Figure 1.

Among 30 provinces of Iran, 21 ones were selected for correcting the misclassification error in registering HCC incidence in neighboring provinces based on their expected coverage percent of cancer cases. In the other nine provinces, the number of cancer cases was about the same as their expected number; so the cancer

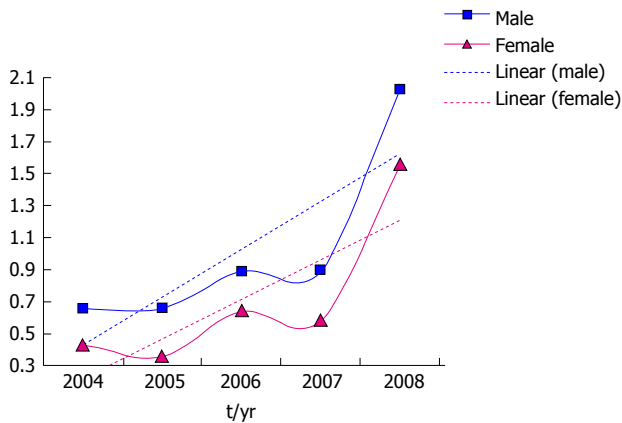


Figure 1 Age standardized rate of hepatocellular carcinoma and its trend for male and female in Iran (2004-2008).

Table 1 Expected coverage of cancer cases in provinces of Iran (2004-2008)

	2004	2005	2006	2007	2008
South khorasan		30.30	45.16	41.02	41.40
Razavi khorasan	106.50	106.50	101.81	117.54	143.74
Tehran	157.11	157.11	162.25	145.74	155.63
Markazi	43.35	43.35	53.07	57.46	69.60
Sistan	25.24	25.24	18.78	18.83	18.44
Qom	53.09	53.09	62.76	60.98	53.90
Ghazvin	65.07	65.07	71.44	72.84	66.30
Khozesta	61.09	61.09	62.68	69.81	101.19
Ilam	28.42	28.42	32.97	41.27	39.40
Bushehr	28.46	28.46	29.10	26.00	25.00
Golestan	50.65	50.65	58.61	58.20	50.80
Mazandaran	148.13	148.13	161.78	163.83	338.45
North khorasan		30.76	40.47	44.87	34.80
Chaharmahal	40.67	40.67	34.39	40.76	37.00
Isfahan	111.51	111.51	114.09	116.93	106.98
Kohgilouye	23.90	23.90	29.00	29.60	25.10
Hormozgan	25.44	25.44	25.11	25.31	19.00
Fars	98.07	98.07	112.01	134.53	127.65
Ardebil	63.73	63.73	72.71	64.99	63.00
East azarbaijan	108.22	108.22	110.98	138.52	123.60
West azarbaijan	81.96	81.96	75.32	82.53	69.00

rates of them remained unchanged. Each time the data of two neighboring provinces that one of them had a more than 100% expected coverage and the other one had a less than 100% of its expected coverage were candidates for entering the Bayesian model for estimating the existed misclassification between them.

For example the reported percent of expected coverage of cancer incidence for East Azerbaijan which is a province with more medical facilities in north-west of Iran, was 123.6% in 2008. It means that East Azerbaijan province have covered 23.6% more cancer cases than its expectation, whereas the West Azerbaijan and Ardebil provinces that are in neighborhood of East Azerbaijan, have just covered 69% and 63% of their expected coverage of cancer incidence respectively; which is a clear indication of existence of misclassification error in registering cancer cases. The expected coverage for the provinces for years 2004 to 2008 are reported in

Table 2 Bayesian estimated from misclassification rate between provinces

		Estimated misclassification rate				
		2004	2005	2006	2007	2008
Razavi khorasan	South khorasan		0.2	0.51	0.44	0.58
Tehran	Markazi	0.31	0.41	0.39	0.38	0.73
Razavi khorasan	Sistan	0.39	0.39	0.65	0.41	0.51
Tehran	Qom	0.18	0.22	0.18	0.28	0.65
Tehran	Ghazvin	0.2	0.25	0.5	0.4	0.74
Khozesta	Ilam	0.19	0.21	0.42	0.5	0.73
Khozesta	Bushehr	0.38	0.4	0.31	0.36	0.72
Mazandaran	Golestan	0.08	0.28	0.21	0.14	0.38
Razavi khorasan	North khorasan		0.16	0.43	0.34	0.42
Isfahan	Chaharmahal	0.16	0.16	0.18	0.39	0.23
Isfahan	Kohgilouye	0.18	0.43	0.16	0.18	0.16
Fars	Hormozgan	0.3	0.34	0.4	0.38	0.64
East azarbaijan	Ardebil	0.36	0.17	0.13	0.46	0.13
East azarbaijan	West azarbaijan	0.28	0.15	0.05	0.25	0.42

Table 1. After implementing the Bayesian method it was estimated to be 0.13% misclassification between East Azerbaijan and Ardebil and 0.42% misclassification between East Azerbaijan and West Azerbaijan in 2008. The estimated misclassification rate among other provinces for years 2004 to 2008 are reported in Table 2. The rate of HCC incidence, before and after Bayesian correction of misclassification for years 2004 to 2008 are reported in Table 3.

## DISCUSSION

There was a non-ignorable misclassification in registering cancer incidence between neighboring provinces in Iran. An increase is observed in trend of HCC during 2004 to 2008. The rate of HCC is even gets higher in some provinces after correcting for misclassification. Higher rates of estimated misclassifications are belonging to provinces with lower facilities like Hormozgan, Bushehr, Ilam, Qom, Markazi, Qazvin, Sistan and South Khorasan. Meanwhile it seems that misclassification rate is increasing during the period under study. It shows that not enough attention is paid to equip low-facilitate provinces.

The incidence of this cancer in many countries such as the United States, Central America and Europe is on the rise<sup>[7]</sup>. The findings of a study on incidence of HCC in Iran, showed that the incidence of this cancer is increasing in the country, especially in males and higher age groups<sup>[1]</sup>. A study on HCC indicated that little is known about the incidence of HCC in Iran, particularly in southeast of the country. Some provinces such as Ardebil, Guilan, Kerman, Fars, Razavi Khorasan, and most notably Tehran as the capital of Iran, have a low but significantly higher incidence proportional to other provinces<sup>[26]</sup>. It is also indicates the presence of misclassification error between neighboring provinces that are expected to have similar incidence rates of cancer.

Knowledge of geographic pattern of diseases is useful to identify the influencing factors on disease incidence and planning for disease control and prevention<sup>[27,28]</sup>.



**Table 3** Age standardized rate of hepatocellular carcinoma

	ASR before Bayesian correction					ASR after Bayesian correction				
	2004	2005	2006	2007	2008	2004	2005	2006	2007	2008
South khorasan		0.50	0.46	0.46	0.47		0.83	0.98	0.95	1.12
Razavi khorasan	0.74	0.35	1.18	0.91	1.57	0.54	0.17	0.61	0.25	0.64
Tehran	0.43	0.44	0.57	0.46	2.23	0.37	0.37	0.51	0.41	2.12
Markazi	0.37	0.25	0.35	0.30	0.32	0.63	0.48	0.61	0.49	0.66
Sistan	0.44	0.21	0.26	0.52	0.63	1.07	0.50	1.14	1.65	1.90
Qom	0.67	0.55	0.95	0.45	0.48	0.90	0.78	1.22	0.65	1.05
Ghazvin	0.62	0.47	0.24	0.27	0.32	0.80	0.65	0.40	0.42	0.67
Khozesta	0.79	0.65	1.00	1.23	5.09	0.62	0.46	0.73	0.93	4.47
Ilam	1.13	0.86	0.54	0.49	0.78	1.88	1.50	1.23	1.07	2.23
Bushehr	0.45	0.36	0.85	0.83	0.82	1.05	0.87	1.75	1.97	3.16
Golestan	0.76	0.40	0.83	0.55	0.66	0.88	0.62	1.13	0.68	1.14
Mazandaran	0.22	0.46	0.61	0.28	1.04	0.18	0.35	0.43	0.20	0.80
North khorasan		0.62	0.62	0.76	0.86		0.94	1.28	1.34	1.89
Chaharmahal	0.84	1.13	1.02	0.52	1.01	1.17	1.57	1.55	1.01	1.62
Isfahan	0.41	0.61	0.64	0.85	0.83	0.27	0.40	0.44	0.68	0.52
Kohgilouye	0.39	0.32	1.15	1.36	1.54	0.68	0.90	1.78	2.18	2.51
Hormozgan	0.26	0.25	0.69	0.68	0.51	0.56	0.57	1.78	1.70	2.23
Fars	0.30	0.35	1.12	0.80	2.21	0.22	0.27	0.67	0.54	1.56
Ardebil	0.47	0.49	0.41	0.41	2.20	0.74	0.61	0.48	0.69	2.65
East azarbaijan	0.69	0.30	0.19	0.92	0.97	0.44	0.16	0.12	0.57	0.61
West azarbaijan	0.64	0.55	0.91	0.98	0.53	0.86	0.64	0.97	1.27	0.84

When a cluster with high incidence is not occurred by chance, this question comes to mind that what could be the underlying causal mechanism. It is natural to initially get focused on risk factors of the disease<sup>[29]</sup>. But major differences in incidence rate of HCC in neighboring provinces that are almost identical in exposure with risk factors, is justifiable with existence of misclassification error in registering patient permanent residence, that are diagnosed and registered in facilitate provinces of the country.

In conclusion there is misclassification error in cancer registry system despite international efforts to standardize cancer incidence data collection processes and elimination of deficiencies in personal and demographic information, especially in developing countries such as Iran<sup>[30]</sup>. So the true incidence rate of HCC is higher than the reported rate in some provinces and consequently lower in some other provinces. Since cancer registry data is used by health policy makers to allocate the facilities and resources to different provinces. To help for making the right decisions, it is necessary to correct for misclassification in cancer incidence between provinces. Otherwise again fewer resources will be assigned to low facility provinces based on the low incidence rate, while they are in need of more healthcare facilities and the true cancer incidence rate is more than taught in that provinces.

Iran is located in Middle East, a region where majority of HCC cases presents with intermediate or advanced stages of the disease<sup>[4,31]</sup>. In most Asian countries, early detection and treatment services are limited. There are many people who have no health insurance and many of them are too poor to go for screening tests or medical treatments. Therefore, it is important for the health organizations and governments in each country to

recognize these groups in order to reduce the incidence and mortality of cancers<sup>[5]</sup>.

The dramatic increase in the forecasted number of deaths due to HCC in the United States is a warning to the research and healthcare systems since it projected to be one of the top three cancer killers in 2030<sup>[32-34]</sup>.

So whereas deaths from liver are projected to increase, changes in treatment and prevention strategies, using screening tests, vaccination, and informing about risk factors and early symptoms of HCC can alter both the incidence and death rates. It requires an unisonant effort by search and health care organizations now for a substantial change in the future<sup>[7,9,34]</sup>. Also employing and training more motivated and educated staff in all sectors of cancer registry program in order to complete the cancer case registry forms accurately and remit them to the appropriate center, Enhancing hardware and software resources, expert researchers in medicine, biostatistics and computer science are needed to qualify the cancer registry program and increasing its completeness; specially in address-related information<sup>[35,36]</sup>.

In the absence of valid data, statistical methods are good alternatives for correcting the existed errors in data. Of course it should be noted that there is always some uncertainty as a potential weakness in statistical models and the statistical model which was used in this study is also not an exception. Thus a small cluster of HCC cases could be misattributed as patients registering in a neighboring province. But the low cost, high speed and efficiency of this model can compensate small errors.

## COMMENTS

### Background

Some patients from deprived provinces prefer to get medical treatment in their

neighboring provinces with more medical facilities without mentioning their permanent residence. It makes misclassification error in cancer registry data. Consequently health policy makers who use cancer registry data for resource allocation and cancer control programs will make mistakes in their decisions. The aim of this study is to investigate the trend of hepatocellular carcinoma incidence after correcting for misclassification between neighboring provinces by means of a Bayesian method.

### Research frontiers

Knowing about geographic spread of cancers is so important for identification the risk factors of cancers for control and prevention purposes. There is misclassification in patient's permanent residence in Iran's cancer registry data that leads to under-estimating the rate of cancer in some provinces and consequently over-estimating in other provinces. While those cancer rates are used in spatial analysis to determine the high risk areas, the existence of misclassification error is usually ignored. The hotspot of this study is accounting and correcting for misclassification in registering cancer incidence using the Bayesian method.

### Innovations and breakthroughs

By using the Bayesian method for estimating the rate of misclassification, that's enough to have prior information about the misclassification rate and there is no need for validating data to explore the misclassification rate which is costly and time consuming. Bayesian method for correcting the misclassification is a faster and more cost effective method in comparison to data validation which in many cases is not achievable.

### Applications

Cancer incidence rates are used for allocating medical resources to different provinces. So to have more accurate estimates from the rates of cancer incidence in each province misclassification error in registering patient's permanent residence should be corrected. Consequently better planning and decisions will be made for interventions for cancer control and prevention.

### Terminology

Bayesian method is a statistical method that assigns a prior distribution to parameters or events, according to expert's idea or previous knowledge from previous studies and updates those distributions with combining prior knowledge by observed data by using Bayes' theorem. Misclassification is one of the measurement error which is defined as disagreement between the observed value and the true value in categorical data.

### Peer-review

This is a very interesting study from Iran aimed at estimating the rate of regional misclassification in registering the incidence of hepatocellular carcinoma in cancer registry system using a Bayesian method. The study is original and very well written. The statistical analysis is well done. The results are consistent.

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## Usefulness of the MESH score in a European hepatocellular carcinoma cohort

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### Abstract

The Barcelona Clinic Liver Cancer classification is the most widely - used hepatocellular carcinoma (HCC) staging system because it is simple, precise and linked to a treatment algorithm based on randomized studies. But each group includes a broad spectrum of tumors, with limited therapeutic options, particularly for intermediate and advanced stages. Consequently, different additional scoring systems have been proposed to refine the prognosis and/or to improve the management. But until now, there is no consensus. Liu *et al* proposes a new scoring system, based on a large HCC cohort, with patients at different stages, treated using diverse modalities. This score includes six parameters used in current practice. It is simple to calculate, reliable, with an ability to predict survival superior to other systems, which also works with our European HCC cohort. The MESH score may be especially useful to differentiate subgroups with different prognosis for each treatment modality.

**Key words:** Hepatocellular carcinoma; Barcelona Clinic Liver Cancer; Scoring system; MESH; NIACE

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**Core tip:** The Barcelona Clinic Liver Cancer system has become the reference classification for hepatocellular carcinoma (HCC). But it has been criticized; each group includes a broad spectrum of tumors with limited therapeutic options. For this reason, different additional scoring systems have been proposed to improve the management. Liu *et al* proposes the MESH score, based on a large HCC cohort. It includes six parameters used in current practice, and in a European HCC cohort, this new score appears to be simple, reliable and useful to differentiate subgroups with different prognosis for each treatment modality.



Adhoute X, Pénaranda G, Raoul JL, Bourlière M. Usefulness of the MESH score in a European hepatocellular carcinoma cohort. *World J Hepatol* 2017; 9(15): 711-714 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i15/711.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i15.711>

## TO THE EDITOR

Hepatocellular carcinoma (HCC) staging system is still a controversial issue, and we have read with interest the article by Hsu *et al*<sup>[1]</sup> who proposed a new survival prognostic score for HCC called MESH. This score has been determined by multivariate analysis within a large HCC cohort ( $n = 1591$ ) mainly related to viral B hepatitis, mostly treated (44%) with curative strategy (surgery or radiofrequency ablation). The MESH score demonstrated a good predictive survival value, superior to other known staging and scoring systems [Barcelona Clinic Liver Cancer (BCLC), Hong Kong Liver Cancer (HKLC), Cancer of the Liver Italian Program (CLIP), Taipei Integrated Scoring system] within a large validation cohort ( $n = 1591$ ), with a lower Akaike information criterion (AIC) value, a higher homogeneity; within each BCLC stage and whatever treatment strategy (curative or palliative).

We have evaluated the prognostic value of the MESH score and compared it to other known staging and scoring systems [BCLC, HKLC, CLIP and NIAACE: Tumor Nodularity, Infiltrative nature of the tumor, serum Alpha-fetoprotein (AFP) level, Child-Pugh stage, Eastern Cooperative Oncology Group Performance Status (ECOG PS)<sup>[2]</sup>] within a French HCC cohort including 581 patients. Demographic and clinical characteristics of the 581 patients with HCC are shown in Table 1. Our patients were mostly male (82%), with a mean age of 67 years. Cirrhosis was present in 87% of our patients, CP A (64%), CP B (36%). Underlying liver disease was mostly related to alcohol abuse (37%) or viral C hepatitis (36%). HCC were multinodular in 61% of cases and vascular invasion or distant metastasis was found in 37% and 10% of patients, respectively. Baseline ECOG PS of our population (as expression of symptomatic tumor) was as follows: PS 0 (48%), PS 1 (23%), PS 2 (24%), PS 3-4 (5%). BCLC distribution was similar to the Liu cohorts: BCLC A 31%, B 16%, C 41% and D 12%. Treatment modalities were as follows: 23% were treated by surgery or radiofrequency ablation (RFA), 30% by transarterial chemoembolization, 26% by Sorafenib and 21% have received supportive care. Mean overall survival for the entire cohort was  $26.0 \pm 1.3$  mo, consistent with the median follow-up duration:  $18.3 \pm 20.3$  mo. Seventy-one percent of patients died. The discriminatory ability (linear trend  $\chi^2$  score), homogeneity ability (likelihood ratio test), prognostic stratification ability (AIC) and C-index were compared among scoring systems. Survivals between groups were compared using log-rank test in case of proportionality of hazards across time; generalized Wilcoxon test was used in case of non-proportionality of

**Table 1** Baseline characteristics in European hepatocellular carcinoma cohort ( $n = 581$ )  $n$  (%)

Patients characteristics	Cohort ( $n = 581$ )
Age, yr, mean $\pm$ SD	67.4 $\pm$ 11.7
Male	475 (82)
Etiology - HCV/HBV/ Alcohol/MS/others	209 (36)/41 (7)/215 (37)/87 (15)/29 (5)
Cirrhosis	505 (87)
Child - Pugh stage <sup>1</sup> A/B	323 (64)/182 (36)
Maximal tumor diameter, mean $\pm$ SD	60.9 $\pm$ 39.1
Tumor nodularities (1/2/ $\geq 3$ ), $n$ (%)	227 (39%)/76 (13%)/278 (48)
Infiltrative tumor	235 (40)
Extrahepatic metastasis	59 (10)
Vascular invasion	213 (37)
Performance status 0/1/2-4	276 (48)/136 (23)/169 (29)
Laboratory values (mean $\pm$ SD)	
Alkaline phosphatase (IU/L) > 200	112 (19)
PT (%), mean $\pm$ SD	78.0 $\pm$ 15.8
Albumin (g/L), mean $\pm$ SD	34.7 $\pm$ 6.1
Aspartate transaminase (IU/L), mean $\pm$ SD	68.7 $\pm$ 60.7
Alpha-fetoprotein (ng/mL), mean $\pm$ SD	5680 $\pm$ 31332
Tumor stages	
BCLC (A/B/C/D), $n$ (%)	181 (31)/92 (16)/241 (41)/67 (12)
Treatment allocation	
Resection or RFA, $n$ (%)	131 (23)
TACE, $n$ (%)	175 (30)
Sorafenib, $n$ (%)	152 (26)
Supportive care, $n$ (%)	123 (21)
Follow-up Time, mo, mean $\pm$ SD	18.3 $\pm$ 20.3
Deaths, $n$ (%)	413 (71)
Overall Survival, mo, mean $\pm$ SD	26.0 $\pm$ 1.3

<sup>1</sup>Cirrhotic patients. HCV: Hepatitis C virus; HBV: Hepatitis B virus; MS: Metabolic syndrom; PT: Prothrombin time; BCLC: Barcelona Clinic Liver Cancer; RFA: Radiofrequency ablation; TACE: Trans arterial chemoembolization.

hazards.

Each staging system showed a significant difference in the probability of survival across the stages ( $P < 0.0001$ ). The MESH score determined subgroups of different survival prognosis in our cohort: MESH 0: 66 (40-68) mo, MESH 1: 37 (22-80) mo, MESH 2: 21 (13-49) mo, MESH 3: 10 (6-20) mo, MESH 4: 5 (4-9) mo, MESH 5 and 6: 4 (2-6) mo;  $P$  (Wilcoxon)  $< 0.0001$ . Its predictive value on survival was higher than other scores or classifications (BCLC, HKLC and CLIP) within this cohort with a lower AIC, a higher homogeneity, a higher c-Index (Table 2). However the NIAACE score obtained the best prognostic information.

The BCLC system has become the reference classification by its simplicity, its prognostic value and a treatment algorithm based on randomized clinical trials. But each BCLC stage includes a broad spectrum of tumors of different prognosis<sup>[2-5]</sup>, with one therapeutic option for stages B and C. Some stage B HCC patients could be good candidates for surgery<sup>[6,7]</sup>, unlike other BCLC B

**Table 2** Comparison of performances of each scoring systems in the entire cohort

Score	Discriminatory ability linear trend test		Homogeneity likelihood ratio test		Akaike Information Criterion	C-index (95%CI)
	LT ( $\chi^2$ )	P value	LR ( $\chi^2$ )	P value		
MESH	145.125	< 0.0001	372.4846	< 0.0001	4145.284	0.830
BCLC	137.845	< 0.0001	327.5024	< 0.0001	4194.266	0.806
HKLC	104.966	< 0.0001	387.2755	< 0.0001	4146.493	0.811
CLIP	108.423	< 0.0001	341.3485	< 0.0001	4101.288	0.816
NIACE	144.998	< 0.0001	425.6698	< 0.0001	4092.099	0.853

MESH: Model to estimate survival for HCC; BCLC: Barcelona Clinic Liver Cancer; HKLC: Hong Kong Liver Cancer; CLIP: Cancer of the Liver Italian Program; NIACE: Tumor Nodularity, Infiltrative nature of the tumor, Serum Alpha-fetoprotein level, Child-Pugh stage, Eastern Cooperative Oncology Group Performance Status; LT: Linear trend; LR: Likelihood ratio.

**Table 3** Comparison of performances of each scoring systems in patients treated by surgery/radiofrequency ablation

Score	Discriminatory ability linear trend test		Homogeneity likelihood ratio test		Akaike Information Criterion	C-index (95%CI)
	LT ( $\chi^2$ )	P value	LR ( $\chi^2$ )	P value		
MESH	21.5588	< 0.0001	23.3342	< 0.0001	346.508	0.719
BCLC	15.5560	< 0.0001	12.4538	0.0020	359.388	0.644
HKLC	5.9647	0.0146	18.9510	0.0020	358.891	0.629
CLIP	9.9391	0.0016	13.1460	0.0003	356.696	0.642
NIACE	19.1701	< 0.0001	23.1937	< 0.0001	346.648	0.672

MESH: Model to estimate survival for HCC; BCLC: Barcelona Clinic Liver Cancer; HKLC: Hong Kong Liver Cancer; CLIP: Cancer of the Liver Italian Program; NIACE: Tumor Nodularity, Infiltrative nature of the tumor, serum Alpha-fetoprotein level, Child-Pugh stage, Eastern Cooperative Oncology Group Performance Status.

HCC patients who do not benefit from the recommended treatment namely the chemoembolization<sup>[8]</sup>. Consequently, different staging or scoring systems have been proposed in the last years, in order to improve its prognostic value<sup>[1]</sup> and/or the decision making process<sup>[8,9]</sup>. A prognostic score needs to be easy to use, reliable and useful, and the MESH score fulfills these conditions. It has a good prognostic value, especially for HCC patients treated by surgery/RFA (Table 3); it is easy to use by adding up the points of each variable, and it includes six parameters used in daily clinical practice, an essential part of HCC management. Actually, it incorporates tumor-related characteristics, general conditions and liver function, as well as two easily available biological variables (AFP, alkaline phosphatase) correlated to the HCC patients' survival, absent from the BCLC and HKLC classifications.

The MESH score could be useful for HCC management. It distinguishes two different prognostic groups within BCLC A HCC patients treated by surgery/RFA in our cohort [MESH  $\leq$  2: 68 (44-74) mo vs MESH > 2: 7 (5-7) mo,  $P$  (Wilcoxon) = 0.0292], within BCLC B HCC patients treated by TACE [MESH  $\leq$  2: 20 (15-50) mo vs MESH > 2: 14 (7-20) mo,  $P$  (Log-Rank) = 0.0078], or within BCLC C HCC patients treated by Sorafenib [MESH  $\leq$  3: 10 (6-26) mo vs MESH > 3: 5 (3-8) mo,  $P$  (Log-Rank) < 0.0001]. Thus, it could help clinicians in the treatment decision. We observed the same findings with the NIACE score whatever HCC stages and treatment modalities<sup>[10]</sup>.

The BCLC treatment recommendations are seldom followed<sup>[11,12]</sup>, related to a strict treatment algorithm and great prognosis heterogeneity within each BCLC stage. In

our cohort, 65% of patients have been treated according to the BCLC recommendations and for some authors other options are possible<sup>[13,14]</sup>.

We have checked that the MESH score provides good prognostic information within a European HCC cohort, whatever the treatment modalities, including HCC patients treated according to the BCLC guidelines. But these findings show once again that additional variables such as AFP and/or tumor morphology may influence HCC prognosis and its therapeutic management<sup>[15]</sup>. If the BCLC system is unavoidable, there are sufficient arguments for a prospective clinical trial to validate the usefulness of this new strategy based on a combination of BCLC system and scores<sup>[16]</sup> such as NIACE or MESH, and to determine which one to use.

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*WJH* covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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## Non-alcoholic fatty liver disease: An expanded review

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### Abstract

Non-alcoholic fatty liver disease (NAFLD) encompasses the simple steatosis to more progressive steatosis with associated hepatitis, fibrosis, cirrhosis, and in some cases hepatocellular carcinoma. NAFLD is a growing epidemic, not only in the United States, but worldwide

in part due to obesity and insulin resistance leading to liver accumulation of triglycerides and free fatty acids. Numerous risk factors for the development of NAFLD have been espoused with most having some form of metabolic derangement or insulin resistance at the core of its pathophysiology. NAFLD patients are at increased risk of liver-related as well as cardiovascular mortality, and NAFLD is rapidly becoming the leading indication for liver transplantation. Liver biopsy remains the gold standard for definitive diagnosis, but the development of noninvasive advanced imaging, biochemical and genetic tests will no doubt provide future clinicians with a great deal of information and opportunity for enhanced understanding of the pathogenesis and targeted treatment. As it currently stands several medications/supplements are being used in the treatment of NAFLD; however, none seem to be the "magic bullet" in curtailing this growing problem yet. In this review we summarized the current knowledge of NAFLD epidemiology, risk factors, diagnosis, pathogenesis, pathologic changes, natural history, and treatment in order to aid in further understanding this disease and better managing NAFLD patients.

**Key words:** Non-alcoholic fatty liver disease; Metabolic syndrome; Steatohepatitis; Hepatocellular carcinoma; Steatosis

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**Core tip:** Non-alcoholic fatty liver disease (NAFLD) is a growing epidemic, not only in the United States, but worldwide in part due to obesity and insulin resistance leading to liver accumulation of triglycerides and free fatty acids. NAFLD patients are at increased risk of liver-related as well as cardiovascular mortality, and NAFLD is rapidly becoming the leading indication for liver transplantation. Numerous risk factors for the development of NAFLD have been espoused with most having some form of metabolic derangement or insulin resistance at the core of its pathophysiology. However, the exact pathogenic mechanism of NAFLD still remains unclear, and there



is no effective treatment yet so far. In this review we summarized the current knowledge of NAFLD epidemiology, risk factors, diagnosis, pathogenesis, pathologic changes, natural history, and treatment.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an umbrella term and encompasses the simple deposition of adipose tissue in the liver to more progressive steatosis with associated hepatitis, fibrosis, cirrhosis, and in some cases hepatocellular carcinoma (HCC)<sup>[1]</sup>. For the sake of terminology, NAFLD is comprised of non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH)<sup>[1]</sup>. NAFL is characterized by steatosis of the liver, involving greater than 5% of parenchyma, with no evidence of hepatocyte injury<sup>[2]</sup>. Whereas, NASH is defined by histologic terms, that is a necroinflammatory process whereby the liver cells become injured in a background of steatosis<sup>[2]</sup>. Although the natural history of NAFLD remains incompletely characterized, what is clear from the published data is a risk of progression to cirrhosis and HCC<sup>[3-7]</sup>. However, whether there is a clear progression of NAFL to NASH is under active investigation, but early evidence suggests this could be the case<sup>[1]</sup>. In terms of epidemiology, several studies have tried to quantify the true worldwide incidence of NAFL/NASH; however, due to extreme variations in study parameters and available testing, a clear and reliable occurrence rate is not currently available<sup>[1]</sup>. With that being said, estimates have been posited suggesting the incidence of NAFLD to be 20%-30% in Western countries and 5%-18% in Asia<sup>[1]</sup>. It is no surprise that the prevalence of NAFLD is increasing worldwide with each passing year, given the current trends in dietary irresponsibility and preponderance of a sedentary lifestyle<sup>[1]</sup>. Additionally, there has been a linear rise of NAFLD with that of diabetes and metabolic syndrome<sup>[3]</sup>. In one study from the United States, it was shown that the incidence of NAFLD was 10% higher in overweight individuals compared to lean persons<sup>[8]</sup>. In fact, NAFLD has been projected, within the next 20 years, to become the major cause of liver related morbidity and mortality as well as a leading indication for liver transplantation<sup>[3]</sup>. As it currently stands, NAFLD represents the second most common reason to be listed for a liver transplant<sup>[9]</sup>. Additionally, not only does NAFLD place a strain on the medical system and its resources, it also is associated with a 34%-69% chance of dying over the next 15 years when compared with the general population<sup>[9]</sup>. The pathogenetic processes that underscore NAFLD typically

lead to death by cardiovascular disease with liver related mortality only accounting for 5% in these individuals<sup>[9,10]</sup>. In the forthcoming sections we will provide context for how and why NAFLD develops, current genetic proposals, histologic criteria, differential diagnoses, and prognosis of this very important disease affecting not only the United States but much of the world.

## RISK FACTORS AND ETIOLOGY

### *Metabolic syndrome and type 2 diabetes mellitus*

Metabolic syndrome is a conglomerate of cardiovascular risk factors which predispose a person to developing type II diabetes and cardiovascular disease<sup>[2]</sup>. The current diagnostic criteria require having 3 of 5 of the following factors: Triglycerides 150 mg/dL or greater, high-density lipoprotein-cholesterol of less than 40 mg/dL in men and less than 50 mg/dL in women, hyperglycemia (fasting glucose of 100 g/dL or greater), an increased waist circumference (defined by population specific data), and hypertension (systolic blood pressure of 130 mmHg or greater or diastolic blood pressure of 85 mmHg or greater)<sup>[2]</sup>. As previously mentioned the incidence of NAFLD has been increasing in concert with the rising rates of metabolic syndrome. In fact it has been stated that the incidence of NAFLD increases with increasing number of metabolic syndrome criteria met<sup>[2]</sup>. When compared to non-diabetic patients (matched for age, sex, and body weight), type 2 diabetes mellitus (T2DM) patients have liver fat contents that are 80% higher<sup>[11]</sup>. Interestingly, it has been shown that T2DM patients with NAFLD can have normal liver function tests, which may lead one to believe that the prevalence of NAFLD in T2DM patients is much higher than reported in this patient population<sup>[11]</sup>. Additionally, T2DM patients display a very high risk of developing NASH as well as a two-to-four-fold increased risk of fatty liver associated complications<sup>[11,12]</sup>.

### *Ethnic differences*

The rate at which NAFLD develops has been shown to be greatest in Hispanic patients<sup>[13]</sup>. Also, NAFLD in the Asian population has been increasing, and interestingly, can be seen in those who have a normal body mass index<sup>[13]</sup>. In a United States based study, the investigators found a lower degree of steatosis in African Americans when compared to whites and also showed a higher degree of NAFLD findings in Asians and Hispanics<sup>[14]</sup>. The Hispanic population also has been shown to have a higher occurrence of steatohepatitis and cirrhosis, while those who are African American enjoy a decreased chance of developing liver failure<sup>[15]</sup>. With further genetic investigation by genome wide association, it was noted that Hispanics had a twofold higher liver fat content if they possessed the homozygous PNPLA3 allele (patatin-like phospholipase domain-containing protein 3 rs738409)<sup>[15]</sup>. The PNPLA3 gene family has been shown to affect lipid metabolism and patients who harbor this polymorphism were found to have increased hepatic fat content, triglyceride stores,

and inflammation<sup>[13]</sup>. In fact, the mutation of *PNPLA3 rs738409* gene (encoding I148M) has revealed more severe histologic features of NAFLD in those carrying the mutation<sup>[13]</sup>. More information on the genetic basis for NAFLD can be found under the “genetics” heading.

### Gender and age

Unfortunately, the role of gender in the development of NAFLD has been met with differing conclusions in the literature. Several studies provide data to suggest a higher prevalence in males while others proposed the opposite<sup>[1]</sup>. However, according to Lonardo *et al.*<sup>[11]</sup> epidemiological review, NAFLD is more common in men and has been shown to increase in those who are younger to middle aged with a decline noted after the age of 50-60 years. In contrast, NAFLD has been shown to spare those women who are pre-menopausal and then a rise in incidence occurs after the age of 50 with a peak at 60-69 years, and the preponderance of evidence does seem to suggest that NASH is histologically more severe in women when compared to men<sup>[11]</sup>. It has been reported that the prevalence of NAFLD increases with age (20% in people younger than age 20) to greater than 40% in those who are older than 60 years of age<sup>[16]</sup>. Not only does the prevalence of NAFLD increase with increasing age, but the incidence of NASH and cirrhosis also increases in those patients who are 50 years of age or greater compared with younger age groups<sup>[1]</sup>. Notably, it has been suggested that NAFLD begins in utero based on several studies, using magnetic resonance spectroscopy, showing steatosis in infants born to mothers with gestational diabetes (GD)<sup>[17]</sup>. In a study using hepatic fat fraction (HFF), performed at 1-3 wk of age in neonates born to normal mothers compared to those with gestational diabetes, neonates born to obese mothers with GD had a mean HFF that was 68% higher than those born to normal weight mothers<sup>[18]</sup>. In another study by Patel *et al.*<sup>[19]</sup>, 33 stillborn babies of diabetic mothers were compared with 48 stillborn babies of mothers without diabetes and there was a markedly increased rate of hepatic steatosis in neonates born to mothers with diabetes (79%) vs controls (17%). A study with 191 Italian children with biopsy confirmed NAFLD, showed hepatic steatosis, inflammation, hepatocyte ballooning, and fibrosis were worse in those children who were not breast-fed compared to those who were<sup>[20]</sup>. Similar to what has been observed in adults, obesity is a considerable risk factor for the development of NAFLD in children<sup>[21]</sup>. According to the Study of Child and Adolescent Liver Epidemiology, approximately one-third of obese children have NAFLD<sup>[22]</sup>. With that being said, a fatty liver is the most common liver abnormality found in children aged 2-19 years<sup>[22]</sup>. Again like that seen in adulthood, there is also an association of pediatric NAFLD and cardiovascular disease with higher levels of total cholesterol, LDL, triglycerides, and systolic blood pressure reported<sup>[21]</sup>. As it currently stands the incidence of HCC in the pediatric population with NAFLD is not

known but thought to be rare<sup>[17]</sup>. Only one case report of HCC with concurrent NAFLD in a 7-year-old boy has been reported<sup>[23]</sup>. Longitudinal outcomes are sparse for pediatric patients with NAFLD; however, what is known is that children can present with cirrhosis at diagnosis and may progress from NASH to cirrhosis<sup>[24]</sup>.

### Diet, smoking and life style

Diet has been thought of as an independent risk factor for the development of NAFLD, specifically, a diet high in fats<sup>[15]</sup>. It has been shown, through energy restriction and manipulation of dietary macronutrients, namely, restriction of carbohydrates, fat, or enrichment with monounsaturated fatty acids, that dietary modifications can reduce metabolic syndrome<sup>[25,26]</sup>. Diets that model after a Westernized pattern, such as those high in red meat consumption, refined grains, pastries, and sugar laden beverages are associated with a greater likelihood for the development of metabolic syndrome and subsequent NAFLD<sup>[15]</sup>. In a retrospective study with 2029 participants, cigarette smoking was found to be an independent risk factor for the onset of NAFLD<sup>[27]</sup>. The use of tobacco predisposes a person for the development of insulin resistance<sup>[28-30]</sup>. Additionally, in a study looking at adolescents in the United States, passive and active smoke exposure are strong independent predictors of metabolic syndrome<sup>[31]</sup>. As to life style, associations have been shown between a person's fitness and sedentary behavior with the risk of developing NAFLD and NASH; the severity of NAFLD also intensifies with lower physical activity<sup>[15]</sup>. In fact, as part of the EASL-EASD-EASO Clinical practice guidelines for the management of NAFLD, a recommendation for the assessment of physical activity habits should be included as part of a comprehensive NAFLD screening exam<sup>[32]</sup>. Additionally, part of the treatment regimen for NAFLD incorporates diet and physical activity to address obesity and insulin resistance. Several studies have evaluated the effect of a balanced diet with gradual weight reduction and their effects of NAFLD biologic parameters. Overwhelmingly, gradual weight reduction through diet, with or without exercise, have shown improvements in serum liver enzymes, reduced hepatic fatty infiltration, decreased hepatic inflammation and reduced levels of fibrosis<sup>[33]</sup>. Also there is a clear benefit of exercise on hepatic fatty infiltration; this benefit is even evident with minimal or no weight loss and exercise levels that fall below those which are recommended for obesity management<sup>[34]</sup>. According to a systematic review, NAFLD is also improved with resistance exercise (as opposed to the therapeutic benefits of aerobic activities such as running), which may be more tolerable for the NAFLD patients who suffer from poor cardiorespiratory fitness and cannot tolerate intense aerobic exercise<sup>[35]</sup>.

### Polycystic ovarian syndrome

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder in reproductive aged women and is typically

characterized by obesity and insulin resistance<sup>[36]</sup>. Hence, women with PCOS are at a heightened risk of developing T2DM<sup>[36]</sup>. In a study that evaluated 600 women with PCOS and 125 body mass index (BMI)-matched healthy control women, the prevalence of NAFLD was found to be higher in those with PCOS<sup>[36]</sup>. Insulin resistance and obesity, as have been previously examined in this paper, are known to contribute to the development of NAFLD. Women with PCOS are typically hyperandrogenemic and insulin resistance worsens the hyperandrogenemia by increasing ovarian androgen synthesis and decreasing liver SHBG production, which results in elevated circulating levels of free androgens<sup>[36]</sup>. The subsequent hyperandrogenemia is associated with a more prominent insulin resistance in patients with PCOS, which endangers these patients for developing NAFLD<sup>[36]</sup>. Numerous other investigations into the association of PCOS and NAFLD have been performed and similar results were obtained<sup>[37-40]</sup>.

### Obstructive sleep apnea

Obstructive sleep apnea (OSA) is characterized by complete or partial airway obstruction caused by pharyngeal collapse during sleep<sup>[41]</sup>. A budding association of OSA with diabetes mellitus, metabolic syndrome, and cardiovascular disease has started to appear in the last few years<sup>[41]</sup>. In the general population, obstructive sleep apnea has a prevalence of around 4% with that number jumping to 35%-45% in obese individuals<sup>[15]</sup>. In a study performed by Tanné *et al.*<sup>[42]</sup>, patients with severe OSA were found to be more insulin resistant and had a higher percentage of steatosis as well as increased necrosis and fibrosis scores (on liver biopsy) when compared to those patients without OSA and a similar BMI. The pathogenic mechanisms that underpin this association is believed to be due to the alteration of gas exchange (repetitive hypoxemic and hypercapnic events), termed chronic intermittent hypoxia, which can lead to an increase in proinflammatory cytokines, endothelial dysfunction, oxidative stress, metabolic dysregulation, and finally insulin resistance<sup>[41]</sup>. Interestingly, OSA may be one of the elements promoting the evolution of NAFLD from steatosis to NASH<sup>[41]</sup>. Additionally, using animal models, OSA was shown to promote the digression of NAFLD to NASH<sup>[15]</sup>. Investigational evidence has suggested that chronic intermittent hypoxia may trigger liver injury, inflammation, and fibrogenesis with several studies showing an intriguing relationship between OSA and NASH<sup>[41,43-48]</sup>.

## GENETICS

Data from numerous studies have given evidence for a heritable component to NAFLD and includes: Familial aggregation, twin studies, and interethnic differences in susceptibility<sup>[49-57]</sup>. Whole exome sequencing studies performed on obese Caucasian participants with NAFLD have revealed deleterious mutations in Bardet-Biedl syndrome 1 gene as well as the Melanocortin 3 receptor gene<sup>[58]</sup>. In 2008, the first genome wide association study was published; it examined hepatic triacylglycerol (HTAG)

accumulation and identified association with increased HTAG and the *PNPLA3* gene<sup>[59]</sup>. This single nucleotide polymorphism is a nonsynonymous cytosine to guanine nucleotide transversion mutation that results in an isoleucine to methionine amino acid change. Subsequent work has confirmed this variant (*PNPLA3* rs738409) in Japanese, Indian, and Chinese NAFLD patients<sup>[60-65]</sup>. In a meta-analysis of 24 studies with 9915 participants, Singal *et al.*<sup>[66]</sup> found that *PNPLA3* was associated with fibrosis severity. Additionally, among nine studies, totaling 2937 participants, the *PNPLA3* was again linked with increased risk for the development of HCC in those with cirrhosis<sup>[66]</sup>. A separate meta-analysis, 16 studies included, revealed the rs738409 GG genotype compared to the CC genotype was linked to a 73% greater liver fat content as well as a 3.24-fold increased risk of more pronounced necroinflammatory scores and a 3.2-fold increased risk of developing fibrosis<sup>[67]</sup>. Xu *et al.*<sup>[68]</sup>, by way of meta-analysis totaling 23 case-control studies (totaling 6071 NAFLD participants and 10366 controls) found the *PNPLA3* rs738409 polymorphism to have a significant association with a high cross-ethnicity risk for NAFLD as well as NASH. Genome-wide association study performed on 236 non-Hispanic white women with NAFLD (324623 single nucleotide polymorphisms in total from 22 autosomal chromosomes) found the NAFLD activity score to be associated with the SNP rs2645424, the degree of fibrosis associated with SNP rs343062, lobular inflammation with SNPs rs1227756, rs6591182, and rs887304, increased levels of ALT was associated with SNPs rs2499604, rs6487679, rs1421201, and finally rs2710833<sup>[69]</sup>. Using exome-wide association, Kozlitina *et al.*<sup>[70]</sup> found three variants to be associated with higher liver fat levels: Two in the aforementioned *PNPLA3* and one in the *TM6SF2* gene, which likely is required for normal VLDL secretion. The variant frequency in *TM6SF2* gene was found to be highest in those of European, African-American, and Hispanic ancestry<sup>[58]</sup>. In a later study by Mahdessian *et al.*<sup>[71]</sup>; the *TM6SF2* gene was found to be a regulator of liver fat metabolism, which influenced triglyceride secretion and hepatic lipid droplet content. As it stands currently, approximately 7 categories of genes have been associated with NAFLD and are broken down as follows: (1) hepatic lipid export/oxidation in steatosis (*PNPLA3*, *TM6SF2*, *NR1H2*, *PPAR-alpha*, *PEMT*, *MTTP*, *APOC3* and *APOE*); (2) glucose metabolism and insulin resistance (*ENPP1/IRS1*, *GCKR*, *SLC2A1*, *GOAT*, *TCF7L2* and *PPARG*); (3) steatosis-hepatic lipid import or synthesis (*SLC27A5*, *FADS1*, and *LPIN1*); (4) steatohepatitis-oxidative stress (*HFE*, *GCLC*/*GCLM*, *ABCC2* and *SOD2*); (5) steatohepatitis-endotoxin response (*TLR4* and *CD14*); (6) cytokines (*TNF* and *IL6*); and (7) fibrosis (*AGTR1* and *KLF6*)<sup>[49,72]</sup>.

## PATHOGENESIS

Non-alcoholic fatty liver disease, not surprisingly, as its name implies revolves around the deposition of fat within the liver. Specifically, free fatty acids and triglyceride accumulation is

the hallmark feature and has been attributed, at least in part, to insulin resistance and obesity<sup>[73]</sup>. With that being said, the pathogenic components of NAFLD are complex and multifactorial with different theories presented in the literature<sup>[74]</sup>. A two-hit model of NAFLD development has been proposed with the first hit consisting of: Hepatic lipid accumulation, sedentary lifestyle, high fat diet, obesity, and insulin resistance<sup>[74]</sup>. The second hit activates an inflammatory event with associated fibrogenesis<sup>[75]</sup>. This two-hit model has lost some favor as it was believed to be too simplistic to fully describe the intricacy of human NAFLD where a multitude of factors are acting in concert with one another in a genetically predisposed individual<sup>[74]</sup>. As was described in the risk factors, a multitude of factors contribute and have some association with the development of NAFLD<sup>[76]</sup>. However, it is insulin resistance that plays a key role in the development of steatosis/NASH, which results in hepatic *de novo* lipogenesis and subsequent reduction of adipose tissue lipolysis, with a consequent increase of fatty acids in the liver<sup>[77]</sup>. Alterations in the production and secretion of adipokines and inflammatory cytokines are a consequence of adipose tissue dysfunction, which is brought about by insulin resistance<sup>[78]</sup>. The production of reactive oxygen species and endoplasmic reticulum stress coupled with mitochondrial dysfunction occurs as a result of fat accumulations in the liver, specifically in the form of triglycerides<sup>[79]</sup>. An excess of nutrients essentially overwhelms the endoplasmic reticulum, which then turns on the unfolded protein response and as a consequence, triggers the development of insulin resistance through a number of mechanisms, including c-jun N-terminal kinase activation and inflammation<sup>[79]</sup>. The gut microbiota has been recognized as one of the key players in the pathogenesis of NAFLD. Gut microbiota not only influences absorption and disposal of nutrients to the liver, but also conditions hepatic inflammation by supplying toll-like receptor ligands, which can stimulate liver cells to produce proinflammatory cytokines. Accordingly, the modification of intestinal bacterial flora by specific probiotics has been proposed as a therapeutic approach for the treatment of NASH<sup>[80]</sup>. Interestingly, dysfunctional adipose tissue, as seen in obesity, T2DM and NAFLD, impairs glucose and lipid metabolism by two mechanisms: One, by acting as an endocrine organ, which is releasing a number of fat-derived cytokines; and two, by free fatty acid-induced ectopic fat deposition and lipotoxicity<sup>[79]</sup>.

Liver transplantation is performed for a variety of reasons: Liver failure, end-stage liver disease, tumors; however, after surgery these patients often develop an increase in body weight, subsequent insulin resistance, and metabolic perturbations<sup>[81]</sup>. Additionally, patients who undergo a liver transplant may also fall prey to diabetes mellitus, hyperlipidemia, and arterial hypertension<sup>[81]</sup>. In part, some the metabolic derangements that occur after liver transplantation are due to medication effects (*i.e.*, corticosteroids, calcineurin inhibitors, and sirolimus promote hyperglycemia, hypertension, and hyperlipidemia)<sup>[81]</sup>.

Many of the effects aforementioned can be found in the diagnostic criteria of metabolic syndrome, and as previously discussed, NAFLD is essentially the liver's manifestation of this syndrome. Hence, it is not surprising to see recurrent or *de novo* NAFLD/NASH after a liver transplant<sup>[82]</sup>. It is important to note that 15.5% and 26.3% of liver transplant patients, at one and three years, respectively, become clinically obese<sup>[83]</sup>. Likewise, post-transplant development of DM is reported to range from 10%-64%, although the underlying mechanisms for this is yet to be entirely worked out<sup>[84]</sup>. However, it does appear that the main risk factors for the development of post liver transplant DM would include: Male gender, obesity, family history, hepatitis C virus (HCV), older age range, and high dose immunosuppressives<sup>[84]</sup>. Additionally, the rate of metabolic syndrome development post liver transplant is approximately 50%-60%<sup>[85]</sup>. In a cohort comprising 170 transplant patients followed for two years, the researchers showed the presence of metabolic syndrome in approximately one-third<sup>[86]</sup>. Not surprisingly, the incidence of NAFLD after having received a liver transplantation ranges from 18%-40% and the incidence of NASH ranges from 9%-13%<sup>[87]</sup>. Intriguingly, post-transplant NAFLD risk has also been tied to polymorphisms in PNPLA3, which has been shown to mediate triglyceride hydrolysis and is also associated with pretransplant obesity and NAFLD<sup>[87]</sup>. Overall, the natural history of post-liver transplant NAFLD is incompletely understood, however, it may contribute to increased cardiovascular disease mortality in these patients<sup>[87]</sup>.

## HISTOPATHOLOGY

Non-alcoholic fatty liver disease shows a wide range of histologic manifestations, which can range from a very mild steatosis (5% or more of hepatocytes involved), to more aggressive forms showing lobular and/or portal inflammation, ballooning hepatocytes, fibrosis, and ultimately cirrhosis<sup>[88]</sup>. The presence of less than 5% of steatosis is not regarded as clinically significant. In adult patients, steatosis typically affects the centrilobular hepatocytes first; whereas in children the periportal or panacinar patterns are more likely seen<sup>[89]</sup>. Steatosis comes in a few morphologic appearances, the macrovesicular terminology is used when large lipid droplets inhabit the cytoplasm and displace the nucleus<sup>[90]</sup>. However, macrovesicular steatosis also encompasses small lipid droplets, which varying in size and keep their nuclear central location<sup>[90]</sup>. Finally, the terminology of microvesicular steatosis denotes the accumulation of innumerable lipid droplets with the hepatocyte nucleus remaining essentially in its original location<sup>[90,91]</sup>. It is important to note that microvesicular steatosis is rare in isolation but has been reported to occur in a patchy distribution (approximately 10% of NAFLD cases)<sup>[90,91]</sup>. With that being said the presence of pure microvesicular steatosis has been reported somewhat more commonly in the diagnosis of alcoholic fatty liver disease (so-named alcoholic foamy degeneration)<sup>[92]</sup>. As was alluded to earlier in this paper,



lipid is a dynamic and metabolically active substance and the same holds true for fatty lipid droplets in the liver. Lipid droplets are comprised of a core of triacylglycerols with or without cholesterol esters and a peripheral monolayer of phospholipids<sup>[93]</sup>. Inactive PNPLA3 has been shown to accumulate on the surface of lipid droplets and is linked to an increase in macrovesicular steatosis<sup>[94]</sup>. Recent studies have espoused that the loss of reticulon seen in those patients with extensive steatosis may not be related to the presence of inflammation or fibrosis; the effects of such a loss in connective framework has yet to be determined, however, this finding should be remembered when HCC enters the differential diagnosis<sup>[95]</sup>.

### **Assessment of the extent of steatosis**

With the starting point of at least 5% steatotic involvement being pathologic, the affected parenchyma is then divided into thirds: 5%-33%, 34%-66% and > 66%<sup>[96]</sup>. The rule of thirds has allowed a three-tiered classification system with 5%-33% designated as mild, 34%-66% designated as moderate, and > 66% corresponding to severe steatosis<sup>[96]</sup>. Steatosis, when not in abundance, is typically centered in a zone 3 distribution but when prominent can be found in a panacinar location<sup>[90]</sup>. In a patient who has resolving hepatic steatosis, the fat droplets can be found in an irregular distribution throughout the acinus<sup>[90]</sup>. In a more rare occurrence, the steatosis may be found in a zone 1 location with disease progression to cirrhosis leading to a more irregular distribution or complete loss of steatotic droplets<sup>[90]</sup>. There has been a documented tendency to overestimate the degree by which the liver parenchyma is involved by steatosis among pathologists, hence more accurate and objective methods have employed the use of digital imaging analysis<sup>[97]</sup>. It is important to point out that conventional imaging (ultrasound, computed tomography, or magnetic resonance imaging), are not sensitive enough to detect hepatic steatosis when the percent involvement is less than 30%<sup>[91]</sup>. More advanced imaging techniques such as controlled attenuation parameter, magnetic resonance imaging-estimated proton density fat fraction, and <sup>1</sup>H-magnetic resonance spectroscopy have been shown to correlate well with histologic steatosis assessment in both the adult and pediatric NAFLD populations<sup>[98,99]</sup>.

### **Steatosis with inflammation and/or fibrosis**

In the realm of NAFLD, steatosis rarely is identified as the only finding and is oftentimes accompanied by a chronic inflammatory infiltrate (typically mononuclear) with varied severity, few plasma cells and monocytes may also be encountered<sup>[91]</sup>. Neutrophils make a rare appearance with occasional eosinophils in the presence of a lipogranuloma (a structure composed of a central steatotic hepatocyte or fat droplet and a peripheral accumulation of mononuclear cells and macrophages)<sup>[91]</sup>. Kupffer cell density in NAFLD has correlated with the degree of necroinflammatory activity, injury, and degree of fibrosis<sup>[100]</sup>. In fact, it is the Kupffer cell that is believed

to play a commanding role in the pathogenesis of NAFLD with its regulation of hepatic triglyceride storage, mediation of inflammatory activity, and hepatocyte injury to include parenchymal fibrosis<sup>[100]</sup>. In the strictest and most traditional of viewpoints of NAFLD, the presence of hepatocyte injury and fibrosis were thought to be a product of disease progression to steatohepatitis<sup>[89]</sup>. However, some mild NAFLD cases encountered in adults have shown a very mild degree of fibrosis, mainly centered on the portal area or occasionally zone 3<sup>[91]</sup>. A note of clarification is in order due to some confusion which may occur with NASH. In NASH, most experts would agree that the most basic criteria of hepatocyte ballooning in addition to steatosis and inflammation must be met in order to render a diagnosis of NASH<sup>[88,101]</sup>. It is, as of yet, still unclear whether these patients with NAFLD (*i.e.*, not NASH) and a mild component of inflammation/fibrosis have as benign of a course when compared with those who have steatosis alone<sup>[90]</sup>. Conflicting reports on progression are found in the literature with some suggesting that these cases may evolve to more severe disease, typically at a slower rate, while others have shown these lesions may stabilize or regress<sup>[102,103]</sup>.

### **Steatohepatitis**

Ballooned hepatocytes with accompanied steatosis and inflammation are typically found in zone 3 of the hepatic microanatomy<sup>[91]</sup>. Some recent work using immunohistochemistry, specifically CK8/18, have shown that ballooned hepatocytes display significantly decreased expression compared to normal hepatocytes<sup>[90]</sup>. As it currently stands, the use of immunohistochemical stains for differentiating ballooned hepatocytes is not currently a common practice<sup>[90]</sup>. Although the exact mechanisms by which a hepatocyte takes on a ballooned appearance are not entirely elucidated, some proposed mechanisms include: Oxidative stress alteration of microtubules, loss of intermediate filament cytoskeleton, retention of fluid, modifications to small droplet fat and endoplasmic reticulum dilatation<sup>[104-108]</sup>. Mallory-Denk bodies, glycogenated nuclei, acinar lipogranulomas, megamitochondria, pericellular fibrosis, and acidophilic bodies are frequently seen in NASH, but are not required for the diagnosis<sup>[101]</sup>. Ductular reaction can be seen in NASH as well and is usually associated with fibrosis<sup>[90]</sup>. It is important to keep in mind that no single feature is entirely specific for the diagnosis of NASH<sup>[91]</sup>.

### **Fibrosis**

The impact of fibrosis cannot be overstated when discussing NAFL/NASH. In fact, literature has shown a substantial impact regarding the stage of fibrosis and overall mortality<sup>[90]</sup>. Fibrosis, when seen in NAFLD, has a characteristic appearance with early lesions showing a perisinusoidal deposition in zone 3<sup>[90]</sup>. Collagen fibers may be seen to encircle hepatocytes with more progressed lesions<sup>[90]</sup>. Additionally, pericellular fibrosis has been shown to progress without any appreciable periportal

fibrosis<sup>[90]</sup>. Periportal fibrosis develops after the perisinusoidal fibrosis and is demonstrated as trapping of hepatocytes around the portal area and extension of short strands of collagen into the parenchyma. Bridging fibrosis may eventually form single bands between the portal area and central vein without hepatocyte trapping or island formation. Evidence suggests that portal fibrosis in association with pericentral fibrosis is a necessary component for bridging fibrosis to develop<sup>[90]</sup>. Masson trichrome stain can highlight the fibrosis and are useful in identifying early fibrosis of steatohepatitis. Of note, NASH may retain all of the active steatohepatitis changes but the steatosis may decrease below the 5% level. On the other hand, the active steatohepatitis changes may disappear in cirrhosis as well, resulting in a diagnosis of "cryptogenic cirrhosis"<sup>[109]</sup>.

### HCC: Steatohepatitic variant

In the United States HCC has increased by 80% in the last twenty years with HCC being the fifth most common malignancy worldwide and the third most common cause of cancer-related death<sup>[110,111]</sup>. Hepatitis B and C, alcoholic liver disease, hemochromatosis, and several others represent the mainstay of risk factors for the development of HCC; recent studies have reported NAFLD to be an underlying cause of HCC in a number of cases even in the absence of cirrhosis<sup>[112-116]</sup>. A new variant of HCC has been described, that is the steatohepatitic variant of HCC, which is reminiscent of steatohepatitis (inflammation, hepatocyte ballooning, Mallory-Denk bodies, and pericellular fibrosis), and was first seen in a population of patients with HCV-related HCCs<sup>[117]</sup>. In one study, examining 118 cases of HCC over a 3.5 year period, 13.5% represented the steatohepatitic HCC with all but one case occurring in patients with underlying steatohepatitis<sup>[116]</sup>. When examining patient characteristics, the steatohepatitic HCC variant patients showed higher numbers of metabolic syndrome risk factors as well as at least 3 components of metabolic syndrome<sup>[116]</sup>. In a separate study, Jain *et al.*<sup>[118]</sup> found the steatohepatitic variant of HCC (SH-HCC) in approximately 19% of their cases over a period of 7 years, with 50% of those cases being seen in NAFLD patients and the other 50% were largely of HCV etiology. It is important to note, in a study performed by Yeh *et al.*<sup>[119]</sup>, that SH-HCC can occur outside the morphology of that seen in fatty liver disease or metabolic syndrome and was posited to be more likely attributable to genetic changes of shared genes or metabolic pathways. Yeh *et al.*<sup>[119]</sup> also found a loss of 9q12-q31.1 in a subset of cases, in this regard more investigation needs to be done to further ascertain the molecular driver for such a morphologic variant.

### Pediatric NAFLD histology

The main histological differences seen in some pediatric NAFLD when compared to adults has been the distribution of hepatocyte lipid droplets, inflammation and fibrosis location<sup>[120]</sup>. In some pediatric patients with NAFLD, the lipid vacuoles are largest in the periportal

hepatocytes and tend to decrease in diameter in pericentral area (zone 3). Similarly, inflammation and fibrosis is also seen around the portal tract (that is zone 1 predominance opposed to zone 3). When bridging fibrosis develops, the bridges connect portal to portal areas, leaving the central veins alone<sup>[120]</sup>. However, these features are not specific for pediatric NAFLD and many cases have similar picture as that of adult NAFLD.

### Grading and staging in NAFLD/NASH

In order to provide a consistent and reproducible assessment of NAFLD, the evaluation of morphological features must be semiquantified *via* an agreed upon scoring system to guide clinical decision making and for use in clinical trials<sup>[96,121-124]</sup>. Three histological scoring systems are currently in place: NASH clinical research network's NAFLD activity score (NASH CRN-NAS), steatosis, activity, and fibrosis (SAF), and the Brunt staging system<sup>[96,121,124]</sup>. The NAS uses numerical scores (Table 1) to develop an activity grade, which includes steatosis (0-3 points), hepatocellular ballooning (0-2 points), and acinar inflammation (0-3 points), as well as a separate fibrosis stage (0-4)<sup>[121]</sup>. Using a threshold of < 3 (activity score), the NAS showed a good correlation with the absence of a histological diagnosis of NASH<sup>[121]</sup>. Likewise, using a threshold of greater than or equal to 5, the NAS showed good correlation with having a diagnosis of NASH<sup>[121]</sup>. In validation by Hjelkrem *et al.*<sup>[125]</sup>, a total of 386 liver biopsies were evaluated, the sensitivity and specificity were 57% and 95%, respectively, when using a NAS  $\geq$  5 (indicating NASH) and NAS < 5 (indicating no NASH). When using an activity score of  $\geq$  4, the sensitivity increased to 85% with a slight decrease in specificity to 81%<sup>[125]</sup>. The  $\geq$  4 threshold has been recommended for any admission to an interventional trial for NASH<sup>[125]</sup>. In contrast, the SAF scoring algorithm (Table 2) was originally intended for the grading and staging of NAFLD in those patients who were morbidly obese about to undergo bariatric surgery<sup>[124]</sup>. Since then it has been used in patients with metabolic syndrome and concomitant NAFLD<sup>[91]</sup>. When using the SAF scoring system, the activity score (consisting of ballooning and lobular inflammation), enabled the discrimination of NASH (NASH patients had A > 2, whereas no patients with an A < 2 had NASH)<sup>[124]</sup>. Finally, the Brunt system uses a three tiered grading system (mild, moderate, and severe) with three parameters under histological investigation: Steatosis, ballooning, and inflammation (Table 3)<sup>[96]</sup>. The Brunt system also uses a four tiered staging system based on the location and degree of fibrosis (Table 3)<sup>[96]</sup>. It should be noted that regardless of every effort to devise a scoring system that is standardized and highly reproducible, the classification of NAFLD will always be plagued by observer bias and a lack of complexity which would be necessary to describe an intricate disease process<sup>[91]</sup>.

## DIFFERENTIAL DIAGNOSIS

As would be intuitive by the name of the disease, non-

**Table 1** Non-alcoholic fatty liver disease activity scoring system<sup>[121]</sup>

Steatosis, grade (0-3)	
< 5%	0
5%-33%	1
34%-66%	2
> 66%	3
Lobular inflammation	
No foci	0
< 2 foci per 200 × field	1
2-4 foci per 200 × field	2
> 4 foci per 200 × field	3
Hepatocyte ballooning	
None	0
Few balloon cells	1
Many cells/prominent ballooning	2
Fibrosis stage	
None	0
Perisinusoidal or periportal	1
Mild, zone 3, perisinusoidal	1A
Moderate, zone 3, perisinusoidal	1B
Portal/periportal	1C
Perisinusoidal and portal/periportal	2
Bridging fibrosis	3
Cirrhosis	4

alcoholic fatty liver disease/non-alcoholic steatohepatitis, the presence of alcohol driving these changes must be ruled out. However, many other disease settings are associated with liver injury which may resemble histological changes that are typically observed in NAFLD/NASH<sup>[91]</sup>. One category that may mimic NAFLD/NASH is termed chemotherapy (CASH)- or drug-associated steatohepatitis<sup>[91,126-128]</sup>.

Alcoholic steatosis, alcoholic steatohepatitis, alcoholic cirrhosis and HCC are the entities that a patient may develop with chronic alcohol use and abuse<sup>[129]</sup>. The distinction of alcoholic liver disease (ALD) and NASH can simply be made by delving into the history for the affirmation of alcohol use; however, there are histologic features that may help differentiate one form over the other in the absence of being able to obtain a detailed history (Table 4)<sup>[129]</sup>. The diagnostic criteria for rendering an ALD diagnosis rests on evidence of liver injury and a reported history of alcohol intake<sup>[101]</sup>. The amount of alcohol ingested is the strongest predictor of ALD development; just 60 g/d of alcohol consumed leads to the develop fatty liver in more than 90% of individuals<sup>[130]</sup>. In fact, the risk of developing alcohol related cirrhosis increases greatly with consumption of > 60-80 g/d for more 10 years in men, and > 20 g/d in women<sup>[130]</sup>.

There has been a rapid increase in the number of novel cytotoxic chemotherapeutic agents over the last few years and with the liver's role of drug metabolism it is not surprising that these drugs wreak havoc and produce hepatic injury<sup>[131]</sup>. Hepatotoxicity is neither predictable nor dose-dependent with most drug reactions occurring in an idiosyncratic manner<sup>[132]</sup>. Drug induced hepatic steatosis is a fairly rare event with several drugs/classes implicated: Methotrexate, amiodarone, tetracycline, glucocorticoids, tamoxifen, chemotherapeutics, and

**Table 2** Steatosis, activity, and fibrosis scoring system<sup>[91,124]</sup>

Steatosis score (S): Assessed the quantities of large or medium-sized lipid droplets (0-3)
S0: < 5%
S1: 5%-33%
S2: 34%-66%
S3: > 67%
Activity grade (0-4): Sum of scores for ballooning and lobular inflammation
A1: Mild activity
A2: Moderate activity
A3 and A4: Severe activity
Hepatocyte ballooning (0-2)
0: None
1: Foci of hepatocytes with rounded shape, pale or reticulated cytoplasm
2: Foci of hepatocytes with rounded shape, pale or reticulated cytoplasm and enlargement (> 2 × normal size)
Lobular inflammation (0-2)
0: None
1: < 2 foci per 20 × field
2: > 2 foci per 20 × field
Fibrosis stage (F)
F0: No relevant fibrosis
F1: 1a - mild zone 3 perisinusoidal fibrosis
1b - moderate zone 3 perisinusoidal fibrosis
1c - portal fibrosis
F2: Zone 3 perisinusoidal fibrosis with periportal fibrosis
F3: Bridging fibrosis
F4: Cirrhosis

nucleoside analogues to name a few<sup>[133]</sup>. Drug-induced hepatic steatosis is thought to result from the exuberant accumulation of intracellular phospholipids due in part by a drug therapy that has lasted several weeks to months<sup>[133]</sup>. Mechanistically, drug-related hepatic injury is due in part to mitochondrial toxicity resulting in inhibition of beta oxidation, oxidative phosphorylation, and mitochondrial respiration<sup>[134]</sup>. Since beta oxidation is one of the main ways lipids are metabolized, drug induced inhibition results in the accumulation lipids within the hepatocytes<sup>[134]</sup>. The steatosis that occurs in the setting of drug/chemotherapeutic treatment often resembles that seen in NAFLD with several notable exceptions<sup>[91]</sup>.

As previously outlined, the prevalence of NAFLD is growing and expanding, which allows the likely overlap of this disease with a concurrent disease, specifically: Chronic hepatitis B, chronic hepatitis C, human immunodeficiency virus, autoimmune hepatitis, biliary diseases, or other inherited metabolic disturbances<sup>[135-141]</sup>. In fact it has been reported that half of patients with human immunodeficiency virus (HIV) who undergo testing for liver test aberrations have concurrent NAFLD, which can result from HIV itself or the HAART therapy used in treatment<sup>[138]</sup>. In terms of autoimmune hepatitis, routine autoantibodies are present in NAFLD patients 23% of the time, necessitating the need for a liver biopsy for differentiation<sup>[139,140]</sup>. When looking at virally infected livers, specifically by HCV, hepatic steatosis has been reported in approximately 40%-85% of infected patients<sup>[142]</sup>. HCV is interesting in terms of its two pathway approach to liver steatosis: Viral and non-viral<sup>[142]</sup>. HCV, especially genotype 3a, has

**Table 3** Brunt grading and staging of nonalcoholic steatohepatitis<sup>[96]</sup>

Grading	Staging
Mild (Grade 1)	Stage 1
Steatosis (mostly macrovesicular)	Zone 3 perisinusoidal/pericellular fibrosis (focal or extensive)
Involves up to 66% of biopsy	
Occasional ballooned zone 3 hepatocytes	Stage 2
Scattered rare intra-acinar neutrophils with/without associated lymphocytes	Zone 3 perisinusoidal/pericellular fibrosis with associated focal or extensive periportal fibrosis
No/mild portal chronic inflammation	
Moderate (Grade 2)	Stage 3
Steatosis-any degree	Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with associated focal or extensive bridging fibrosis
Ballooning hepatocytes-zone 3	
Intra-acinar neutrophils-may be associated with zone 3 pericellular fibrosis	Stage 4
Portal and intra-acinar chronic inflammation	Cirrhosis
Severe (Grade 3)	
Panacinar steatosis	
Ballooning-zone 3	
Intra-acinar inflammation with scattered neutrophils	
Neutrophils associated with ballooned hepatocytes with/without chronic inflammation	
Chronic portal inflammation-mild or moderate	

**Table 4** Histologic comparison of non-alcoholic fatty liver disease/non-alcoholic steatohepatitis and alcoholic liver disease<sup>[129]</sup>

Characteristic	NAFLD and NASH	Alcoholic liver disease
Disease severity	Mild	Varying
Mallory-Denk body	Poorly formed	Well formed
Glycogenated nuclei	Common	Less common
Ductular proliferation	Less prominent	More prominent
Fibrosis/cirrhosis	Less common	More common
Sclerosing hyaline necrosis	None/rare	Present
Phlebosclerosis	None/rare	Present
Canalicular cholestasis	None/rare	Present
Foamy degeneration	None/rare	Present

NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

been reported to up-regulate the expression of fatty acid synthase in infected hepatocytes leading to increased fatty acids, impaired beta oxidation and reduced export of triglycerides<sup>[143]</sup>. As a part of its pathogenesis, HCV causes the inhibition of the microsomal triglyceride transfer protein, which is involved in the release of triglycerides from hepatocytes and as a consequence leads to triglyceride accumulation<sup>[142]</sup>. The non-viral approach to liver steatosis is typified by interference of insulin signaling resulting in insulin resistance<sup>[142]</sup>. The mode by which hepatitis B virus (HBV) causes hepatic steatosis is not entirely agreed upon<sup>[142]</sup>. It is postulated that HBV X protein may lead to lipid accumulation in hepatocytes with inhibition of apolipoprotein B secretion while at the same time PPARgamma and SREBP-1c activation with resultant nuclear factor-kappa B activation and TNF production<sup>[144]</sup>.

## PROGNOSIS, PROGRESSION AND CLINICAL COURSE

Numerous studies have tracked the progression of steatosis, steatohepatitis, and fibrosis in NAFLD patients

through paired liver biopsies<sup>[103,145-151]</sup>. Wong *et al.*<sup>[145]</sup>, via a prospective longitudinal hospital based cohort study, found that of patients with simple steatosis, 39% developed a borderline NASH picture and 23% developed full blown NASH. In another study, totaling 108 patients (81 with NASH and 27 with NAFL), 42% had fibrosis progression, 40% had no change in fibrosis, and 18% had fibrosis regression<sup>[103]</sup>. Interestingly, 22% of patients with NAFL at baseline developed stage 3 fibrosis at follow-up biopsy (median biopsy interval 6.6 years, range of 1.3-22.6)<sup>[103]</sup>. Overall, when evaluating the bulk of progression data it appears as though 33% of patients with NAFL and NASH will progress to fibrosis and up to 20% may have some regression of their disease<sup>[3]</sup>. Progressive fibrosis in NASH has been shown to be as high as 2 times that of NAFL and some patients with NASH and NAFL may progress rapidly from no fibrosis to severe fibrosis over the course of several years<sup>[102]</sup>. Clinically, cirrhosis and liver decompensation in NAFLD patients has been shown to be on the order of 3.1% over a mean 7.6 years<sup>[152]</sup>. The development of complications, specifically portal hypertension, with the development of cirrhosis is 17% (at one year), 23% (at three years), and 52% (at 10 years)<sup>[153]</sup>. A median survival of two years is seen in those patients with NASH who have experienced decompensation<sup>[154]</sup>.

Several investigations have found that men, post-menopausal women, those who underwent early menopause, and duration of menopause have an increased chance of fibrosis<sup>[155,156]</sup>. Although Hispanic patients have an increase prevalence of NAFLD, this feature does not seem to confer an increased risk of progression of their disease<sup>[57,157]</sup>. In contrast, Asian patients have been shown in some studies to have a more severe histologic picture<sup>[14]</sup>. Single nucleotide polymorphisms, namely, PNPLA3 rs738409 and rs58542926 are associated with severe histology to include NASH and cirrhosis<sup>[158,159]</sup>. Although increasing age is shown to be prone for the development of more severe fibrosis in NASH, it is



unclear whether this finding just underscores the fact that these patients have cumulative metabolic insults and a longer duration of disease exposure<sup>[160]</sup>. Additionally, higher rates of fibrosis progression have been seen in diabetics, those who are obese, hypertension (although several studies looking at NASH patients found no increased risk of progression due to hypertension), and degree of inflammation found on biopsy<sup>[102,103,145,147,161]</sup>.

In studies where biopsies were taken at the time of bariatric surgery and after subsequent weight loss, changes in hepatic histology were reported to improve<sup>[162,163]</sup>. However, some degree of worsening of either the fibrosis or steatosis has also been documented<sup>[164]</sup>. In an extreme case, one patient was reported to progress from mild fatty change before surgery to severe NASH and death due to liver failure<sup>[165]</sup>. The obvious mechanisms by which bariatric surgery improved the features of NAFLD would be related to weight loss, improvements in T2DM, reduced insulin resistance, reduced hyperlipidemia, and improved components of metabolic syndrome<sup>[162]</sup>. Other proposed mechanisms would include the altered route of food delivery, which results in changes to the release of gut and pancreatic hormones, changes in fat distribution, hepatic insulin and free fatty acid metabolism, and changes in adipocytokines and other cytokines<sup>[166]</sup>. These alterations in hormone secretion affect carbohydrate and lipid metabolism and interfere with hepatic glucose release<sup>[166]</sup>. Changes in gene expression may also play a pivotal role. In a study of 28 severely obese participants, PNPLA3 expression was measured by rtPCR before and after gastric banding-induced weight loss with the results showing a restoration of PNPLA3 expression in adipose tissue, but not in liver specimens<sup>[167]</sup>.

A study, evaluating NASH and steatosis improvement by weight loss, found that NASH resolution was obtained in 25% and NAS score improvement was seen in 47% of participants<sup>[168]</sup>. Likewise, 48% had improvement of their steatosis, 39% reduced the ballooning hepatocyte score and 50% showed improved lobular inflammation<sup>[168]</sup>. In terms of fibrosis, 65% had no change, 19% showed improvement, and 16% progressed<sup>[168]</sup>. Not altogether surprising, those participants who had the greatest weight loss also showed the most improvement of their histologic endpoint<sup>[168]</sup>. In another study with 180 participants, those who showed weight reduction had a 18.37-fold increase in the odds of NAFLD resolution<sup>[169]</sup>. One recommendation is a weight loss of at least 5% to decrease the burden of steatosis and 10% weight reduction to have an effect on liver necroinflammation<sup>[170]</sup>.

Investigations have proposed a link between metabolic syndrome, T2DM, obesity and the development of HCC<sup>[171,172]</sup>. NAFLD, even in the absence of fibrosis, provides a nurturing environment for the development of HCC with insulin resistance and steatosis providing the inflammation, adipokines, oxidative stress, and lipotoxicity needed for hepatocellular carcinogenesis<sup>[172,173]</sup>. In a study examining 1500 American veterans, NASH was found to be the third most common risk factor for the development of HCC<sup>[174]</sup>. With that being said, the

appearance of HCC is relatively rare in NAFLD, on the order of 0.2% (after eight year follow-up); however, the development of HCC in NASH cirrhosis ranges from 2.4% and 12.8% over a 3.2 and 7.2-year period, respectively<sup>[175,176]</sup>. In fact, once HCC develops in these cirrhotic patients their survival appears to be shorter than that seen in patients with HCV induced HCC<sup>[114]</sup>.

## DIAGNOSIS, TREATMENT AND SCREENING

Non-alcoholic fatty liver disease, in most instances, represents an incidental diagnosis due to alterations noted on a chemistry profile or when imaging for other purposes finds a steatosis pattern in the liver<sup>[9]</sup>. In the absence of incidental discovery, often patients are asymptomatic until liver decompensation occurs; however, if the evaluation of the patient reveals such factors as insulin resistance, obesity, or factors associated with metabolic syndrome, the diagnosis can be achieved much earlier than decompensation<sup>[9]</sup>. In the physical evaluation of the patient, BMI and visceral adiposity are helpful clues to the possible presence of NAFLD; however, in lean patients the diagnosis becomes much more challenging<sup>[9]</sup>. Screening of patients who are at risk for the development of NAFLD seems to be a worthy undertaking, but liver function tests can be in the normal range in patients with NAFLD/NASH and ultrasound is too expensive and burdensome for use in screening large portions of a population (although it is a good starting point when suspicion is high)<sup>[177]</sup>. The diagnosis of NAFLD is a four-pronged approach (Table 5): (1) hepatic steatosis (*via* imaging or histology); (2) alcohol consumption is ruled out; (3) there are no rival etiologies; and (4) no other causes for chronic liver disease are identified<sup>[177]</sup>. The entities discussed in the differential diagnosis section of this paper should be ruled out, namely, alcohol use, chronic hepatitis B and C, medication use, parenteral nutrition, Wilson's disease, biliary disease, autoimmune hepatitis, and malnutrition to name a few of the major considerations. Although mild elevations in serum ferritin can be seen in NAFLD, marked increases should be worked-up for hemochromatosis and *HFE* gene mutations (*i.e.*, C282Y)<sup>[177]</sup>. As mentioned previously, NAFLD patients may have elevations in serum autoantibodies; however, increased serum autoantibodies in the presence of features to suggest an autoimmune liver disease should result in a more complete work-up for autoimmune disease/autoimmune liver disease<sup>[177]</sup>. Biomarker development in NAFLD has been a topic of great interest and research. Numerous potential biomarkers have been investigated, for example, cytokeratin 18 fragments were evaluated in potential NAFLD patients at the time of liver biopsy and then correlated with histologic findings<sup>[178]</sup>. In this study, CK18 fragments found in the plasma showed a significant ( $P < 0.001$ ) and marked increase in patients with NASH when compared with those having steatosis

**Table 5** Factors to be assessed in the evaluation of a patient with suspected non-alcoholic fatty liver disease<sup>[32]</sup>

Factor
Personal and family history of diabetes, hypertension and CVD
Alcohol use: < 20 g/d (women), < 30 g/d (man)
Waist circumference, BMI, change in body weight
Hepatitis B/C infection
Liver enzymes
History of steatosis-associated drug use
Fast blood glucose, hemoglobin A1c
Serum total and HDL-cholesterol, triacylglycerol, uric acid
Undertaken due to clinical suspicion
Ultrasound
Hemochromatosis testing: Ferritin and transferrin saturation
Celiac disease: IgA and tissue transglutaminase
Thyroid disease: TSH level (T3/T4)
Polycystic ovarian syndrome
Wilson's disease: Ceruloplasmin
Autoimmune disease: ANA, AMA, SMA
Alpha-1 antitrypsin deficiency: Alpha-1-antitrypsin level

ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; SMA: Anti-smooth muscle antibody; CVD: Cardiovascular disease; BMI: Body mass index; HDL: High density lipoprotein; TSH: Thyroid stimulating hormone.

or normal findings (median 765.7 U/L vs 202.4 U/L vs 215.5 U/L, respectively)<sup>[178]</sup>. These findings were further investigated by several subsequent studies and a meta-analysis revealed CK18 fragment levels to have a sensitivity and specificity of 78% and 87%, respectively, for steatohepatitis in those with NAFLD<sup>[179]</sup>. Other studies have offered insight into miRNAs as a biomarker for NAFLD and HCC spectrum; however, more investigation is needed to determine its true place in the diagnostic algorithm of NAFLD<sup>[180]</sup>. Extracellular vesicles shed from the liver have also caught the attention of many investigators and they are being actively researched for a possible role in NAFLD detection<sup>[181]</sup>.

Perhaps the most important treatment option, lifestyle modification (to include diet and exercise), as well as surgical interventions for the treatment of NAFLD have already been discussed. Medications and supplements are also part of the treatment consideration when dealing with NAFLD. Hence, there are four main pathways currently available in the treatment of NAFLD. First, targeting hepatic fat accumulation (pioglitazone, elafibranor, saroglitazar), bile acid-farnesoid X receptor axis (obeticholic acid), *de novo* lipogenesis inhibitors (aramchol, NDI-010976), incretins (liraglutide) and fibroblast growth factor FGF-21 or FGF-19 analogues<sup>[182]</sup>. Second, oxidative stress alleviation through the use of antioxidants and medications that target the tumor necrosis factor alpha pathway (emricasan, pentoxifylline) as well as immune modulators (amlexanox, cenicriviroc)<sup>[182]</sup>. Third, antiobesity medications such as orlistat and finally antifibrotics (simtuzumab and GRMD-02) will be important players in therapeutic management of NAFLD<sup>[182]</sup>. Insulin resistance, as a major player in the pathogenesis of NAFLD, is an obvious target of therapeutic intervention by way of insulin sensitizing

agents<sup>[177]</sup>. With that being said, several studies have looked at the effects of metformin on liver function test levels and histology in those with NASH. In initial work, use of metformin showed a reduction in insulin resistance and aminotransferase levels; however, no changes were noted in the participants liver histology<sup>[183,184]</sup>. A meta-analysis found that in combination with lifestyle changes, metformin did not improve liver function test profiles or liver histology compared with lifestyle modification alone<sup>[177]</sup>. Although some evidence exists of NASH's histological improvement by metformin intervention (study confounded by weight loss), the current AASLD practice guideline recommendation is not to use metformin for the specific treatment of liver disease in adults with NASH<sup>[177,185]</sup>. The thiazolidinediones (TZDs), specifically pioglitazone, was shown in meta-analysis to improve steatosis and inflammation but not fibrosis with the caveat that TZDs long term safety profile is still under investigation<sup>[177]</sup>. The current recommendation, according to the AASLD Practice Guideline for NAFLD, Pioglitazone can be used in the treatment of steatohepatitis in those who have biopsy confirmed NASH with the understanding that trials were conducted in NASH patients without diabetes<sup>[177]</sup>. Vitamin E, an anti-oxidant, has been investigated for use in the treatment of NASH as oxidative stress is considered to be a major player in hepatocyte injury and disease progression<sup>[186,187]</sup>. Several studies have produced data to suggest that the use of vitamin E leads to improved steatosis, reduced inflammation and ballooning, decreased liver function test values, resolution of steatohepatitis with no effect on hepatic fibrosis<sup>[177]</sup>. However, concerns over the use of vitamin E and associated increases in all-cause mortality and an increased risk of prostate cancer in men have been raised<sup>[188,189]</sup>. As it currently stands, vitamin E should be considered in the therapeutic regimen of patients with biopsy proven NASH who also are non-diabetics<sup>[177]</sup>. Other therapies such as Pentoxifylline (shown to improve hepatic steatosis with no effect on insulin resistance), obeticholic acid (improves insulin resistance, hepatic steatosis, hepatic inflammation, and hepatic fibrosis), Orlistat (improves insulin resistance), ursodeoxycholic acid (improves insulin resistance and hepatic steatosis), Statins (improves hepatic steatosis), and Omega-3 (improves hepatic steatosis), and glucagon-like peptide 1 receptor agonists (improves hepatic steatosis) have been investigated and have shown varying and often limited benefit<sup>[190]</sup>. Finally, up and coming agents to be aware of: PPAR $\alpha/\delta$  agonists, chemokine receptor (CCR)2/CCR5 antagonists and numerous fatty acid/bile acid conjugates and antifibrotic agents are being investigated for use in NASH and the results of these studies/trials will reveal what benefit if any they will have on the NAFLD landscape<sup>[32]</sup>.

According to the most recent American College of Gastroenterology and American Gastroenterological Association guidelines, the screening of adults in primary care clinics or high-risk groups (*i.e.*, those attending diabetes or obesity clinics) for NAFLD is not recommended and the

systematic screening of family members for NAFLD is also discouraged<sup>[191]</sup>. This due to the lack of evidence or current understanding regarding the long-term benefits and cost effectiveness of screening and the current uncertainties related to diagnostic tests and treatment options<sup>[191]</sup>. However, other screening guidelines suggest the implementation of a screening policy in those who are at high risk for NAFLD identified by the presence of metabolic risk factors and/or IR<sup>[191]</sup>.

## CONCLUSION

NAFLD is a growing epidemic, not only in the United States, but worldwide in part due to obesity and insulin resistance leading to liver accumulation of triglycerides and free fatty acids. Liver steatosis may be innocuous in most occasions but the progression and development of fibrosis is not and often heralds a poor prognosis. Numerous risk factors for the development of NAFLD have been espoused with most having some form of metabolic derangement or insulin resistance at the core of its pathophysiology. Additionally, access and decreasing cost for high quality and powered genetic scrutiny will no doubt provide future clinicians with a great deal of information and opportunity for enhanced targeted treatment. The same can be said for the development of advanced imaging and biochemical tests. As it currently stands several medications/supplements may be used in the treatment of NAFLD; however, none seem to be the “magic bullet” in curtailing this growing problem. Not enough can be said about the importance of lifestyle coupled with proper diet and appropriate exercise in the defense of developing NAFLD.

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## Abdominal cross-sectional imaging of the associating liver partition and portal vein ligation for staged hepatectomy procedure

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**Author contributions:** Girometti R and Lorenzin D projected the paper; Zerial M, Girometti R and Zuiani C planned the review structure; Lorenzin D and Risaliti A performed surgeries and collected surgical images; Zerial M reviewed radiological literature; Lorenzin D and Risaliti A reviewed surgical literature; Zerial M, Zuiani C and Girometti R reviewed radiological images and selected them; Zerial M wrote the paper; Zuiani C and Girometti R prepared the tables; Zerial M and Lorenzin M prepared the figures; Girometti R, Lorenzin D, Zuiani C and Risaliti A reviewed the paper for structure and content; Girometti R supervised the work.

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### Abstract

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a recently introduced technique aimed to perform two-stage hepatectomy in patients with a variety of primary or secondary neoplastic lesions. ALPPS is based on a preliminary liver resection associated with ligation of the portal branch directed to the diseased hemiliver (DH), followed by hepatectomy after an interval of time in which the future liver remnant (FLR) hypertrophied adequately (partly because of preserved arterialization of the DH). Multidetector computed tomography (MDCT) and magnetic resonance imaging (MRI) play a pivotal role in patients' selection and FLR assessment before and after the procedure, as well as in monitoring early and late complications, as we aim to review in this paper. Moreover, we illustrate main abdominal MDCT and MRI findings related to ALPPS.

**Key words:** Hepatectomy; Computed tomography; Magnetic resonance imaging; Associating liver partition and portal vein ligation for staged hepatectomy; Liver surgery

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**Core tip:** Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a variant

of two-stage hepatectomy aimed to obtain rapid hypertrophy of the future liver remnant. Given its recent introduction, there are still controversies on indications and safety issues. Cross-sectional imaging by means of multidetector computed tomography (MDCT) and magnetic resonance imaging (MRI) play a key role in the multidisciplinary process of patients' selection and postoperative management. This review aims to emphasize such a role and illustrate main abdominal ALPPS-related findings on MDCT or MRI.

Zerial M, Lorenzin D, Risaliti A, Zuiani C, Girometti R. Abdominal cross-sectional imaging of the associating liver partition and portal vein ligation for staged hepatectomy procedure. *World J Hepatol* 2017; 9(16): 733-745 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i16/733.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i16.733>

## INTRODUCTION

Resection is the only treatment proven to achieve long-term survival in patients with primary hepatic malignancies or selected liver metastases<sup>[1,2]</sup>. Over the last years, advances in surgical techniques, systemic chemotherapy and intensive care improved the outcome of liver resection, leading to wider criteria for operability compared to the past<sup>[3]</sup>. However, adequate future liver remnant (FLR) (*i.e.*, the liver remnant planned to be left *in situ*) is still a critical factor in selecting patients when extended hepatectomy is required, given the need to minimize the risk of postoperative liver failure<sup>[4,5]</sup>. FLR should be at least 25%-30% of the liver volume in patients with normal preoperative liver function, 30% in chronic liver disease, and 40% in the setting of chemotherapy-related injury or cirrhosis<sup>[6,7]</sup>. Borderline FLR volumes pose the dilemma of whether attempting radical surgery vs performing palliative treatments<sup>[7]</sup>.

In the 2000s, two-stage hepatectomy after preoperative percutaneous portal vein embolization (PVE) or portal vein ligation (PVL) has been proposed as a strategy to resect primarily inoperable tumors after having increased the FLR<sup>[8,9]</sup>. This approach combines the technical advantages of two-stage hepatectomy (*i.e.*, wedge resections of lesions in the FLR in the case of bilobar tumors) with the compensatory hypertrophy of the FLR induced by PVE or PVL performed at the time of first surgery<sup>[10]</sup>. The mechanism with which PVE and PVL lead to hypertrophic FLR is complex, involving both the diversion of portal blood flow and release of growth factors<sup>[7]</sup>. Since hypertrophy usually takes at least 4 wk to be completed, this technique shows high failure rate because of insufficient FLR growth and/or tumor progression during the interval of time between the two stages<sup>[11,12]</sup>.

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a two-stage hepatectomy

procedure introduced in September 2007 by Schnitzbauer *et al.*<sup>[13]</sup> to obtain more rapid and larger increase of the FLR volume compared to conventional staged hepatectomy (40%-80% within 6-9 d vs 8%-27% within up to 60 d, respectively)<sup>[4,6,7,13-15]</sup>. The key technical point in ALPPS is the preservation of hepatic artery blood flow to the diseased hemiliver (DH) at the time of first surgical stage. Preserved arterialization leads the DH to act as a vital auxiliary liver and assist the growth of FLR through metabolic and synthetic functions<sup>[16,17]</sup>. ALPPS achieves a high rate of tumor complete resection (83%)<sup>[18]</sup>, given the successful rate of adequate FLR growth (78%-91%)<sup>[19]</sup>. Additionally, the reduced interval of time between surgical steps translates into lower tumor progression rate, less adhesions during second surgery, faster patients recovery and prompter starting of adjuvant chemotherapy<sup>[4,15,20,21]</sup>.

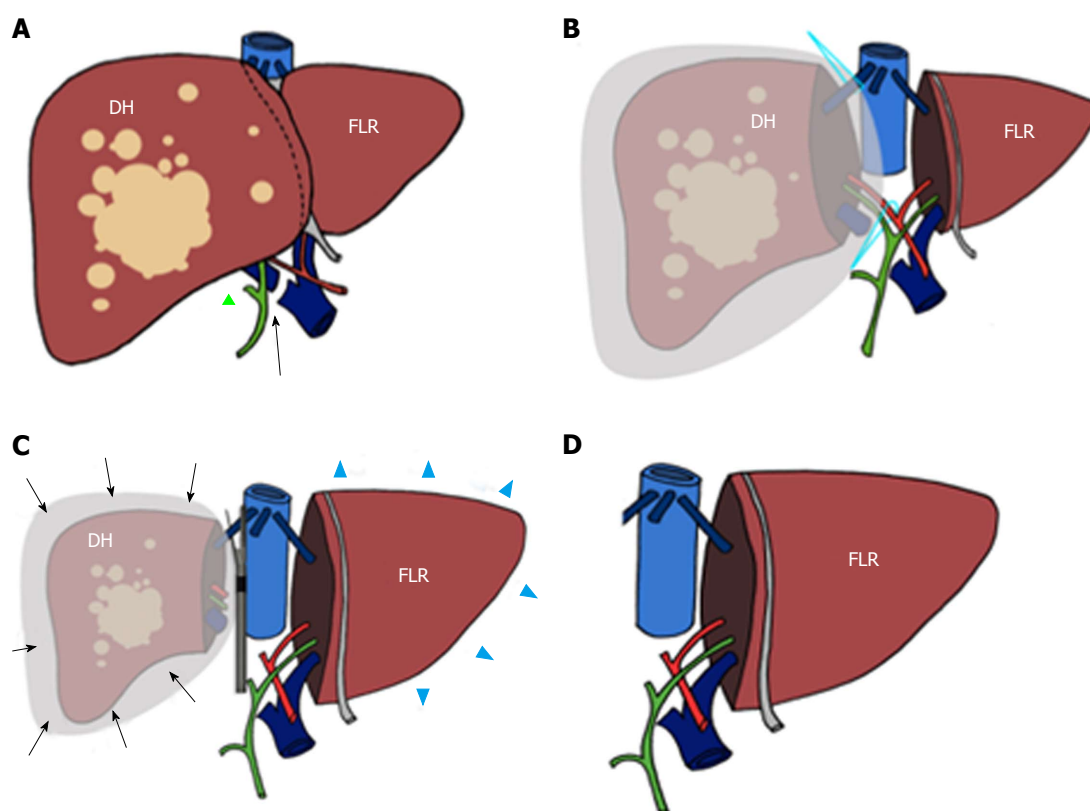
ALPPS is becoming increasingly popular in patients candidate to extended hepatectomy. To our knowledge, though imaging plays a key-role in planning the procedure and monitoring the results of both surgical stages, radiological findings related to ALPPS have been poorly reported. In this review, we aimed to summarize the current role for multidetector computed tomography (MDCT) and magnetic resonance imaging (MRI) in the procedure, which enable detailed view of pre- and postoperative anatomy, as well as prompt and reliable identification of complications. We also illustrated main cross-sectional imaging findings related to ALPPS, with special emphasis on normal aspects.

## ALPPS: INDICATIONS AND TECHNIQUE

### Indications

There is controversy on which lesions should be treated with ALPPS<sup>[6]</sup>, given initially reported high mortality rates (up to 22% in some series)<sup>[22]</sup>. It should be kept in mind that ALPPS is an "extrema ratio" procedure to be proposed after careful, multidisciplinary patient selection<sup>[6,23,24]</sup>. Morbidity and mortality amount up to 14% and 6.6% in experienced centers applying strict selection criteria<sup>[10,25-27]</sup>. Best results have been obtained in patients with bilobar metastases from colorectal cancer with predictable radical resection, absence of extrahepatic disease and partial or complete response to chemotherapy<sup>[2]</sup>. Other treatable lesions include hepatocellular carcinoma, cholangiocarcinoma (intrahepatic or hilar), gallbladder carcinoma, and metastases from breast cancer or neuroendocrine tumors<sup>[7,25,26]</sup>. However, higher postoperative mortality was reported for non-colorectal liver metastases<sup>[7]</sup>. ALPPS can be also offered as first-line treatment or salvage-therapy after failed PVE<sup>[20,25,28-32]</sup>.

Contraindications to ALPPS include unresectable lesions in the FLR, unresectable extrahepatic metastases, infiltration of the retrohepatic avascular space, severe portal hypertension, high anesthesiology risk, medical contraindications to major hepatectomy, impossibility to achieve negative margins, and unresectable primary



**Figure 1** Scheme of trisectionectomy associating liver partition and portal vein ligation procedure. During surgical stage 1 the right portal vein is sectioned and sutured (arrow in A) after performing cholecystectomy (green triangle in A). Subsequently, the diseased hemiliver (DH) is sectioned from the future liver remnant (FLR) and wrapped with a bag (B). At the time of surgical stage 2 (C), hypertrophy of the (FLR) (blue arrowheads in D) and atrophy of the DH (arrows) have been obtained. Associating liver partition and portal vein ligation for staged hepatectomy procedure is then completed by removing the DH (D).

tumor in extrahepatic locations<sup>[26]</sup>. ALPPS is not recommended in patients with advanced liver cirrhosis, because liver regeneration in the context of chronic liver disease is less predictable<sup>[7,33]</sup>. On the other hand, some Authors attempted ALPPS in selected cirrhotic patients<sup>[34]</sup>.

### Technique

Elective indication to ALPPS is right trisectionectomy<sup>[7]</sup>, in which FLR and DH consist of Couinaud segments 2-3 vs 4-8 (Figures 1 and 2), respectively. Other technical approaches include right hepatectomy (leaving a segments 2-4 FLR), left hepatectomy (leaving segments 5-8 FLR), central hepatectomy (segments 4, 5 and 8 FLR) or monosegmental ALPPS<sup>[35-37]</sup>.

ALPPS includes two consecutive surgical stages (stage 1 and stage 2). During stage 1, the portal branch directed to the DH side is sectioned and sutured in order to divert the portal flow to the FLR. Hepatectomy is subsequently performed to separate the FLR from DH completely (complete ALPPS) or partially (partial ALPPS)<sup>[32,38]</sup>. If affected by metastases, the FLR is cleaned up by wedge resections and/or intraoperative radiofrequency ablation<sup>[17,26]</sup>. At the end of the procedure, DH is left in situ, often after having enveloped it into a hermetic bag made of plastic or a biodegradable type- I acellular collagen membrane<sup>[39]</sup>. The rationale for using the bag is to avoid adhesions and obtain an easier removal of DH on surgical stage 2,

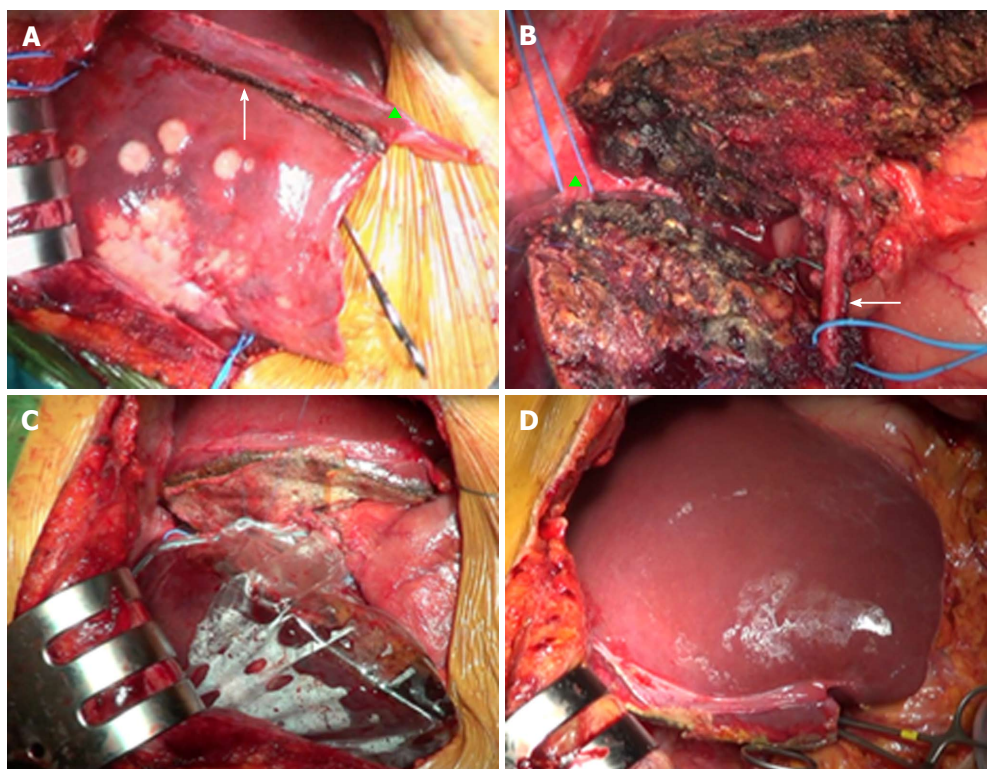
as well as better drainage or identification of collections (Figures 1 and 2)<sup>[7]</sup>. The purpose of stage 1 is to induce hypertrophy of the FLR (in which arterial and portal vascular supply is preserved) and atrophy of the DH (in which arterial supply alone is preserved). Cholecystectomy is also performed<sup>[40]</sup>. In the case of perihilar cholangiocarcinoma, biliary continuity is obtained by performing Roux-en-Y bilioenteric anastomosis<sup>[26]</sup>. After stage 1 completion, two drains are placed along the transection line and within the plastic bag, respectively.

Stage 2 is scheduled 7-14 d from stage 1<sup>[2]</sup>. Hepatectomy is completed by removing atrophic DH after transecting the serving hepatic artery, hepatic duct and hepatic veins (e.g., right hepatic and middle hepatic veins in the case of right trisectionectomy, or right hepatic vein only in the case of right hepatectomy).

### IMAGING TECHNIQUES

First-line imaging after both surgical stages 1 and 2 is represented by ultrasonography (US) with Color-Doppler examination. In our experience, US permits a "quick-and-dirty" evaluation at patient's bedside to screen for gross complications (e.g., collections) and assess the patency of FLR portal vein, hepatic artery branches and hepatic vein. However, early postoperative US is limited by lack of patients' collaboration and reduced acoustic





**Figure 2** Surgical overview of the associating liver partition and portal vein ligation for staged hepatectomy procedure. A: Intraoperative findings during stage 1, with evidence of resection line (arrow) on the right side of ligamentum falciforme (green triangle); B: Resected liver with right hepatic vein (green triangle) and right hepatic artery (arrow) encircled by a vessel loop to simplify their identification during stage 2; C: After transection, diseased hemiliver is enveloped with a plastic bag; D: Pronounced hypertrophy of future liver remnant during intraoperative stage 2.

windows because of bowel gas and surgical dressing material<sup>[41]</sup>. Furthermore, US lacks panoramcity, *i.e.*, the capability to represent a section or a 3D reconstruction of the entire liver within a single image. Consequently, though this technique is useful in initial diagnosis of liver abnormalities, it has no direct role in selecting patients for ALPPS (*e.g.*, by assessing the number of lesions in the FLR or estimating its volume). Thus, cross-sectional imaging with MDCT and/or MRI is mandatory in the preoperative patients' selection, in evaluating postoperative increase in FLR volume and in assessing complications.

Because of wide panoramcity, fast acquisition time and lesser costs, MDCT should be regarded as the cross-sectional modality of choice to image patients before and after ALPPS. Our institutional protocol is summarized in Table 1. Fast acquisition makes MDCT feasible in less collaborating patients, with the possibility to extend the examination to the thorax and/or the lower abdomen if needed. Moreover, the multiphasic MDCT protocol has the advantage of providing all-in-one evaluation of liver neoplasms (in terms of both tumor burden and characterization), extrahepatic disease or complications, and the status of arterial, portal and venous structures for the purpose of preoperative planning and complications assessment. Multiplanar reformations and 3D reconstruction are of help in interpreting images and communicating imaging results to referring clinicians.

Given limited availability and longer acquisition

times, MRI should be reserved to inconclusive MDCT cases, especially in the preoperative phase, *i.e.*, when there is less risk of image quality degradation because of reduced patients' collaboration. Similarly to other liver applications<sup>[41-44]</sup>, MRI should be performed with 1.5 Tesla or 3.0 Tesla magnets, equipped with highly performing gradients and multi-element surface coils (preferably 8-16 elements) implementing parallel imaging. Our MRI protocol is illustrated in Table 2.

Hepatobiliary contrast agents such as gadoxetic acid and/or gadobenate dimeglumine improve the detection and characterization of focal liver lesions by representing the vascularity and the presence/absence of hepatocellular contrast uptake at one time<sup>[45,46]</sup>. When liver metastases are the cause for ALPPS, preoperative MRI with diffusion-weighted imaging and hepatobiliary contrast agents should be regarded as the method of choice for detailed identification of small lesions potentially affecting ALPPS feasibility or FLR cleaning up<sup>[47]</sup>. Furthermore, hepatobiliary contrast agents are of help in assessing tumor relapse after surgery.

Magnetic resonance cholangiopancreatography (MRCP) should be used preoperatively to evaluate whether biliary tree anatomic variants are at risk of increasing surgical difficulty, or to assign the Bismuth category of cholangiocarcinoma extension<sup>[48]</sup>. In the postoperative phases, this technique can be of help in assessing the content of fluid collections (fluid vs hemorrhagic) or early and late biliary complications. In

**Table 1 Institutional multiphase multidetector computed tomography protocol for evaluating associating liver partition and portal vein ligation for staged hepatectomy patients before and after surgery (LightSpeed HD, General Electric, Milwaukee, United States)**

Scan phase (timing from contrast injection)	Scan length	Scanning parameters	Rationale in the preoperative phase	Rationale in the postoperative phases
Unenhanced	Upper abdomen	KVp 120 mA modulated between 200-450 Tube rotation 0.6 s Pitch 0.984 Noise index 16.10 Collimation 1.25 mm (0.625 for the angiographic phase)	Identifying potential confounders in image interpretation ( <i>e.g.</i> , lesion's or vascular calcifications). Measuring baseline attenuation of target lesions ( <i>e.g.</i> , fat-containing HCC) or in diffuse liver disease ( <i>e.g.</i> , steatosis). This phase is not required if recent prior imaging is available.	Identifying potential confounders in image interpretation ( <i>e.g.</i> , surgical clips). Measuring the attenuation of intra-abdominal collections (biloma <i>vs</i> hematoma). This phase is not mandatory in repeated follow-up examinations
Angiographic phase (20)	Upper abdomen	Image reconstruction thickness 1.25 mm	Assessing the patency and anatomic variants of the hepatic artery and its branches, both on source images and MIP reconstructions	Assessing the sources of suspicious active postoperative bleeding
Delayed arterial (35-40 s)	Upper abdomen		Assessing hypervascular focal liver lesions (malignant and benign ones)	Assessing the patency of the hepatic artery and its branches. Identifying the recurrence of hypervascular tumors in the delayed post-operative period
Venous (70 s)	Whole abdomen		Assessing lesions' enhancement pattern for the purpose of identification/characterization. Assessing the patency and anatomic variants of the portal trunk and intrahepatic branches, both on source images and MIP reconstructions. Identifying additional abdominal findings potentially contraindicating ALPSS. Assessing for signs of chronic liver disease (including splenomegaly, venous collaterals and ascites)	Assessing the portal status (absence of flow in the ligated portal branch and patency of the FLR branch). Assessing successful tumor cleaning up in the FLR before surgical stage 2. Ruling out thrombosis of the portal branches, hepatic veins and inferior vena cava. Identifying tumor relapse
Delayed (3-5 min)	Upper or whole abdomen, depending on findings on previous scans		Assessing lesions' enhancement pattern for the purpose of identification/characterization. Identifying additional findings potentially contraindicating ALPSS ( <i>e.g.</i> , peritoneal carcinosis). This phase is not mandatory	Assessing venous bleeding. This phase is not mandatory

MIP: Maximum intensity projection; FLR: Future liver remnant; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; HCC: Hepatocellular carcinoma.

particular, 3D T1-weighted MRCP acquired in the delayed phase after gadoxetic acid administration is useful in confirming clinical suspicion of biliary leakage (*e.g.*, persisting postoperative fluid collections associated with clinical sign of biliary sepsis) by showing active contrast extravasation<sup>[44]</sup>. The presence of endobag after surgical stage 1 can avoid gadoxetic acid-based MRCP, since bile leakage can be actively monitored through the internal surgical drainage.

## ROLE FOR IMAGING

### Preoperative imaging

Preoperative findings are essential to understand whether ALPPS is feasible or not based on tumor burden, liver status and presence of ancillary findings with potential surgical significance. There are five main goals of cross-sectional imaging in this setting.

The first task for imaging is accurate detection and

characterization of liver lesions. Radiologists should carefully report the number, size, and location of individual lesions, as well as their relationship with surgically relevant anatomic structures, including the hepatic artery, main portal branches, hepatic veins, and first- to second-order biliary branches (Figure 3). This will help the surgeon to establish lesions resectability and the risk for intraoperative complications (*e.g.*, lesions close to the retrohepatic course of inferior vena cava, a region at higher risk of intraoperative bleeding). Second, imaging aims to evaluate the status of liver parenchyma, looking for signs of cirrhosis, cholestasis, steatosis or any other pathologic change attributable to the effects of lesions, diffuse liver disease or chemotherapy. Liver status may influence operability, regardless of the FLR volume (see below). Third, it is crucial to identify vascular and biliary anatomy variants of potential surgical significance (*e.g.*, aberrant and/or accessory branches)<sup>[49]</sup>. Fourth, any extrahepatic finding potentially affecting the feasibility of ALPPS should be

**Table 2** Institutional magnetic resonance imaging protocol with *i.v.* administration gadoxetic acid (0.025 mmol/kg at an injection rate of 1 mL/s) for evaluating associating liver partition and portal vein ligation for staged hepatectomy patients before and after surgery

Sequence	Weightening	Acquisition plane	Technical clues	Rationale in the preoperative phase	Rationale in the postoperative phase
Half fourier acquisition single-shot turbo spin echo/ single shot fast spin echo	T2	Coronal, transverse	-	Ruling out signs of chronic liver disease, including splenomegaly and/or ascites. Detection of parenchymal low signal intensity in iron accumulation	Detection of perihepatic/abdominal collection and/or ascites
GE in-phase/out-of-phase	T1	Transverse	Dual echo, breath hold sequence with slice thickness 6 mm	Characterization of fat-containing lesions. Detection of signal intensity patterns of liver steatosis or hemochromatosis	Evaluation of the postoperative status of liver parenchyma. Characterization of tumor recurrence
MRCP	T2	Radial coronal acquisition (2D) or oblique coronal (3D)	2D and/or 3D technique	Evaluation of anatomic variants complicating or contraindicating surgery. Assessing the Bismuth category of hilar cholangiocarcinoma	Assessment of biliary strictures (site, extent) and biliary dilation upstream
Dynamic study with fat saturated 3D GE	T1	Transverse	Thin slice thickness (3 mm). Baseline acquisition followed by early arterial, late arterial, venous and delayed phases	Detection and characterization of liver lesions	Detection and characterization of parenchymal abnormalities, including tumor recurrence
Single-shot echoplanar imaging	Diffusion	Transverse	<i>b</i> values 50 and 400 and 800 s/mm <sup>2</sup> (1.5T) or 50 and 800 and 1200 s/mm <sup>2</sup> (3.0T). Nominal acquisition time about 3 min (1.5T) and 4 min (3T)	Detection and characterization of smaller lesions (< 1 cm in size)	Detection of parenchymal/periportal edema. Detection and characterization of smaller lesions (< 1 cm in size)
Fat saturated Turbo spin echo	T2	Transverse	Respiratory triggered, with slice thickness 6 mm. Nominal acquisition time 1.50 min	Detection and characterization of liver lesions.	Detection of parenchymal/periportal edema. Detection and characterization of liver lesions. Assessment of collections
GE in-phase/out-of-phase	T1	Transverse	Same sequence as (2), acquired in the hepatobiliary phase (15-20 min after contrast injection)	Detection and characterization of liver lesions	Detection and characterization of liver abnormalities
Fat saturated 3D GE	T1	Transverse	Same sequence as (4), with modified flip angle (35°) to increase lesion-to-parenchyma conspicuity. Acquired in the hepatobiliary phase		
Contrast-enhanced MRCP	T1	Oblique coronal	Thin-slice (1 mm) fat saturated 3D fast low angle shot (FLASH) sequence acquired	Functional evaluation of biliary obstruction (if present)	Detection of active bile leakage. Functional assessment of bile duct strictures and patency of bilioenteric anastomosis

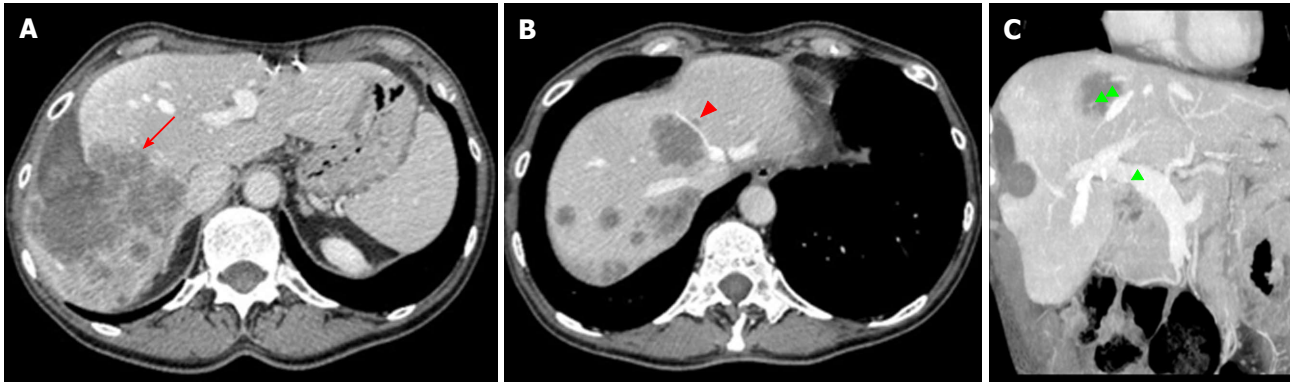
GE: Gradient echo; MRCP: Magnetic resonance cholangiopancreatography.

evaluated, including large, inoperable primary cancer on other sites, as well as portal hypertension (including splenomegaly and venous collaterals).

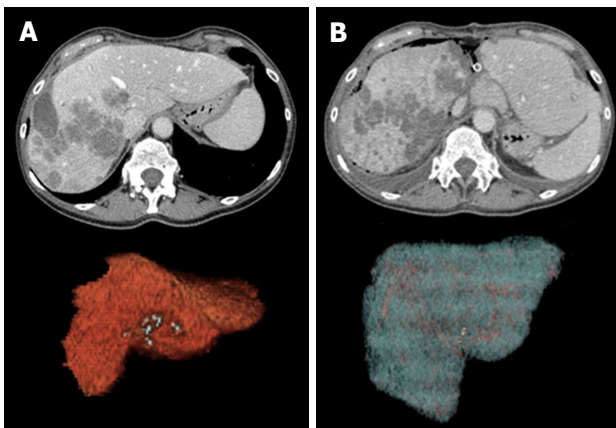
The final key step in preoperative imaging is liver volumetry (LV) of the FLR and the whole liver. FLR volume should be calculated by excluding major vessels and FLLs, in order to obtain a reasonable estimate of final viable liver tissue supporting liver function. FLR should be no lower than 25%-30% of preoperative liver

volume in patients with normal liver function, and no lower than 40% in patients with underlying chronic liver disease or liver dysfunction (including the effects of chemotherapy)<sup>[7,23,50-52]</sup>. Many dedicated liver volumetry software are currently available, most times implemented in the picture archive and communication systems used for routine image analysis. In our Institution, abdominal radiologists perform LV together with liver surgeons, with the objective of reliable volumes definition according to





**Figure 3** Radiologists should carefully report the number, size, and location of individual lesions, as well as their relationship with surgically relevant anatomic structures, including the hepatic artery, main portal branches, hepatic veins, and first- to second-order biliary branches. A: Preoperative assessment with computed tomography in a 64 male years old patient showing colorectal metastases on the right hepatic lobe (red arrow). Right trisectionectomy associating liver partition and portal vein ligation for staged hepatectomy was planned; B: One small satellite lesion was found on the left side of the middle hepatic vein (arrowhead), indicating the need for future liver remnant clean-up during stage 1; C: No vascular involvement was shown, as exemplified by patent main portal trunk and intrahepatic branches (green triangle), except for infiltration of the middle hepatic vein (double green triangle). Based on this finding, a wide free margin between the line of resection and the middle hepatic vein was obtained.



**Figure 4** Evolution of the future liver remnant (liver segments 2 + 3) before (A) and after stage 1 surgery (B). Future liver remnant remnant almost doubled in volume (from 280 cm<sup>3</sup> to 468 cm<sup>3</sup>), showing clear enlargement on 2D images and volume rendering reconstructions.

the intended lines of resection.

### Imaging after surgical stage 1

**Goals:** In uncomplicated patients, post-stage 1 imaging is performed at the time adequate FLR hypertrophy is expectedly achieved (about 6-9 d from surgery)<sup>[7]</sup>. Cross-sectional imaging is mandatory to calculate the increase in volume of the FLR using LV (Figure 4), to confirm tumor-free status of the FLR, and to verify the expected changes in the DH (atrophy and persistent portal devascularization).

In the case of US and/or clinical suspicion, MDCT or MRI must be anticipated to guarantee early assessment and intervention. Cross-sectional imaging is also of help in ruling-out surgical complications or insufficient FLR volume as a cause for postoperative liver failure.

**Normal findings:** Normal hypertrophic FLR is represented in Figure 4. Enlargement can be easily appreciated

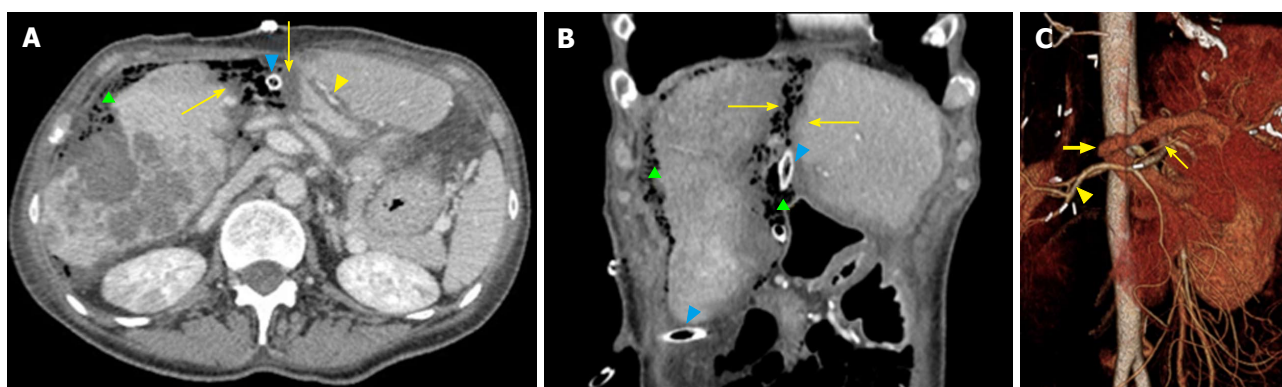
on transverse and reformatted 2D images, though precise estimation should be always performed on 3D reconstructions obtained with LV. The magnitude of expected FLR increase ranges between 61% and 93% compared to the baseline volume<sup>[50]</sup>. In our center, a minimum increase of 40% is needed for completing the procedure. It is of paramount importance to distinguish between true parenchymal hypertrophy and liver enlargement from postoperative liver edema or congestion. Measurement of Hounsfield units (HU) on MDCT can be of help in the distinction, since edematous parenchyma shows significantly lower attenuation compared to unaffected liver<sup>[20]</sup>. In rare cases in which doubts persist, MRI can be of help in differential diagnosis by showing parenchymal and/or prominent periportal edema.

FLR and DH are often surrounded by a thin rim of free fluid, which is usually more prominent around the DH when the endobag is on site. Of note, thin walls make the endobag usually not directly visible on images. Small air bubbles are frequently mixed within the perihepatic fluid, sometimes at a larger extent along the line of hepatectomy (Figure 5). It is crucial not to misdiagnose this normal finding with an infected collection, which is usually larger, lenticular or round in shape and sometimes well-encapsulated on contrast-enhanced images. Mild periportal edema is commonly present as a thin hypodense (on MDCT) or hyperintense (on T2-weighted MRI images) halo surrounding the intrahepatic portal branches.

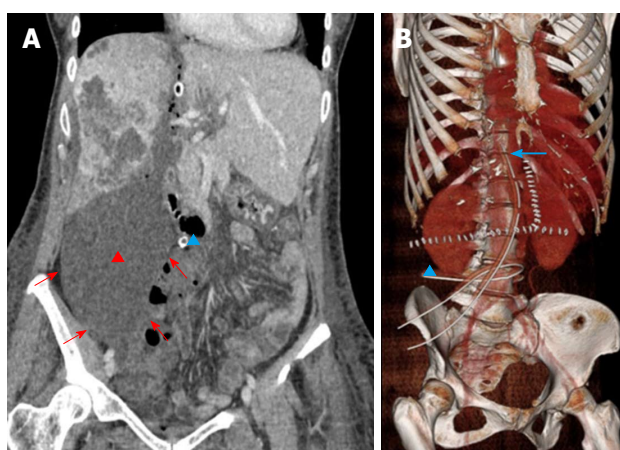
Except for the portal branches of the DH, the vascular supply to the liver is preserved, with the hepatic artery for the DH appearing slightly hypertrophic compared to the baseline examination to compensate for portal occlusion. No biliary dilation should be observed, in both the DH and FLR.

**Main complications:** Postoperative complications of ALPPS include bleeding, bile leakage, fluid or bile collections,





**Figure 5** Normal findings on computed tomography after stage 1 surgery on transverse (A) and coronally-reformatted images (B), as well as volume rendering 3D reconstruction (C) (right trisectionectomy associating liver partition and portal vein ligation for staged hepatectomy). A thin rim of free fluid with air bubbles is visible along the surface of diseased hemiliver (right liver lobe), suggesting its accumulation within the plastic bag (green triangle on A and B). A similar finding can be appreciated along the line of transection. Mild periportal edema (yellow arrowhead in A), thin hypodense bands along the edges of surgical resection (arrows on both A and B) and drains (blue arrowheads) are visible. Main right portal branch was ligated and transected (thick arrow in C). Hepatic artery branches are patent (C), including right hepatic artery, which shows mild hypertrophy (arrowhead), and left hepatic artery (thin arrow).

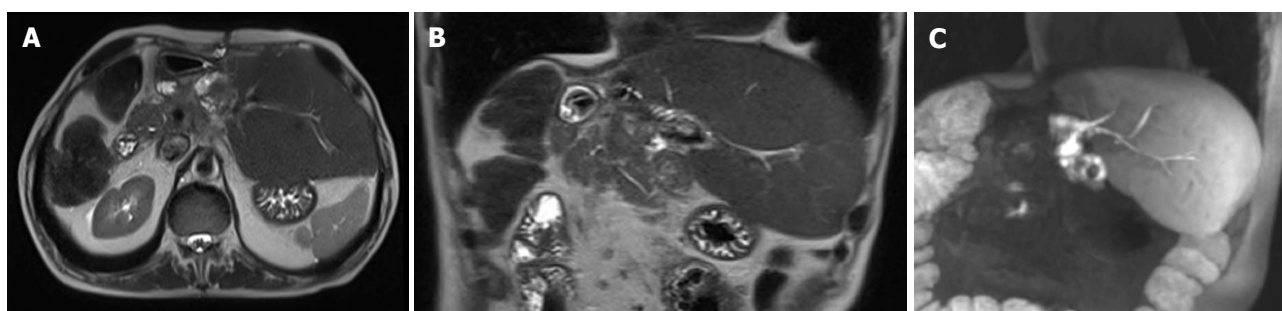


**Figure 6** Biloma in a 49-year-old female patient who underwent associating liver partition and portal vein ligation for staged hepatectomy because of peripheral cholangiocarcinoma of the right liver lobe. A: Computed tomography was performed because of bile flowing from the right drainage (blue triangle). The examination confirmed a large fluid collection beneath the DH (red triangle), which distended the plastic bag (red arrows). Biloma was removed with the DH during stage 2 surgery, resolving the biliary leakage originating from right transection surface; B: Normal position of the two drains on volume rendering reconstruction. Left drain has a vertical course along the line of transection up to the inferior margin of the diaphragm (arrow). Right drain has an horizontal course beneath DH (triangle), with its his placed within the plastic bag, in order to drain collections. DH: Diseased hemiliver.

biliary fistula, cholangitis, portal vein thrombosis (PVT), hepatic vein and hepatic arterial thrombosis, hepatic dysfunction, liver failure, persistent postoperative ascites, pleural effusion, prolonged ileus, coagulation disorders, cardiovascular, respiratory, and renal system dysfunction, encephalopathy and infection<sup>[5,53]</sup>. Clinical presentation is often challenging, since patient's signs and symptoms tend to be non-specific. They include fever, abdominal pain, jaundice, ascites, pleural effusion, abnormal liver tests and bleeding or bile within the drains<sup>[54]</sup>. Post-hepatectomy liver failure has been specifically defined according to so called 50-50 criteria (prothrombin time < 50% and total serum bilirubin > 50 mmol/L on postoperative day

5 or after)<sup>[55]</sup>. Imaging is recommended in symptomatic patients to rule-out vascular, biliary or parenchymal causes. The most common ALPPS complications encountered on abdominal cross-sectional imaging are collections, hemorrhage and vascular thrombosis.

Collections are represented by hematoma (up to 50% of cases), biloma (25%) and infected collections (25%)<sup>[56]</sup>. Collections tend to origin from the resection surfaces, *i.e.*, (assuming right trisectionectomy) in the subphrenic space if originating from the FLR, and within the endobag if originating from DH. Small bilomas and/or transient hematomas are common during the first post-operative days, being rapidly reabsorbed or showing no tendency to increase. On the contrary, collections with large size or increasing in volume over a few days should be regarded as pathological (Figure 6). Bilomas are virtually indistinguishable from serous collections on MDCT, since they present homogeneous fluid content (< 30 HU) without contrast-enhancement. Active biliary leakage can be shown on gadoxetic acid-enhanced MRCP because of contrast extravasation from bile ducts or liver surface into the collection<sup>[57,58]</sup>. Early diagnosis of biliary leakage is important to prevent biliary sepsis. In this case, stage 2 might be anticipated before the FLR is sufficiently hypertrophied, even if at risk of subsequent insufficient liver function. Hematomas usually show more heterogeneous content than bilomas, with mixed internal areas of low and high attenuation (> 30 HU) on MDCT reflecting the presence of fibrin septa and clots. On MRI, bilomas appears as fluid collections with hypointensity on T1-weighted images and hyperintensity on T2-weighted images, whereas hematomas show typical hyperintensity on T1-weighted fat suppressed images. Treatment options for collections include drainage under sonographic or MDCT guidance, as well as surgical toilette in more extensive cases<sup>[3,59]</sup>. Infected collections typically show small air bubbles from anaerobic bacteria, and may be surrounded by thickened contrast-enhancing walls of peripheral inflammatory tissue.



**Figure 7** Bilioenteric anastomosis between the jejunum and biliary branches for hepatic segments 2-3 after right trisectionectomy associating liver partition and portal vein ligation for staged hepatectomy performed for hilar colangiocarcinoma showing. A, B: Magnetic resonance imaging single-shot turbo spin echo T2 weighted images acquired on transverse (A) and coronal planes (B) show absence of biliary dilatation; C: This finding was confirmed on thick maximum intensity projection coronally-reformatted image acquired on the hepatobiliary phase after gadolinic acid administration, demonstrating regular flow of hyperintense bile.

Postoperative hemorrhage generally arises within 48 h from intervention, commonly originating from the resection margins (e.g., because of an arterial branch truncation or congestion of the hepatic vein due to stenosis or ligation), incomplete intraoperative hemostasis or dehiscence of vascular sutures<sup>[3]</sup>. MDCT with angiographic phase should be promptly performed to identify the site of bleeding and guide embolization or surgery.

The most threatening vascular complication after stage 1 is portal thrombosis. This rare condition may affect the portal trunk and/or the FLR branch, thus affecting the hypertrophy process. Not surprisingly, patients showing extensive PVT are at high risk of liver failure and death<sup>[7,26,28]</sup>. Color Doppler US has a primary role in detecting thrombosis. Similarly to other postoperative scenarios<sup>[41]</sup>, thrombosis manifests with absent flow, with or without direct demonstration of an intraluminal echogenic thrombus on B-mode. Although no specific data on ALPPS have been reported, to our knowledge, contrast-enhanced US is supposedly of help in confirming absent contrast arrival in thrombotic vessels<sup>[41]</sup>. Post-contrast MDCT and/or MRI acquired on venous and delayed phases are useful to confirm color Doppler findings, as well as to map the extent of thrombosis (portal trunk and/or FLR main branch and/or intrahepatic branches) and the degree of occlusion (partial or complete filling defects). Contrast enhancement of vascular walls is an additional findings of thrombosis, likely representing contrast engorgement within dilated vasa vasorum<sup>[60,61]</sup>. Partial thrombosis may benefit from medical therapy, whereas complete thrombosis requires thrombolysis.

### Imaging after surgical stage 2

**Goals:** Early cross-sectional imaging is usually not required in the case of an uncomplicated clinical course. Chest X-ray and abdominal US with color Doppler interrogation of major vessels are usually sufficient to monitor the patient in the first weeks after the intervention. MDCT and/or MRI should be ordered in the case of suspicious complications and/or inconclusive findings on US. On the contrary, cross-sectional imaging has a major role in the

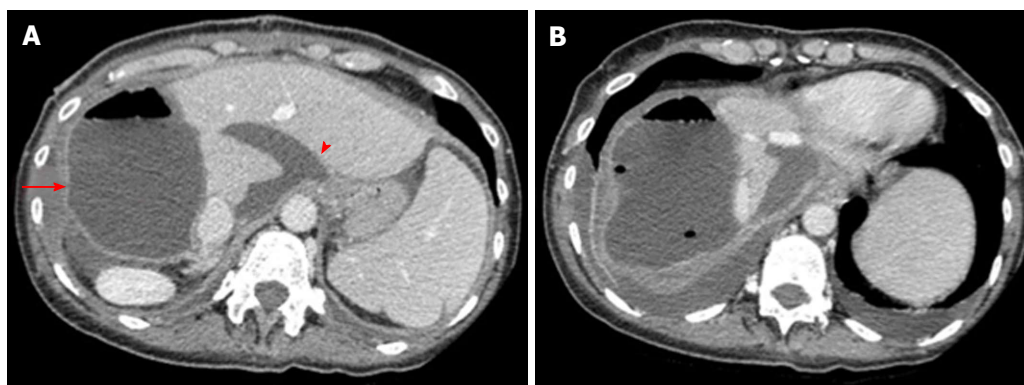
delayed postoperative period, mainly in assessing tumor recurrence and/or late complications with or without prior US.

Recommended imaging follow-up includes US and MDCT or MRI scan after 3 and 6-12 mo from surgery, respectively<sup>[54]</sup>. However, there is no definite schedule for imaging controls, which should be tailored to patients according to the type and extent of the operated tumor, concomitant chemotherapy and history of major complications after surgical stage 1 and/or 2. MRI is reserved to cases of suspicious biliary complications or for characterizing ambiguous CT findings.

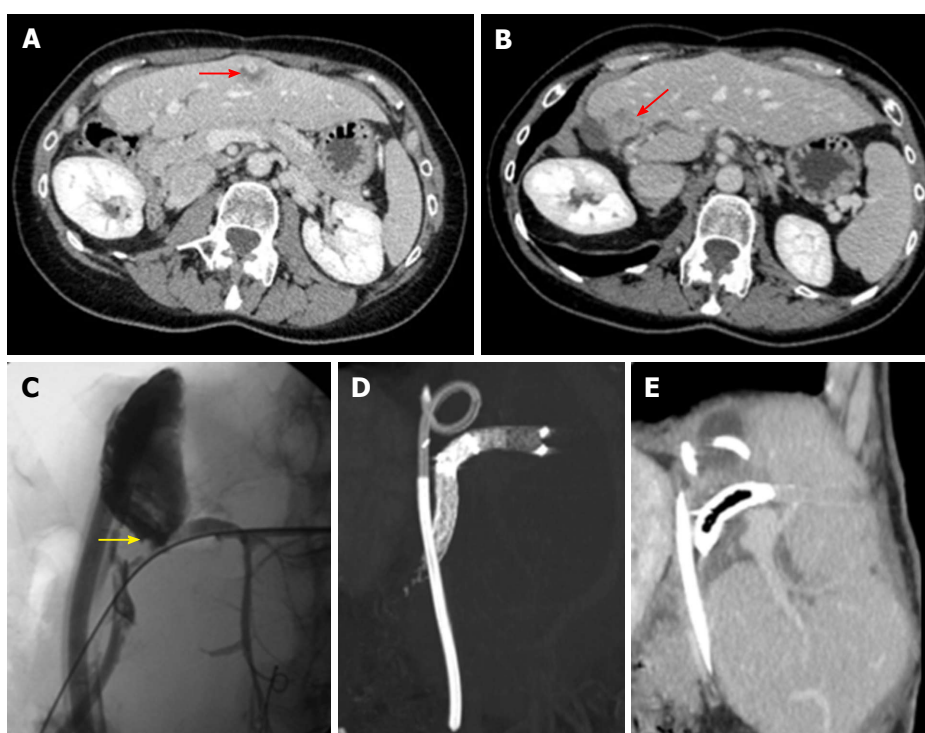
**Normal findings:** Asymptomatic, small amounts of intra-abdominal air or small fluid collections are common findings in the postoperative phase. Air is usually reabsorbed early, whereas collections can persist up to two months after surgery<sup>[56]</sup>. Another frequent finding is represented by a hypoattenuating linear band adjacent to liver raw surface (about 30%-50% of cases), which has been related to the effects of parenchymal devascularization or bile/blood accumulation<sup>[56]</sup>. No vascular or biliary abnormalities should be found (Figure 7).

Of note, transitory splenic enlargement is commonly encountered within 6 mo from hepatectomy. The degree of splenomegaly is generally proportional to the volume of liver resection, with average increase in splenic volume of about 40% compared to the preoperative period<sup>[62-64]</sup>.

**Main complications:** Complications after stage 2 may be classified into early and late, depending on the onset from surgery. Early complications occur within a few weeks from stage 2, and manifest with a clinical and radiological spectrum similar to that following stage 1 surgery. Thus, hematomas/bilomas (Figure 8), bleeding, vascular thrombosis and pleural effusion represent main expected findings, presenting as described above. Late complications are stage 2 specific, and tend to occur from 3 to about 6 mo after this surgical step. The most frequent and relevant ones are tumor recurrence and biliary complications. The treatment of late complications may be challenging, especially if further surgery is



**Figure 8** Intrabdominal collection 9 d after stage 2 surgery in a 49-year-old female patient with fever and altered liver function tests. A: Large, encapsulated collection with fluid-air level was shown after diseased hemiliver removal, with mild parietal enhancement (arrow). Part of the collection surrounded liver segment I (arrow head); B: Bilateral pleural effusion coexisted.



**Figure 9** Recurrence appears with multidetector computed tomography and/or magnetic resonance imaging signs of the original tumor, though recurrence can manifest with pleomorphic, nonspecific appearance in our experience. A, B: Multifocal recurrent cholangiocarcinoma presenting 16 mo after right trisectionectomy associating liver partition and portal vein ligation for staged hepatectomy. Lesions showed atypical persistently hypovascular appearance on dynamic contrast-enhanced multidetector computed tomography; C-E: Wedge resection of recurrences was complicated by biliary leakage, as shown on percutaneous transhepatic cholangiography (arrow in C), treated by positioning a drainage within the biloma and a biliary stent graft, as shown on maximum intensity projection reconstruction in D and oblique sagittal reformation in E.

needed. Indeed, additional interventions may in turn increase the risk of morbidity (Figure 9).

The International ALPPS registry<sup>[27]</sup> reported disease-free survival of 73% and 59% at 1 and 2 years after ALPPS, respectively, with median survival of 14 mo. Recurrence appears with MDCT and/or MRI signs of the original tumor, though recurrence can manifest with pleomorphic, nonspecific appearance in our experience (Figure 9). Suspicious solid lesions should be regarded as tumor recurrence, regardless of the fact they mimic preoperative lesions or not.

Late biliary complications include stricture and fistula. Because of the recent introduction of ALPPS, it is difficult to quantify the prevalence of these complications, which are generally rare in experienced centers. Strictures are multifactorial in origin, having been related to mechanical stress from FLR enlargement and rotation, as well as to iatrogenic causes (inaccurately placed clips, injury, periductal bile leakage and ischemia due to injured FLR hepatic artery)<sup>[65,66]</sup>.

MRCP is the elective tool to assess the site of obstruction, which appears as a focal zone of absent signal on fluid-



**Table 3** Overview of normal and abnormal findings after surgical stages 1 and 2

Postoperative phase	Normal findings		Abnormal findings
	Goals of ALPPS	Findings not to be confused with pathological aspects	prompting intervention
After surgical stage 1	Hypertrophic FLR ( $\geq 40\%$ of baseline preoperative volume)	Thin rim of free fluid around both FLR and DH Air bubbles within the perihepatic fluid, especially on the hepatectomy line Mild periportal edema Hypertrophy of hepatic artery for the DH	Large, persisting collections (hematoma, bilomas, infected collections) Bleeding Biliary dilation Bile leakage/fistula Portal vein thrombosis
After surgical stage 2	Uncomplicated appearance of the FLR (e.g., no relapsing focal liver lesions)	Thin rim of free fluid around FLR Air bubbles Hypoattenuating linear band adjacent to liver raw surface Rotation of hypertrophic FLR Transitory splenomegaly	Early complications see surgical stage 1 Late complications (3-6 mo) Biliary stricture Biliary fistula Tumor recurrence

ALPPS: Associated liver partition and portal vein ligation for staged hepatectomy; FLR: Future liver remnant; DH: Diseased hemiliver.

sensitive images, as well as the degree of proximal biliary dilation<sup>[42]</sup>. Strictures of the bilioenteric anastomosis should be evaluated with gadoxetic acid-based MRCP, which shows lack of contrast flow from the biliary tree to the anastomotic bowel loop<sup>[65,67]</sup>. This technique is of help also in identifying the site of bile extravasation when chronic biliary fistula is suspected. Similarly to other clinical scenarios<sup>[42]</sup>, MRCP is electively ordered in patients with low pre-test probability of biliary complications, since a negative result is reliable enough to avoid invasive procedures of direct cholangiography. On the other hand, MRCP is effective also in patients with high pre-test probability of disease, since it provides a panoramic and detailed representation of pathological findings, *i.e.*, an accurate road-map for planning the most appropriate interventional approach. Most bilomas and strictures are treated with endoscopic sphincterotomy and balloon dilation followed by endoprosthesis placement.

An overall view of normal postoperative findings and complications after both surgical stages 1 and 2 is provided in Table 3.

## CONCLUSION

ALPSS is an increasingly popular two-stage hepatectomy technique associated with portal ligation aimed to obtain rapid and adequate FLR hypertrophy, thus extending operability in patients with massive primary or secondary neoplastic liver involvement.

Cross-sectional imaging, especially MDCT, plays a key role in planning ALPPS procedure and monitoring different surgical stages. In particular, MDCT is the main instrument to provide liver volumetry, which is of special importance in assessing technique feasibility and assessing variation in volume of the FLR between surgical stages. MDCT also confirm a clinical or sonographic suspicion of complications, including collections, bilomas, hematomas, post-surgical bleeding, PVT, and tumor recurrence. MRI should be used as a problem-solving tool in both preoperative and postoperative phases, whereas MRCP has an elective role in assessing biliary

complications.

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Prospective Study

## Low bone mineral density and the severity of cholestasis in biliary atresia

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## Abstract

### AIM

To investigate the prevalence of osteopenia and osteoporosis in postoperative biliary atresia (BA) children and the association of bone mineral density (BMD) and biochemical parameters in postKasai BA subjects.

### METHODS

A total of 70 patients with postKasai BA were enrolled in this prospective study. The patients were classified into two groups according to their jaundice status. BMD of the lumbar spine was analyzed using dual energy

X-ray absorptiometry.

## RESULTS

The prevalence of low bone mass (osteopenia and osteoporosis) in BA patients were 51.4% (36 out of 70). Ten patients (35.7%) in the jaundice group and 8 patients (19.0%) in the non-jaundice group had osteopenia. Sixteen patients (57.1%) in the jaundice group and 2 patients (4.8%) in the no jaundice group had osteoporosis. In addition, lumbar spine BMD Z-score was substantially lower in the jaundice BA patients compared with non-jaundice patients. BA subjects with persistent jaundice had significantly lower serum 25-hydroxyvitamin D than those without jaundice. Further analysis revealed that lumbar spine BMD was correlated with age ( $r = 0.774$ ,  $P < 0.001$ ), serum albumin ( $r = 0.333$ ,  $P = 0.005$ ), total bilirubin ( $r = -0.476$ ,  $P < 0.001$ ), aspartate aminotransferase ( $r = -0.583$ ,  $P < 0.001$ ), alanine aminotransferase ( $r = -0.428$ ,  $P < 0.001$ ), and alkaline phosphatase ( $r = -0.456$ ,  $P < 0.001$ ).

## CONCLUSION

Low BMD was associated with biochemical parameters reflecting the severity of cholestasis in postKasai BA patients.

**Key words:** Bone mineral density; Jaundice; Biliary atresia; Cholestasis; Severity

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**Core tip:** Recent evidences have highlighted the importance of bone mineral density (BMD) in chronic liver disease including biliary atresia (BA). This study revealed that BA patients with persistent jaundice had significantly lower BMD and 25-hydroxyvitamin D than those without jaundice. Furthermore, lumbar spine BMD was correlated with hepatic dysfunction suggesting that low BMD was associated with outcome parameters reflecting the severity of cholestasis in postoperative BA patients.

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## INTRODUCTION

Biliary atresia (BA) is a progressive, idiopathic, necro-inflammatory process resulting in obliteration of the extrahepatic biliary tree resulting in intrahepatic cholestasis, hepatic fibrosis, biliary cirrhosis, and advanced chronic liver failure<sup>[1]</sup>. It is a rare disease, with the reported prevalence ranging from 1 in 5000 to 1 in 19000 live births<sup>[2]</sup>. It is

the most common cause of neonatal jaundice for which surgery is indicated and also the most common indication for liver transplantation in children. The pathogenesis of BA has remained a mystery. Most of the causal theories include defects resulting from a viral infection or toxin exposure, defects in morphogenesis, genetic predisposition, defects in prenatal circulation and immune dysregulation<sup>[3-5]</sup>.

Low bone mass is frequent in patients with chronic liver disorder including BA. Metabolic bone disease is a common disorder that can be found in patients with hepatic osteodystrophy, particularly those affected by chronic cholestasis<sup>[6,7]</sup>. Its etiology is complex and multifactorial and presents as osteopenia and osteoporosis which should be investigated and diagnosed early in patients with chronic liver disease in order to minimize the risk of fractures and improve their quality of life<sup>[8,9]</sup>. The purpose of this study was to determine bone mineral density (BMD) from postKasai BA children and to investigate the association of BMD and outcome parameters in postoperative BA patients.

## MATERIALS AND METHODS

### Patients

This investigation was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University and was conducted in compliance with the Declaration of Helsinki. All parents of BA children were informed of the study's objectives, and written informed consent was derived from the parents prior to the participants entering the study.

A total of 70 postKasai BA subjects (30 males and 40 females; mean age  $7.6 \pm 0.5$  years) who attended the follow-up visit in Pediatric Liver Clinic at King Chulalongkorn Memorial Hospital were recruited in the present study. Among the 70 BA children in this study, none of them had any evidence of residual infection or ascending cholangitis or clotting abnormalities during venipuncture. None had experienced liver transplantation. To compare the clinical outcomes among BA subjects, they were allocated into two groups corresponding to their levels of serum total bilirubin (TB): Non-jaundiced group (TB  $< 2.0$  mg/dL,  $n = 42$ ) and persistently jaundiced group (TB  $\geq 2.0$  mg/dL,  $n = 28$ ).

### Laboratory tests

Venous blood specimens were procured from each subject, centrifuged, and then kept at  $-80^\circ\text{C}$  until measurement. Liver function tests including TB, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were assessed using Hitachi 912 automated chemical analyzer at the central laboratory of our hospital. Serum 25-hydroxyvitamin D [25(OH)D] levels were analyzed using automated chemiluminescent immunoassay (Diasorin, Saluggia, Italy).

### BMD assessments

Dual-energy X-ray absorptiometry scans (Hologic QDR



**Table 1** Demographic data and laboratory parameters of biliary atresia patients based on status of jaundice

BA patients	Total	Jaundice	No jaundice	P-value
<i>n</i>	70	28	42	
Gender (male/female)	30:40	12:16	18:24	0.5
Age (yr)	7.6 ± 0.5	6.3 ± 0.8	8.6 ± 0.6	0.01
Albumin (g/dL)	3.9 ± 0.1	3.2 ± 0.3	4.3 ± 0.1	< 0.001
Total bilirubin (mg/dL)	3.8 ± 0.7	8.2 ± 1.5	0.9 ± 0.1	< 0.001
Direct bilirubin (mg/dL)	2.5 ± 0.6	5.8 ± 1.1	0.2 ± 0.1	< 0.001
AST (IU/L)	148.8 ± 13.7	235.9 ± 20.9	90.8 ± 11.3	< 0.001
ALT (IU/L)	133.3 ± 12.8	183.4 ± 18.4	99.8 ± 15.7	0.001
ALP (IU/L)	501.7 ± 36.3	681.6 ± 46.3	381.8 ± 43.3	< 0.001
25(OH)D (ng/mL)	25.3 ± 1.1	16.0 ± 1.8	30.1 ± 0.7	< 0.001
Lumbar BMD (g/cm <sup>2</sup> )	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	< 0.001
Lumbar BMD Z-score	-1.2 ± 0.2	-2.3 ± 0.2	-0.4 ± 0.1	< 0.001

Data are expressed as mean and SEM. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BA: Biliary atresia; BMD: Bone mineral density; 25(OH)D: 25-hydroxyvitamin D.

2000, Hologic Inc., Waltham, MA, United States) were performed on the lumbar spine (anteroposterior lumbar vertebrae L1-L4) of every subject for BMD assessments. BMD was reported as grams of mineral per square centimeter (g/cm<sup>2</sup>) and Z-scores. Z-scores of BMD were expressed as numbers of standard deviations from the mean BMD of age matched norms. Children were categorized into normal, osteopenia, and osteoporosis based on World Health Organization (WHO) criteria. Osteoporosis was designated as a lumbar spine BMD equal to or exceeding 2.5 standard deviations (SD) below the average values (Z score ≤ -2.5). Osteopenia was designated as a lumbar spine BMD below 2.5 SD but above 1 SD under the average values (-2.5 < Z score < -1.0). Normal BMD was designated as a lumbar spine BMD equal to or below 1 SD under the average values (Z score ≥ -1.0).

### Statistical analysis

Statistical analysis was performed using the statistical package for social sciences software, version 22.0 for Windows. All values are expressed as a mean ± standard error. Demographic and clinical data between groups were compared by  $\chi^2$  tests and unpaired Student's *t* tests, where appropriate. Comparisons of clinical data and biochemical markers among patients with normal, osteopenia, and osteoporosis were analyzed using one-way analysis of variance (ANOVA) with Tukey post hoc test if ANOVA showed significance. Correlations between numerical data were acquired using the Pearson correlation coefficient (*r*). A *P*-value < 0.05 indicated statistically significant.

## RESULTS

### Comparisons between BA subjects with and without persistent jaundice

Seventy postKasai BA patients were enrolled in this prospective study. The characteristics and laboratory parameters of BA children with persistent jaundice compared to BA children without jaundice are described

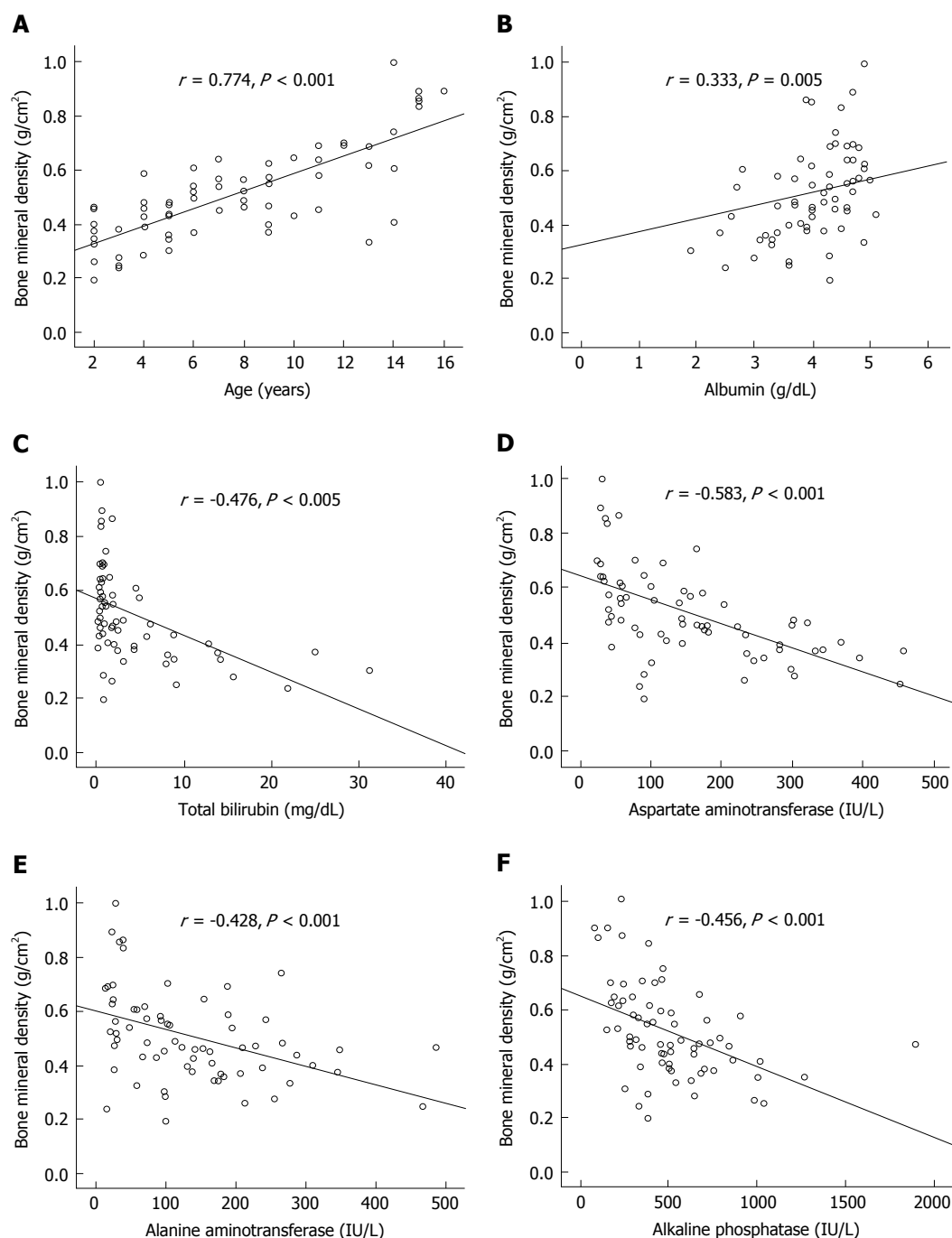
in Table 1. Jaundice BA subjects had markedly lower serum albumin levels than non-jaundice BA children. On the other hand, serum bilirubin, AST, ALT, ALP were considerably higher in BA cases with jaundice than those without jaundice. Subsequent analysis demonstrated that lumbar spine BMD and serum 25-hydroxyvitamin D values of jaundice BA subjects were significantly lower than those of non-jaundice BA subjects (*P* < 0.001).

### Correlation of lumbar spine BMD and outcome parameters in BA subjects

The prevalence of low bone mass (osteopenia and osteoporosis) in BA subjects were 51.4% (36 out of 70). Ten patients (35.7%) in the jaundice group and 8 patients (19.0%) in the non-jaundice group had osteopenia. Sixteen patients (57.1%) in the jaundice group and 2 patients (4.8%) in the no jaundice group had osteoporosis. Subsequently, BA patients were divided into tertiles based on the WHO criteria. The first tertile included 34 patients with BMD Z-scores from 0 to -1 (considered as normal), the second tertile included 18 patients with Z-scores from -1.0 to -2.5 (considered as osteopenia), and the third tertile included 18 patients with Z-score lower than -2.5 (considered as osteoporosis). There was no statistically significant difference in gender and age distribution among the three tertiles (Table 2). However, serum albumin, serum bilirubin, AST, ALT, serum 25(OH)D and lumbar spine BMD were significantly different between the three tertiles. Further analysis revealed that lumbar spine BMD was correlated with age (*r* = 0.774, *P* < 0.001), serum albumin (*r* = 0.333, *P* = 0.005), TB (*r* = -0.476, *P* < 0.001), AST (*r* = -0.583, *P* < 0.001), ALT (*r* = -0.428, *P* < 0.001), and ALP (*r* = -0.456, *P* < 0.001). The correlations between lumbar spine BMD, age, serum albumin, serum TB, AST, ALT, ALP are illustrated in Figure 1.

## DISCUSSION

BA is a serious cholestatic liver disease in neonates. The obstruction of bile flow in BA results in worsening



**Figure 1** Scatter diagram and correlation analysis in biliary atresia patients. Lumbar spine bone mineral density are correlated with age (A), serum albumin (B), total bilirubin (C), aspartate aminotransferase (D), alanine aminotransferase (E), alkaline phosphatase (F).

cholestasis, liver fibrosis and cirrhosis, which lead to portal hypertension and eventually end-stage liver failure in children. Early diagnosis and timely Kasai porto-enterostomy to restore bile flow can help avoid the need of liver transplantation during childhood in a number of patients<sup>[10]</sup>. Despite a number of extensive clinical research studies on BA, the etiology and pathogenesis of BA are largely unknown.

In the recent years, serum 25-hydroxyvitamin D level was decreased in BA patients with low BMD<sup>[11]</sup>. Additionally, circulating leptin and osteoprotegerin levels has been shown to be correlated with BMD and

the presence of jaundice in BA, suggesting that leptin and osteoprotegerin could play a potential role in maintaining bone mass of BA patients<sup>[12,13]</sup>.

The current study showed that postoperative BA patients with jaundice had significantly lower lumbar spine BMD than those without jaundice. Moreover, we have illustrated that the prevalence rates of osteopenia and osteoporosis in jaundiced BA subjects were higher in comparison with those in non-jaundiced children. Further analysis revealed an inverse association between lumbar spine BMD and serum TB and liver synthetic function. The explanation for these findings may be attributable to

**Table 2** Comparison of clinical characteristics and laboratory parameters among biliary atresia patients with normal, osteopenic, and osteoporotic bone mineral density Z-scores at the lumbar spine

Characteristics	Normal	Osteopenia	Osteoporosis	P-value
<i>n</i>	34	18	18	
Gender (male/female)	15/19	7/11	8/10	0.3
Age (yr)	8.2 ± 0.7	7.7 ± 1.1	6.5 ± 1.0	0.4
Albumin (g/dL)	4.1 ± 0.2	4.0 ± 0.1	3.3 ± 0.2	< 0.05
Total bilirubin (mg/dL)	1.0 ± 0.2	2.8 ± 0.7	10.0 ± 2.1	< 0.001
Direct bilirubin (mg/dL)	0.4 ± 0.1	1.6 ± 0.5	7.3 ± 1.7	< 0.001
AST (IU/L)	95.6 ± 13.7	177.1 ± 24.8	221.2 ± 31.2	< 0.001
ALT (IU/L)	104.2 ± 18.2	164.6 ± 23.7	156.8 ± 25.1	< 0.001
ALP (IU/L)	429.1 ± 55.7	538.4 ± 55.2	602.3 ± 71.3	0.08
25(OH)D (ng/mL)	33.2 ± 0.7	26.3 ± 0.5	14.3 ± 1.5	< 0.01
Lumbar BMD (g/cm <sup>2</sup> )	0.6 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	< 0.001

Data are expressed as mean and SEM. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BA: Biliary atresia; BMD: Bone mineral density; 25(OH)D: 25-hydroxyvitamin D.

decreased osteoblastic function or increased osteoclastic resorption in BA patients. It has been documented that osteoblast proliferation was inhibited by unconjugated bilirubin *in vitro* and by the serum of jaundiced patients, indicating that bilirubin might have a direct effect on bone metabolism<sup>[14,15]</sup>. A number of BA cases eventually become advanced stage of liver disease and pediatric liver transplantation is the treatment strategy of choice for improving quality of life in BA children. Recent study has reported that successful liver transplantation could improve biochemical markers of bone formation and resorption suggesting acceleration of growth process in BA children<sup>[16]</sup>. However, the connection between cholestasis and low bone mass in BA patients merits further investigations.

Some caveats need to be acknowledged regarding the current study. First, the number of patients and controls enrolled in the present study was relative small. This could reduce the statistical power of these results. Accordingly, prospective longitudinal study with a larger population is warranted to elucidate the exact relationship between BMD, outcome parameters, and the severity in BA subjects. Secondly, inadequate measurement of plausible confounding factors including comorbidities needed to be taken under advisement. Moreover, another limitation of our study is the lack of Child-Pugh and Model for End-Stage Liver Disease (MELD) scores. Future study is also required to evaluate the Child-Pugh and MELD values for predicting of chronic liver disease severity. Ultimately, the paucity of quantitative bone histomorphometry analysis which may render evidence as to whether bone was correlated with BMD data. Therefore, more research will be needed in order to better comprehend the precise role of bone mass in the severity of postKasai BA.

To summarize, the current study demonstrated that BA subjects with persistent jaundice had significantly lower BMD than those without jaundice. Additionally, lumbar spine BMD was correlated with hepatic dysfunction suggesting that low BMD was associated with outcome parameters reflecting the severity of cholestasis in postKasai BA patients.

## COMMENTS

### Background

Biliary atresia (BA) is a severe congenital cholestatic liver disease with an unknown etiology. Metabolic bone disorder (osteopenia and osteoporosis) can be complicated by existing chronic liver diseases including BA. There is evidence that serum markers of bone metabolism correlated with the degree of jaundice in BA.

### Research frontiers

In recent years, much research has revealed that vitamin D deficiency is associated with the severity of hepatic fibrosis or reduced bone mineral density (BMD) in patients with chronic liver disease. This study showed that lumbar spine BMD and 25-hydroxyvitamin D level in BA patients with jaundice were lower than those without jaundice. Moreover, low BMD was associated with serum bilirubin and liver function.

### Innovations and breakthroughs

Jaundiced BA patients showed significantly lower lumbar spine BMD and 25-hydroxyvitamin D than in non-jaundiced BA patients. Additionally, lumbar spine BMD correlated with hepatic function markers, which reflect the severity of cholestasis in postKasai BA patients.

### Applications

BMD could be used to assist clinicians in assessing the progression of cholestasis. This study highlights the need of vitamin D supplementation and its potential in maintaining bone mass in persistently jaundiced BA children.

### Terminology

BMD is the amount of bone mineral per unit volume of the bone tissue and is used as an indirect parameter of bone health. BMD measurements of the patients are generally compared to those from age-matched population and are expressed as Z-score. Osteopenia is defined as Z-score between -1 and -2.5, and osteoporosis as Z-score < -2.5.

### Peer-review

A very interesting study to explore the prevalence of osteopenia and osteoporosis in post-Kasai BA children and the association of bone mineral density and biochemical parameters in postoperative BA patients.

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## Successful surgical resection of ruptured cholangiolocellular carcinoma: A rare case of a primary hepatic tumor

Shota Akabane, Takushiro Ban, Shunsaku Kouriki, Hiroyuki Tanemura, Haruhiro Nakazaki, Masayuki Nakano, Nobuaki Shinozaki

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### Abstract

Spontaneous rupture is one of the most fatal complications of hepatic tumors such as hepatocellular carcinoma. In fact, many studies have shown that the in-hospital and 30-d mortality rates are as high as 25%-100%. Cholangiolocellular carcinoma (CoCC) is a rare primary hepatic tumor, usually small in size, that is thought to originate from the ductules and/or canals of Hering. Here, we present a case of spontaneous rupture of a CoCC that was successfully resected by radical surgery. Although CoCC is a rare primary hepatic tumor, it demonstrates certain specific clinical features, including a better prognosis than for other primary liver cancers, and thus should be distinguished from those other cancers. Moreover, CoCC can appear as a ruptured huge tumor, and when it does, radical hepatectomy can be an effective measure to achieve both absolute hemostasis and curability of tumor.

**Key words:** Hepatic tumor; Rupture; Cholangiolocellular carcinoma; Resection; Pathology

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**Core tip:** Spontaneous rupture is one of the most fatal complications of hepatic tumors such as hepatocellular carcinoma. Here, we present a case of spontaneous rupture of a cholangiolocellular carcinoma (CoCC) that was successfully resected by radical surgery. Although CoCC is a rare primary hepatic tumor, it demonstrates certain specific clinical features, including a better prognosis

than for other primary liver cancers, and thus should be distinguished from those other cancers. Moreover, CoCC can appear as a ruptured huge tumor, and when it does, radical hepatectomy can be an effective measure to achieve both absolute hemostasis and curability of tumors.

Akabane S, Ban T, Kouriki S, Tanemura H, Nakazaki H, Nakano M, Shinozaki N. Successful surgical resection of ruptured cholangiolocellular carcinoma: A rare case of a primary hepatic tumor. *World J Hepatol* 2017; 9(16): 752-756 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i16/752.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i16.752>

## INTRODUCTION

Spontaneous tumor rupture is one of the most fatal complications of hepatic tumors such as hepatocellular carcinoma (HCC). In fact, many studies have shown that the in-hospital and 30-d mortality rates are as high as 25%-100%<sup>[1]</sup>. Cholangiolocellular carcinoma (CoCC) is a rare primary hepatic tumor first described by Steiner and Higinson<sup>[2]</sup>. Subsequent reports characterized it based on small cords resembling cholangioles (canals of Hering). Although CoCC was previously classified as a special type of intrahepatic cholangiocarcinoma (ICC), as a result of recent advancements in the field, it is now considered to originate from hepatic stem/progenitor cells.

Here, we present a case of spontaneous rupture of a CoCC that was successfully resected by radical surgery.

## CASE REPORT

An 80-year-old Japanese woman presented with right upper abdominal pain that had developed within 2-3 h along with hypotension. She had not experienced vomiting or diarrhea. Her medical history included hypertension and dyslipidemia. On physical examination, there was tenderness in the right upper abdomen, and her body temperature was 36.5 °C. Laboratory tests were negative for anemia and thrombocytopenia and revealed bilirubin, transaminase and albumin levels in the normal range. A contrast-enhanced computed tomography (CT) scan showed a huge tumor (12 cm × 7 cm × 9 cm) located in the right anterior segment of the liver along with extrahepatic hematoma (Figure 1).

The tumor was hyperattenuating relative to the noncancerous liver parenchyma in the arterial phase and was hypo- or isoattenuating in the delayed phase, with central necrosis appearing as a low-density area. The axial T1-weighted gradient-echo image showed a hypointense mass in the right anterior segment of the liver, and the axial T2-weighted spin-echo image with fat suppression showed an isointense mass with a large central hyperintense area (Figure 2). In addition, the penetrating portal tract showed hyperintensity.

The patient was subsequently diagnosed with a ruptured hepatic tumor. Although emergency transcatheter

arterial embolization (TAE) was considered, she was hemodynamically stable due to fluid resuscitation and blood transfusion. As a result, primary right hemihepatectomy was performed. Following surgery, the patient was admitted to the intensive care unit and transferred to the general ward 5 d after surgery. Although it took time to improve her nutritional status and to rehabilitate her, she was discharged 30 d later without any complications.

Histologically, the tumor was mainly composed of small, monotonous glands formed into antler-like anastomosing patterns, embedded in the fibrous stroma to various degrees of fibrous stroma and lacking mucin production (Figure 3). The presence of CoCC cells was confirmed by positive staining for cytokeratin 19 (CK19) and membranous positive staining for epithelial membrane antigen (EMA), but no positive staining for hepatocyte paraffin 1 (HepPar1) was present (Figure 4).

So far, the patient has attended the outpatient clinic for follow-up for 1 year after surgery, with no signs of recurrence detected on CT scans.

## DISCUSSION

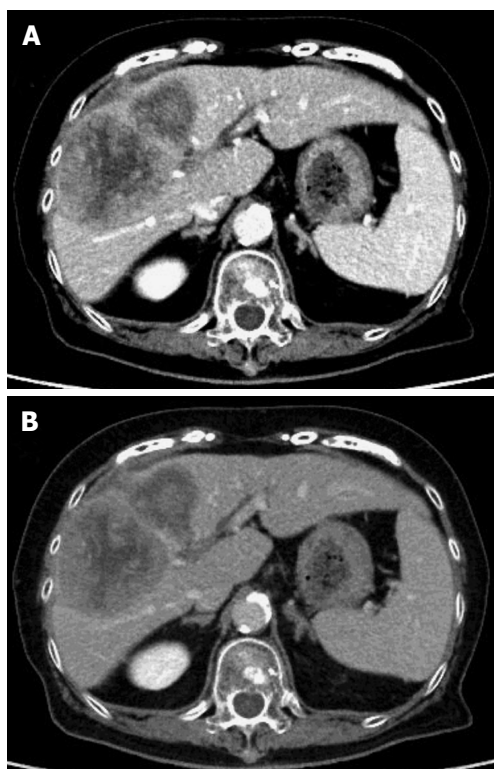
This case highlights two important considerations. First, although CoCC is a rare primary hepatic tumor, it demonstrates certain specific clinical features and hence should be distinguished from other primary liver cancers, such as HCC or ICC. Second, to the best of our knowledge, this is the first report describing the presentation of a CoCC with spontaneous tumor rupture that was successfully resected.

CoCC is derived from the cholangioles or canals of Hering and is characterized by small cords resembling cholangioles and ductular reaction-like anastomosing glands in abundant fibrous stroma. The canals of Hering are found in portal tracts of all sizes, where the canals connect with the bile duct. The ductules contain hepatic progenitor cells that can differentiate into both hepatocytes and cholangiocytes. Therefore, in the case of tumors derived from hepatic progenitor cells, characteristics of hepatocytic and cholangiocytic differentiation can be alternately displayed within the same tumor.

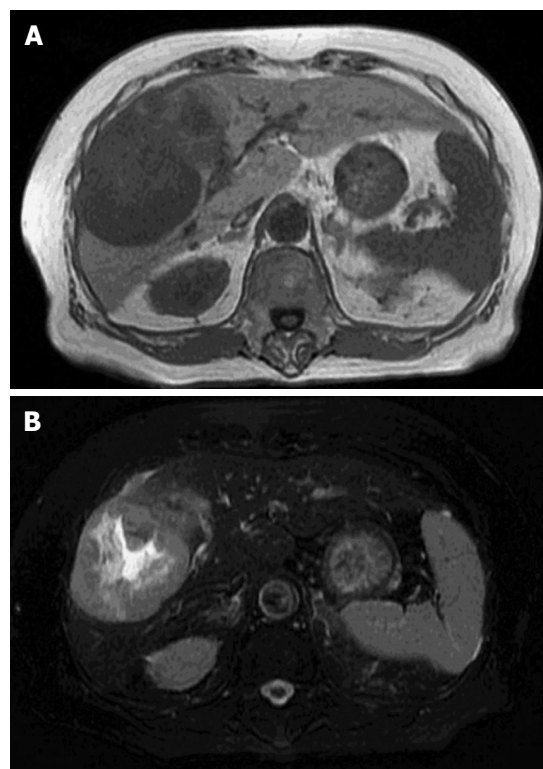
The clinical characteristics and imaging features of CoCC are similar to those of HCC and ICC. Many CoCC patients are infected with hepatitis C virus or hepatitis B virus, and angiographical hypervascularity is one of the characteristics of CoCC<sup>[3]</sup>; therefore, it is not surprising that CoCC has often been mistaken for HCC in the clinic<sup>[4]</sup>.

Histologically, the presence of CoCC cells is further confirmed by either positive staining for CK19 or membranous positive staining for mucin core protein 1 and/or membranous positive staining for EMA but negative staining for HepPar1.

Patients with CoCC demonstrate favorable long-term survival after curative surgery. CoCC has been shown to be less invasive in the portal vein, as the number of patients with remaining portal tracts within their tumors was significantly higher in a CoCC group than in an



**Figure 1** Contrast enhanced computed tomography scan showed a tumor located in the right anterior compartment of the liver with extrahepatic hematoma. The tumors were hyperattenuating relative to the noncancerous liver parenchyma on arterial-phase (A) and hypo- or isoattenuating on delayed phase (B).



**Figure 2** The axial T1-weighted gradient-echo image showed a hypointense mass in the right anterior segment of the liver (A), and the axial T2-weighted spin-echo image with fat suppression showed an isointense mass with a large central hyperintense area (B).

ICC group<sup>[5]</sup>. Moreover, the number of patients with intrahepatic metastasis was significantly lower in a CoCC group than in an ICC group<sup>[6]</sup>. Furthermore, the 5-year overall survival rate and recurrence-free survival rate were significantly higher in a CoCC group than in an ICC group<sup>[7]</sup>.

Spontaneous tumor rupture is one of the most fatal complications of hepatic tumors and is mainly determined by the growth characteristics of the tumor. The mechanism of tumor rupture has not been fully characterized; however, the literature<sup>[8]</sup> suggests that the pathogenesis may be associated with expansive growth and intratumoral pressure, which may cause tumor vein compression and congestion. The rapid growth of tumors also results in an insufficient blood supply to the tumors *in vivo*, causing tumor hypoxia-ischemia to occur, in turn resulting in significant necrosis.

As mentioned previously, CoCC grows relatively slowly and is less invasive, which results in a smaller tumor size (mean: 3.5 cm) than for other hepatic tumors<sup>[9]</sup>. To the best of our knowledge, this is the first report describing CoCC presenting as a huge tumor that had ruptured.

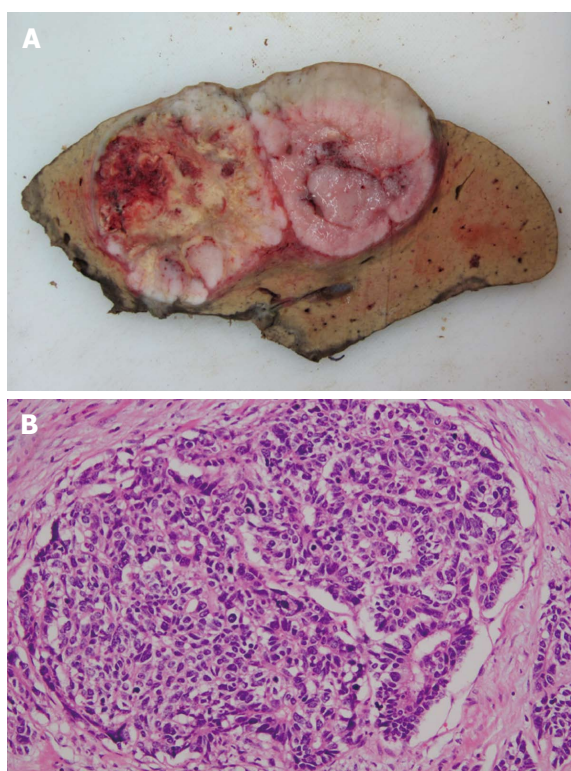
Emergency fluid resuscitation is a key therapeutic step for patients with hepatic tumor rupture. In particular, patients admitted to hospitals are treated with fast rehydration, anti-shock treatment, blood transfusion, and other supporting treatments to stabilize their circulation

so that transhepatic artery angiography and embolization (that is, TAE) can be performed for hemostasis. For patients with continued bleeding, primary surgeries are performed to stop the bleeding. Hepatectomy, however, not only can stop bleeding immediately but also can make the radical resection of hepatic lesions possible as well as allowing for better long-term outcomes. There is still a possibility of rebleeding, even with temporarily controlled bleeding, in the case of spontaneous hepatic tumor ruptures<sup>[10,11]</sup>; hence, radical hepatectomy is an effective measure to address such emergencies. However, liver resection would be risky for patients with relatively poor liver function and/or severe liver cirrhosis<sup>[12]</sup>. Thus, TAE is more advantageous during initial hemostasis in emergency procedures for patients with hepatic tumor ruptures, especially in the case of high-surgical-risk patients. Once initial hemostasis is achieved using active supportive therapy, the patients may undergo staged hepatectomy.

In the case described here, the tumor was so huge that TAE was assumed to be anatomically difficult. In addition, the patient was hemodynamically stable due to fluid resuscitation, and her hepatic function was competent; thus, primary right hemi-hepatectomy was performed as a radical treatment.

CoCC is a rare primary hepatic tumor that demonstrates a better prognosis than for other primary liver cancers, such as HCC or ICC, and thus should be dis-





**Figure 3** Histologically, the tumor was mainly composed of small, monotonous glands formed into antler-like anastomosing patterns, embedded in the fibrous stroma to various degrees of fibrous stroma and lacking mucin production. A: Macroscopically, an expanding tumor located in the right anterior compartment of the liver with extrahepatic hematoma with central necrosis; B: HE staining demonstrated the tumors mainly composed of small, monotonous glands and embedded in various degrees of fibrous stroma without mucin production.

tinguished from those other cancers.

Moreover, CoCC can appear as a ruptured huge tumor, and when it does, radical hepatectomy can be an effective measure to achieve both absolute hemostasis and tumor cure.

## COMMENTS

### Case characteristics

An 80-year-old Japanese woman presented with right upper abdominal pain that had developed within 2-3 h along with hypotension.

### Clinical diagnosis

There was tenderness in the right upper abdomen, and her body temperature was 36.5 °C.

### Differential diagnosis

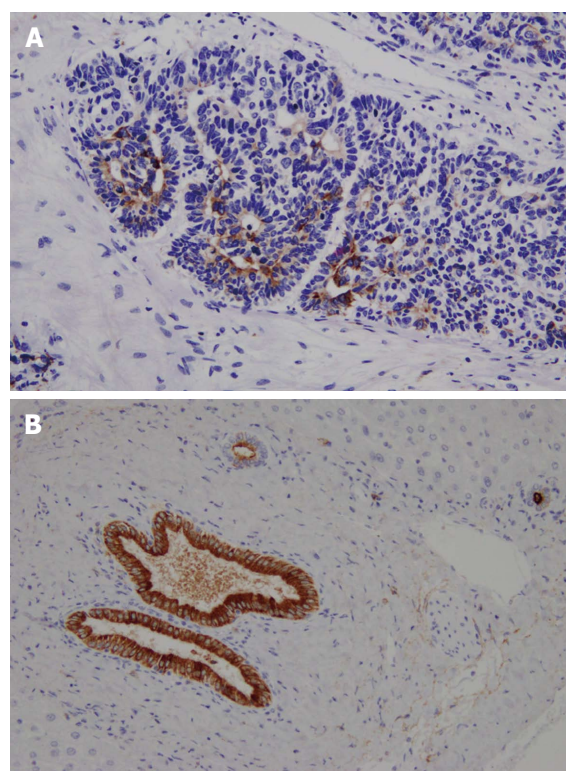
Hepatocellular carcinoma, intrahepatic cholangiocarcinoma, metastatic hepatic tumor or hepatic abscess.

### Laboratory diagnosis

Laboratory test results were within the normal range. In particular, the patient was negative for anemia and thrombocytopenia, and her bilirubin, transaminase and albumin levels were in the normal range.

### Imaging diagnosis

A contrast-enhanced computed tomography scan showed a huge tumor located in the right anterior segment of the liver along with extrahepatic hematoma.



**Figure 4** Cholangiolocellular carcinoma cells were confirmed by positive staining for cytokeratin 19 (A) and membranous positive staining for epithelial membrane antigen (B).

### Pathological diagnosis

The tumor was mainly composed of small, monotonous glands formed into antler-like anastomosing patterns, embedded in the fibrous stroma to various degrees and lacking mucin production.

### Treatment

Fluid resuscitation, blood transfusion and primary right hemi-hepatectomy.

### Related reports

Spontaneous tumor rupture is one of the most fatal complications of hepatic tumors, and it is reported that the in-hospital and 30-d mortality rates are as high as 25%-100%.

### Term explanation

Cholangiolocellular carcinoma (CoCC) was previously classified as a special type of intrahepatic cholangiocarcinoma. However, as a result of recent advancements in the field, CoCC is now considered to originate from hepatic stem/progenitor cells.

### Experiences and lessons

CoCC can appear as a ruptured huge tumor, and when it does, radical hepatectomy can be an effective measure to achieve both absolute hemostasis and tumor cure.

### Peer-review

Nice case report of successful surgical treatment of rare primary hepatic tumor. Excellent illustrations.

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## Liver immunology and herbal treatment

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### Abstract

Beyond the metabolic functions, the liver recently has been defined as an organ of immune system (IS), which have central regulatory role for innate and adaptive immunity. The liver keeps a delicate balance between hepatic screening of pathogenic antigens and immune tolerance to self-antigens. Herbal treatments with immunological effects have potential to alter this hepatic immune balance towards either therapeutic side or diseases side by inducing liver injury *via* hepatotoxicity or initiation of autoimmune diseases. Most commonly known herbal treatments, which have therapeutic effect on liver and IS, have proven *via in vitro*, *in vivo*, and/or clinical studies were summarized in this review.

**Key words:** Herbal treatments; Hepatic immunology; Drug induced liver injury; Adaptive immunity; Innate immunity

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**Core tip:** Herbal treatment is the mother of modern medicine. The ancient habit of treating diseases with plants still goes on as either primary or complementary to conventional medical treatment. The other side of medallion is the fact that the liver is number one target organ for herbal toxicity. Furthermore, liver has been recently defined as an active organ of immune system, which have central regulatory role on innate and adaptive immune response. The delicate homeostasis between immediate and efficient defense against threats (immune surveillance of antigens) without triggering harmful immune response towards self-structures (peripheral immune tolerance to self antigens) is controlled by liver. Herbal formulas are not a single plant extract, but is an interacting mixture of ingredients that determines the final clinical outcome as therapeutic and hepatotoxic effect. This review aimed to drive attention on both potentials of herbals from the point of immunology, in order to initiate a motivation for future studies defining

the mechanisms of immunological interaction between herbals and liver.

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## INTRODUCTION

Herbal treatment is the mother of modern medicine. Because of cultural, economical and practical reasons, the ancient habit of treating diseases with plants still goes on as either primary or complementary to conventional medical treatment. The other side of medallion is the fact that the liver is number one target organ of herbal and dietary supplements (HDS) induced toxicity. The Food and Drug Administration (FDA) under the Dietary Supplement Health and Education Act (DSHEA) regulate herbal treatments since 1994<sup>[1]</sup>. The submissions of new herbal products to FDA require the dose and list of ingredients to be written on its bottle, however, documentation of safety and efficacy is not need to be reported. Furthermore, HDS can be obtained without prescription, medical advice or monitoring. Although the actual size of the problem is not well defined, HDS-induced hepatotoxicity accounts for 20% of cases of hepatotoxicity in the United States and the rates differ from 2.5% in India to 70% in Singapore<sup>[2]</sup>.

The liver is prone to drug-induced liver injury (DILI) because of its functions on metabolizing chemicals and regulating immune response. DILI can develop either by dose related direct drug toxicity, or - much more commonly - as idiosyncratic reactions due to individual susceptibility to ingredients. The complex composition of HDS eases the both direct toxicity and idiosyncratic reactions during their metabolism in liver. Idiosyncratic DILI (IDILI) is in most instances characterized by a mild injury (ALT < 3 times upper normal limit) which normalized with continuous drug treatment. This phenomenon of clinical adaptation is a biochemical adaptive response of organelles such as endoplasmic reticulum and mitochondria metabolizing chemicals. It is hypothesized that defective clinical adaptation mechanisms result in severe IDILI with jaundice and liver failure, in < 0.1% population with susceptible human leucocyte antigen (HLA) type. Microbiota is the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space<sup>[3]</sup>. The microbiota of gut also determinates IDILI susceptibly by regulating hepatic immune-tolerance though lipopolysaccharides (LPS) induced T-cell response in liver. According to theory, haptens of metabolized chemicals covalently bind to proteins, and become antigenic peptides. While 70%-90% of population has immune tolerance, the rest develop adaptive immune response due to their susceptible type HLA and/or dysfunctional microbiota.

If biochemical adaptation mechanisms cannot control this initiated mild injury, acute liver failure develops<sup>[4]</sup>. There are supporting evidences to this theory. The many top IDILI drugs are antibiotics changing the normal microbiota. The "immune check point therapy" for cancer treatments are FDA approved antibodies that aim to inhibit T cell immune-tolerant states, such as ipilimumab (anti-CTLA4), pembrolizumab (anti-PD-1) and nivolumab (anti-PD-1). The major side effect of these treatments is to make individuals to susceptible to haptens (so inducing IDILI) and auto-antigens (so inducing autoimmunity).

This review aimed to drive attention on both therapeutic mechanisms and hepatotoxic potentials of herbal treatments from the point of immunology. After defining basic immune system (IS), we summarized the role of liver as an immune regulatory organ and then the herbal treatments with therapeutic potential on liver.

## IS

IS has evolved to recognize and eliminate internal insults (*i.e.*, cancer cells) or external invading pathogens (*i.e.*, infections) by developing local or systemic response. IS composed of "classical lymphoid organs"; thymus, bone marrow, spleen, tonsils, lymph nodes and "peripheral immune organs"; skin, respiratory and gut mucosa-associated lymphoid tissues, adrenal glands. Additionally, the gut and liver are recently defined as active organs of IS. Although the primary functions of liver parenchymal cells are methabolical, they also carry out essential immune tasks. Beside all metabolic functions, liver has important role as an organ of IS.

IS begins to develop during intrauterine life. However, maturation of IS depends on antigenic stimulations from environment. The gut microbiota is initiated by maternal microorganisms gained during passage through birth channel and it dynamically change according to external conditions. The gut microbiota is necessary for proper "education" of IS. Although IS completes its maturation around teenage, lifelong antigenic stimulations from microbiota is needed for normal functioning of IS. Epidemiological observations and then, experimental data from germ-free animals leads to "hygiene hypothesis" and its modern extension called "microflora hypothesis". According to these hypotheses, the higher levels of cleanliness and decreased exposure to microorganisms (driven by factors such as antibiotic use, xenobiotics, infection, or diet) during early childhood disrupt maturation if IS. In other words, the dysbiotic gut microbiota, which arisen during critical window of IS maturation, turns the differentiation of naïve immune gut dendritic cells (DCs) from generation of Treg (regulatory) cells by tolerogenic DCs into generation of effector T cells by immunogenic DCs. This shifts TH response from Th1 type (IFN- $\gamma$  mediated) to Th2 type (IL-4 mediated). As a result, the risk for autoimmune and allergic diseases increases<sup>[5,6]</sup>.

By definition IS has 2 parts; the innate IS and the adaptive IS. Although this division simplifies the understanding of immune processes, IS orchestrates

whole immune cells during local or systemic responses.

### Innate IS

The innate IS initiates first defense against insults, and is characterized by its ability to distinguish self from non-self. Its members are classic immune cells such as polymorphic nuclear leukocytes (neutrophils), monocytes, macrophages and DCs, natural killer (NK) cells, and innate lymphoid cells (ILCs), beside epithelial, endothelial and mesenchymal cells which are non-immune cells.

The inflammation during innate immune response is triggered by pattern-recognition receptors (PRRs). The 3 families of PRR, according to the structure they can recognize, are Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors and nucleotide-binding oligomerization domain-like receptors. The cells expressing PRR can recognize conserved structures. For instance, miRNAs controls multiple immune processes such as regulating the innate immune responses of macrophages, dendritic cells and NK cells; involving in T-cell differentiation and function. Furthermore defective PRR function might lead to autoimmune or auto-inflammatory diseases, since nucleic acids (DNA and RNA) is commonly shared by the pathogen and host. Additionally, damage associated molecular patterns (DAMP), pathogen associated molecular patterns (PAMP), microbiome associated molecular patterns are the subtypes of PRR<sup>[6,7]</sup>.

Mononuclear phagocyte system (MPS) composed by monocytes, macrophages, and DCs that have phenotypical and functional overlapping boundaries leading to uncertainty in differentiating them from each other. Antigen-presenting cells (APCs) express PRR and characterized by their ability to recognize, process and present antigens for activation of innate and adaptive immunity. The classical APCs include DC, monocytes and macrophages, although parenchymal cells can also act as APCs. MPS may be precursor of some APCs of liver, namely DCs and Kupffer cells (KCs)<sup>[8]</sup>.

ILCs are a recently identified family of heterogeneous variety of T cells and non-T cells, including NK cells, CD56+ T cells, natural killer T cells (NKT), gamma/delta T cells, mucosal-associated invariant T cells, lymphoid tissue-inducer cells and cells that produce IL-5, IL-13, IL-17 and IL-22. ILCs not only regulate innate and adaptive immune responses by promoting DC maturation into APCs, they have function in lymphoid tissue formation and the homeostasis of tissue stromal cells remodeling the tissues<sup>[9,10]</sup>.

### Adaptive IS

The adaptive immunity evolutionarily developed later than innate immunity in high-class vertebrates. The adaptive immune response occurs as second phase of immune response, mediated mainly by lymphocytes, and characterized by the features of antigen-specific response and memory response. It is initiated by antigen presentation lymphocytes. The main lymphoid repertoire includes T-cells, B cells. B cells produce specific antibodies in response to

a specific antigen. These antibodies are crucial for T cells activation against bacterial infections and development of active immunization after vaccination<sup>[7,11]</sup>. However, the major mediator of adaptive immune response is the T cells, which control both the establishment and regulation of adaptive immunity.

T cells are identified by CD3 and T cell receptors (TCRs) positivity, and have vital importance in the adaptive and innate immunity. Conventional T cells express alpha-beta type TCR. Gamma-delta T cells are located in skin, genitourinary tract mucosa and gut, as well as liver. The naive T cells produced in bone marrow migrates to thymus and differentiate into 2 main subtypes are Th (helper) and Ts (suppressor). The differentiated T cells are exported to periphery, where they become effector T cells upon activation by APCs or B cells. T cell activation requires binding of TCR to major histocompatibility complex (MHC), as well as binding of co-stimulatory molecule present on T cells to its co-receptor on APCs (e.g., binding of CD28 to B7). Th cells express CD4, which recognizes antigens in the context of MHC class II, and are mainly regulatory cells. Ts are cytotoxic cells carrying CD8 receptors, which are activated by MHC class I molecules<sup>[11]</sup>. Recently, CD4+ cells have been divided into subsets according to their distinct cytokine production and function; Th1, Th2, T17, Treg, Tfh (follicular T helper). Some features of CD4+ cells are as shown on Table 1. Treg cells express CD4+CD25+ and are essential for maintaining immune homeostasis and self-tolerance. Treg cells either naturally produced from CD4+ thymocytes in the thymus or iTreg cells are induced at periphery from naive CD4+ T cells in response to the low-dose stimulation of TCR, TGF-beta and IL-2. Beside all these effector T cells, there are also memory T cells. Id3 is the key transcriptional regulator for controlling T-cell differentiation into either effector T cells or memory T cells by its action through mTORC signaling<sup>[7,9,12]</sup>.

DCs are professional APC, which can recognize foreign antigens by their PRR, initiate immune response and constitute a bridge between innate and adaptive immunity. They primary screen surrounding microenvironment by antigen sampling and direct IS towards pro- or anti-inflammatory response<sup>[6]</sup>. DCs are found throughout the body as immature DCs and subdivided as plasmacytoid (or lymphoid) DCs and myeloid DCs. Plasmacytoid DCs mediate anti-viral immunity by its capability of viral recognition and type 1 interferons secretion. The myeloid DCs constitute conventional MPS derived DCs in blood, interstitial DCs in tissues, Langerhan cells in skin and monocyte-derived DCs. mDC can internalise antigens by phagocytosis, pinocytosis or receptor-mediated endocytosis. After generation of peptide by proteolytic degradation within endocytic vesicles, it complexes with newly synthesized MHC class II molecule within endocytic compartment, and then is carried *via* the trans-Golgi network to the cell surface. The recognition and internalization of pathogens by DCs leads to maturation of them into professional APCs, which have altered adhesion molecule and chemokine receptor expression.



**Table 1** Features of CD4+ cell subsets

	Th1	Th2	T17	Treg	Tfh
Produced Cytokines	IFN-gama, TNF-alpha, IL-2	IL-4, IL-5, IL-9, IL-10, IL-13	IL-17A, IL-17F, IL-21, IL-22, IL-26	TGF-beta, IL-10	CXCR5, IL-21
Immune response mediated against	Intracellular pathogens	Extracellular parasites, allergy, humoral response	Extracellular bacteria and fungi, autoimmunity	IgA secretion, self-tolerance	Differentiation of B cells
Master transcription factors for differentiation	T-bet	GATA-3	RORct	Foxp3	Bcl6
Effected cells	Macrophages, cytotoxic cells activated	Eosinophils, mast cells activated	Neutrophils activated	B cells activated Th1, Th2, Th17 suppressed	B cells activated

IFN: Interferons; IL: Interleukin; TGF: transforming growth factor; TNF: Tumor necrosis factor.

After maturation DCs leave primary site of infection through lymphatics to carry the internalized pathogen to secondary lymphoid organ. The professional APCs can be either immunogenic DCs which express high levels of MHC and co-stimulatory molecules, and secrete IL-12, IL-18, IL-21 and IL-23 or tolerogenic DCs having low expression levels, express inhibitory receptors, such as programmed death ligand-1, and releasing suppressive cytokines, such as IL-10, IL-27 and TGF-beta. Immunogenic DCs stimulate naïve CD4+ T cells to differentiation into effector cell mediating adaptive immunity against specific pathogen. On the other hand, if the antigenic peptid is presented to naïve CD4+ T cell by tolerogenic DCs, immune tolerance develops either at thymus or periphery. The consequent result in thymus is either T cell apoptosis or T cell maturation into natural Treg cells. The mechanisms for peripheral immune tolerance are anergy of T cells and exhaustion of T cells. The anergy arises when T cells are inactivated due to lack of co-stimulation. The exhaustion of T cells is characterized by expression of inhibitory receptors, namely programmed death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA4) and T cell immunoglobulin mucin-3. Interestingly in mice and humans, the lipid content of DCs in liver determines the maturation type of APCs, as lipid content decreases tolerant immune response is favoured. This phenomenon might be important for progression of simple steatosis into steatohepatitis. The relationship of autoimmune diseases with infection and environmental pollution and is very well known fact. It is thought that the similarity between insulting antigen and self antigens of individuals with susceptible HLA haplotypes causes a shift during APCs maturation from tolerogenic DCs towards to immunogenic DCs, leading to differentiation of naïve T cells into effector rather than tolerogenic cells, and ending in loss of self-immune tolerance<sup>[1,9,11]</sup>.

NK express CD56 in the absence of CD3, but NKT express both of them. NKT mediate anti-tumor effect by activating CD8+ T cells cytotoxicity or overriding the tolerogenic mechanisms through counter-regulation of Treg cells. NKT can identify glycolipid antigens and subtyped into two according to TCR expression profile. Type 1 or invariant NKT (iNKT) carry an invariant TCR alpha-chain pairing with a limited number of beta-chains, whereas type 2 NKT cells express a diverse array of TCRs

that recognize CD1d which is MHC class I-like molecule. Innate T lymphocytes (ITLs) is composed of iNKT cells and gamma-delta T cells. ITLs regulate adaptive immune response through its key roles in initiation and polarization of APCs and other cells of IS. This feature of ITLs has made them target as immunomodulation for treatment of autoimmune diseases<sup>[1,9]</sup>.

## LIVER AS AN ORGAN OF IS

In order to keep homeostasis for survival, the immune response had to continuously adapt according to age, sex, dietary antigens, hormones (*i.e.*, pregnancy and lactation), and external stress factors such as microbiota, environmental flora or exposed chemicals<sup>[13]</sup>. Therefore IS has a dynamic nature and has a wide "range of normal". Beyond being a metabolic organ attached to gut, liver recently has been defined as central axis in IS controlling local and systemic immune reactions and tolerance. All types of liver cells have active immune function, including both parenchymal cells (hepatocytes, cholangiocytes) and non-parenchymal cells [liver sinusoidal endothelial cells (LSECs), hepatic satellite cells (HSCs) or Ito cells, KCs, neutrophils, mononuclear cells, lymphocytes (B cells, T cells, NK cells, NKT cells, ITL)]. Parenchymal cells occupy most of liver volume (78%-80%). Non-parenchymal cells and extracellular space represent the remaining 5%-6% and 14%-17%, respectively<sup>[11,14]</sup>.

The unique anatomical and histological features of liver are important for its immune functions. The liver is located at the junction between systemic and portal circulation. It is supplied by approximately 1.5 L of blood every minute; 2/3 *via* the portal vein and 1/3 *via* the hepatic artery. The double blood supply carries a massive antigenic load from the gastrointestinal tract and systemic circulation to liver. The blood, coming from these two sources mixes within sinusoids, and then flows through hepatic lobule from peri-portal area towards central vein. The fenestrated structures of sinusoids enable intimate interaction of antigens and blood immune cells with hepatocytes, KCs and HSCs at space of Disse. The abundant cells of the innate and adaptive ISs are located in hepatic sinusoids, and have ability for pathogen sensing, phagocytosis, cytotoxicity, cytokine release and antigen presentation to T cells.

The antigen-rich blood passing through the liver sinusoids is “scanned” by IS, which is tightly regulated between activation and tolerance. The liver remains tolerant to harmless dietary antigens, products of commensal gut microbiota and auto-antigens, while responds to exogen toxins, a variety of blood-borne or gut originated viruses, bacteria and parasites, as well as to metastatic cells, which try to home to the liver. Therefore, immune roles of liver can be divided into 2 groups; immune surveillance and induction of peripheral immune tolerance<sup>[3,15]</sup>. Indeed, the hepatic IS plays predominantly tolerogenic role. This can clinically be observed in liver transplant patients, *e.g.*, liver allograft from major MHC or even ABO mismatched donors can be transplanted; if combined transplantation is done with organs from the same donor, non-liver allografts are more likely to be accepted; “operational tolerance”, which describes a patient with clinically normal graft function without needing immunosuppression, developments in up to 50% of hepatic transplantations<sup>[16]</sup>.

### **Immune surveillance function of liver**

The liver relies on its strong immunity for its immediate and efficient defense against potentially toxic agents without triggering harmful immune response towards self-structures. Liver, primarily hepatocytes, synthesizes the major amount of proteins involved in local and systemic immune responses. These proteins are called acute phase reactants such as fibrinogen, proteinase inhibitors, complement proteins, PRRs [*e.g.*, C reactive protein, lipopolysaccharide (LPS)-binding protein, peptidoglycan-recognition protein, soluble CD14], opsonizing proteins (*e.g.*, mannose-binding lectin, serum amyloids), cytokines [*e.g.*, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta$  (TGF- $\beta$ )], and hepcidin. The acute phase reactants function during innate immune response, mediate inflammation as well as tissue repair and regeneration. Their expression in hepatocytes is controlled by liver-enriched transcription factors (*e.g.*, HNFs, C/EBPs), pro-inflammatory cytokines (*e.g.*, IL-6, IL-22, IL-1 $\beta$ , TNF- $\alpha$ ), and downstream signaling pathways (*e.g.*, STAT3, NF- $\kappa$ B)<sup>[7,15]</sup>.

Neutrophils are short-lived, circulating, phagocytic cells, which are recruited to site of infection by cytokines and chemokines, mainly IL-1 and IL-8. They are the first responders to infections and act by three main mechanisms; phagocytosis (requiring opsonization), generation of reactive oxygen species and degranulation (releasing enzymes and antimicrobial peptides), and formation of neutrophil extracellular traps (NETs). NETs are another mechanism of microbe killing. Nuclear DNA ligated with various microbicidal proteins released by activated neutrophils forms these webs. Under normal conditions the liver have few neutrophils, but they rapidly accumulate following necrosis. Neutrophils can rapidly shift their adhesive mechanisms in order to regrade and form NETs in liver as a response to both endotoxin and bacteria<sup>[14]</sup>.

Macrophages have been classified as classically activated macrophages (M1, secretes TNF- $\alpha$ , IL-1, IL-6,

IL-8 and IL-12) or alternatively activated macrophages (M2, secretes IL-10 and TGF- $\beta$ ) based on their cytokine secretory patterns and proinflammatory vs immunoregulatory activity which however, are interchangeable functional states depending on the microenvironment the macrophages encounter<sup>[6,11]</sup>. KCs are fixed macrophages specialized at eliminating insoluble waste by phagocytosis and capable of processing and presenting antigens to T cells and participate in the regulation of the adaptive immune response. KCs reside on intravascular side of LSECs, and capture bacteria and able to bind component 3b under shear conditions while flowing through sinusoids. The role of KCs in microbial killing depends on the nature of the pathogen and on the recruited immune cells to the inflammation side. The characteristic feature of PRRs expression in liver is their constitutive expression and continuous low-level stimulation by endotoxins from gut. TLR4 is an PRRS expressed on all liver cells and it binds and clears endotoxins, and so initiates secretion of pro-inflammatory and anti-inflammatory cytokines<sup>[7]</sup>.

Although, more than 80% of the CD3+ T cells are alpha-betaT-cells, the liver is also enriched by NKs and unconventional lymphocytes (NKT and gamma-delta T cells). The gamma-delta T cells is 5 times higher in the liver (15%) than the periphery<sup>[4]</sup>.

Hepatocytes, which constitutively express intercellular adhesion molecule-1, can directly interact with T cells through the fenestrations of LSECs. IFN- $\gamma$  primes hepatocytes to APCs by dose dependently enhancing HLA expression; from moderate HLA class I expression to enhanced HLA class II expression at low to high IFN- $\gamma$  levels. Hepatocyte primed naïve T cells either become effector T cells or undergoes apoptosis in the absence of co-stimulatory signals. On the other hand, cholangiocytes are relatively spared from antigenic stimulation from blood, but not from those one secreted into bile. Cholangiocytes can express TLRs, HLA class I at a low frequency and co-stimulatory molecules. Hepatotropic viruses (*i.e.*, CMV) enhances HLA class I expression without inducing HLA class II. In pathological conditions such as that of PBC, cholangiocytes act as APC by overexpress HLA class II, as well as CD80 and CD86 co-stimulatory molecules. The limited experimental data supports that HSCs have capacity to act as APCs. The presentation of lipids to T-cells and NKT cells by HSCs can cause activation or tolerance in IS depending on co-stimulation<sup>[4,7]</sup>.

### **Liver mediated peripheral immune tolerance**

Besides conferring strong local innate immunity, the liver regulates immune homeostasis as being a major site for induction of T cell mediated local and systemic adaptive immune response. Both resident and transiting T and B cells scattered throughout the parenchyma and the portal tracts become important effector cells of defensive adaptive immune in liver after activation by APCs. Both hepatic parenchymal and non-parenchymal cells can act as APCs depending on the stimulus and special cytokine milieu. The classical hepatic APCs are

DCs and reticulo-endothelial system (including KCs and LSECs). However, hepatocytes and cholangiocytes become non-conventional APCs by expressing MHC II, if there is under pathological insult or persistent inflammation. Classical hepatic APCs constitutively express MHC class I - II, co-stimulatory receptors and molecules that promote antigen uptake (e.g., mannose and scavenger receptors). Under the physiological liver conditions, DCs are at immature developmental status and there is high production of an anti-inflammatory cytokine (IL-10, TGF- $\beta$ , TNF- $\alpha$  and prostaglandins) from reticulo-endothelial cells. This reduces capacity of APCs to activate effector T cells and lead to generation of anergic T cells and Treg cells. In other words, the tolerogenic nature of the liver by preferentially suppressing adaptive immunity is created by APCs, which kill or suppress effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells and induce maturation of naïve T cells into Treg cells. Since diversion of portal flow results in the loss of immune-tolerance, it is hypothesized that physiological concentration of endotoxin is essential for maintain hepatic immune tolerance. LPS from gut microbiota drained to the liver by portal vein, modulates LSECs mediated CD4<sup>+</sup> T cell activation by inducing secretion of IL-10 from LSECs and by down-regulating expression of MHC class II, CD80 and CD86 on LSEC. Another proposed mechanism for hepatic induction of peripheral immune-tolerance is clonal deletion/apoptosis of antigen-specific T cell at liver. HSCs may have a role in creation of tolerogenic micro-environment. HSCs have a capacity to serve as APCs, expand Treg cells, and promote T cell apoptosis (*via* B7-H1, PDL-1) or inhibit cytotoxic CD8<sup>+</sup> T cells<sup>[4,9,15]</sup>.

The local and systemic self-tolerance can be overridden and so autoimmune diseases can be initiated by several pathological immune mechanisms developed in liver. First of all, pathological antigen presentation might generate of auto reactive T cells and B cells due to defective clonal deletion (apoptosis of antigen-specific T cells). Similarly ILCs also switch on the autoimmunity by promoting antigen presentation with classical APCs, by releasing cytokines that polarize immune response towards effector T cells. ILCs may also be important mediators autoimmune liver injury by killing hepatocytes and/or bile duct epithelial cells. Pathological endotoxemia caused by dysbiotic microbiota may switch immune response from Th2 to Th1 predominance. Treg cells regulate both innate and adaptive immunity through regulation of CD4<sup>+</sup> cells, KCs and LSECs. Therefore, defective function of Treg cells impairs hepatic immune tolerance leading to autoimmune hepatitis<sup>[4,7]</sup>.

## SPECTRUM OF IMMUNE HOMEOSTASIS IN LIVER

Hepatic IS is always active, regardless the overall response outcome. The nature of insult to liver and spectrum of activated cells determines the clinical picture. Healthy individuals have balanced immune surveillance of pathogens together with immune tolerance towards

self-antigens. The over immune tolerance in liver leads to chronic infections with viruses or hepatic metastasis of cancer cells. In contrast the over activation of hepatic immune response causes fulminant hepatitis, allograft rejection or autoimmune diseases.

Hepatic immune homeostasis is continuously re-balanced during clinical courses of cirrhosis. Patients with compensated cirrhosis have hyperactivated IS depending on underlying etiology of the liver diseases. Hepatic decompensation is associated with increased intestinal permeability. The episodic translocation of gut microbiota and their endotoxins into portal circulation triggers systemic and hepatic inflammation. PAMPs recognizing LPS, lipopeptides, glycopolymers, flagellin and bacterial DNA/RNA, activate innate and adaptive immunity. The released pro-inflammatory cytokines and chemokines cause hepatic injury and activation of DAMP. The vicious cycle between members of PRR, namely PAMP and DAMP exhausts IS and so, switches immune response from a predominantly "pro-inflammatory" to wards "immunodeficient" status. This very late stage of cirrhosis is clinically defined as acute- on-chronic liver failure (ACLF). The immune deficient state in ACLF patients is called cirrhosis associated immune deficiency<sup>[15]</sup>.

## HERBAL TREATMENTS WITH POTENTIAL THERAPEUTIC EFFECT ON LIVER

Herbal treatments are very often multifaceted blends of slightly processed medicinal plants, parts of the plants or products of the medicinal plants, which are traditionally accepted, comparatively low side-effects, and naturally compatible with the human body. Herbal remedies are applied for the treatment of a variety of indications and disorders, including hepatic as well as immunological problems (Table 2). Since scientific studies on herbal treatments have shown that they might effect cytokine and immunoglobulin secretion, cellular co-receptor expression, histamine release, lymphocyte proliferation, and cytotoxic activity, thus, herbal preparations might modify immune functions. In this study, literature was surveyed based on *in vitro*, *in vivo* and clinical studies on hepatoprotective, as well as immunostimulant and/or immunomodulator effective medicinal plants, which are lead by ethnopharmacological data. Table 2 was established, which covers common name, scientific name, effective part, known phytochemical content of the plant, ethnopharmacological/clinical effects, and medicinal preparation with their corresponding references. Due to complexity of the herbal treatments, the complete scientific data on mechanism of action is lacking, although clinical outcomes of herbal treatment are promising and leading the researchers to take on demanding scientific studies on the immune activity of herbal remedies (Table 3). The most applied medical plants in herbal treatments were selected and their mechanism of action on liver diseases and on immun system were searched to establish the Table 3. Flavonoid

**Table 2** Plants are effective on liver disorders and immune system

Common name	Scientific name	Effective part	Phytochemical content	Preparation on liver disorders <sup>1</sup>	Ref.
Chaff-flower	<i>Achyranthes aspera</i> L.	Whole plant	Ecdysterone, achyranthine, betaine, pentatriacontane, 6-pentatriacontanone, hexatriacontane and tritriacontane	Natrossil natiris	[28-30]
Fennel	<i>Foeniculum vulgare</i> Mill.	Root	Coumarins (bergapten, isopimpinellin, anthotoxin), flavonoids (quercetin, rutin)	Presselin dyspeptikum presselin, bupleurum compound phytomedicine, epagest lampugnani	[18,25,31]
Korean Ginseng, Chinese Red Ginseng	<i>Panax ginseng</i> Mey.	Root	Polysaccharides, saponins, ginsenoside	Tripid teguhsindo	[32-34]
Yarrow	<i>Achillea</i> sp.	Flower	Volatile oils, flavonoids, terpenoids, alkaloids, saponins, sesquiterpenolactones	Liv-52 drops, cheiranthol klein	[35-38]
Carqueja	<i>Baccharis trimera</i> (Less) DC	Epigeous part	Flavonoids, diterpenoids	Boldina Plata	[39-41]
Chicory	<i>Cichorium intybus</i> L.	Aerial part, root, leaf	Saccharides, methoxycoumarin, cichorine, flavonoids, essential oils, anthocyanins	Natusor hepavesical soria natural, Liv-52 drops	[42-46]
Globe artichoke	<i>Cynara cardunculus</i> var. <i>scolymus</i> L.	Leaf	Sesquiterpenes lactones (cynaropicrin), flavonoids (cynaropicrin), phenolic acids (mainly caffeic acid derivatives)	Livstim mediherb, livton complex mediherb, lorbihepatic bioquimico, olocynan makros, rapacholin C herbapol wroclaw, farmasa, sylcynar herbapol poznan, alcafelol luper, bagohepat bago, armstrong, benevolus schwabe, boldina plata, cinarepa cristalfarma, colachofra EMS, cynarex roux-ocefa, herbapol wroclaw, cynarzym N altana, digestron loprofar, epagest lampugnani, figatil catarinense, salus, hecrosine B12 ortoquimica, hepatofalk falk, jurubileno ibefar	[47,48]
Pale purple coneflower	<i>Echinacea pallida</i> (Nutt.) Nutt. <i>Echinacea angustifolia</i> (DC.) Hell. <i>Echinacea purpurea</i> (L.) Moench	Whole plant	Alkamides, polysaccharides, glycoproteins, cichoric acid (a derivative of caffeic acid)	Andrographis complex mediherb, kalbe, hepatin lapi, imudator pyridam, herbal cleanse vitaplex	[49-52]
Faise daisy	<i>Eclipta alba</i> (syn <i>E. prostrata</i> L.)	Aerial part	Tannins, flavonoids, coumestans, saponins, alkaloids	Dipana promed	[53,54]
Chamomile	<i>Matricaria chamomilla</i> L.	Flower	Coumarin (herniarin and umbelliferone), phenylpropanoids (chlorogenic acid and caffeic acid), flavonoids (apigenin, apigenin-7-o-glucoside, luteolin, luteolin-7-o-glucoside, quercetin, rutin, naringenin), blue essential oils	Presselin dyspeptikum presselin, cholesol herbapol wroclaw, gotas digestivas bunker	[42,55-57]
Milk thistle	<i>Silybum marianum</i> (L.) Gaertn.	Seed	Polyphenolic flavonoids (silymarin, isosilylins, silibinins, silydianin, silychristin)	Liverine cardinal, livermin korean ginseng, liverton siffra, livosil-b centaur, livstim mediherb, livton complex mediherb, lomacholan lomapharm, phytohepar steigerwald, poikicholan lomapharm, prol procare, samarin berlin pharm, schwohepan S schworer, silegon teva, silibene merckle, silicur hexal, silimalon nikkho, silimarin benedetti, silimarit bionorica, silimax filofarm, silirex lampugnani, siliver farmasa, silliver abbott, silmar hennig, silvaysan sanum-kehlbeck, silybon micro, silygal ivax, silyhexal hexal, sily-sabona sabona, mepha, sivyler ranbaxy, sylcaps herbapol lublin, sylcynar herbapol poznan, sylimarol herbapol pruszkow	[18,19,58,59]



				Syliverin aflofarm, sylivit herbapol poznan, solas, vionin nf tempo scan pacific, alepa duopharm, apihepar madaus, <i>via</i> tris, aptivium liver support cynergen, ardeyhepan emonta, ardeypharm, bibol leloup hexa, bilisan duo repha Bioglan liver-vite bioglan, bupleurum complex mediherb, bupleurum compound phytomedicine, carsil sopharma, cefasliymarin cefak, cheiranthol klein, soho capsule/syrup, depatox progen, durasilymarin merck dura, eleparon sankyo, epagest lampugnani, flavobion zentiva, hegrimarin strathmann, strathmann, hepabene ratiopharm, merckle, ratiopharm, hepabesch strathmann, hepadigenor baliarda, hepaduran v otw, loges Capsule, hepamax dankos, hepa-merz sil merz, hepar-pasc pascoe, heparsyx n syxyl, heparviton bode, tempo scan pacific, hepatin lapi, kalbe, falk, darya-varia, hepato, Yung shin, heplant spitzner, herbal liver formula faulding, worwag, legalon-madaus, laragon roemmers, ifet, leveron vesco, limarin serum institute, herbal cleanse vitaplex	
Dandelion	<i>Taraxacum officinale</i> G.	Root	Sesquiterpenes, saponins, phenolic compounds, flavonoids, sugars	Naturica DFM ikapharmindo, livstim mediherb, livton complex mediherb, berberis complex blackmores, cholesol herbapol wroclaw, cinarepa cristalfarma, hepatofalk falk, herbal cleanse vitaplex	[60-63]
Radish	<i>Raphanus sativus</i> L.	Leaf, root	Flavanoids, terpenoids, alkaloids, saponins, sterols	Rapacholin AC herbapol wroclaw, rapacholin c herbapol wroclaw	[64-67]
Caper	<i>Capparis spinosa</i> L.	Root bark	Sugars (glucose, arabinose, mannose, galactose), lipid, volatile oils	Liv-52 drops	[68-71]
Kinkéliba	<i>Combretum micranthum</i> G. Don	Leaf	catechins, glycosylflavones, flavans, galloylated c-glycosylflavone derivatives, flavan-piperidine alkaloid	Tisane mediflor N°5 hepaticque	[72-74]
Arjuna	<i>Terminalia arjuna</i> (Roxb.) Wight and Arn.	Bark	Arjunolic acid, tomentonic acid, arjunin, $\beta$ -sitosterol, ellagic acid, leucodelphinidin, tannins	Liv-52 drops	[75-77]
Coffee senna	<i>Cassia occidentalis</i> L. (Senna occidentalis)	Leaf	Anthraquinones, saponins, sterols, triterpenes, quinines, tannins, flavonoids	Tisane mediflor N°5 hepaticque, Liv-52 drops	[78-80]
Liquorice, Licorice	<i>Glycyrrhiza glabra</i> L.	Root	Triterpene saponins, flavonoids, isoflavonoids and chalcones, glycyrrhizic acid	Tisane mediflor N°5 hepaticque, neominophagen C dexa, torii, curliv soho, soho capsule/syrup	[18,26,81]
Holy basil	<i>Ocimum sanctum</i> Linn.	Leaf	Volatile oils (eugenol, euginal, urosolic acid, carvacrol, linalool, limatrol, caryophyllene, methyl carvicol), anthocyanins, alkaloids, flavonoids, tannins, carbohydrates, xylose, polysaccharides	Andrographis complex mediherb	[22,82-85]
Rosemary	<i>Rosmarinus officinalis</i> L.	Leaf	Diterpenoids, triterpenoids, phenolic acids, and flavonoids, carnolic acid, carnosol, rosmarinic acid	Tisane mediflor N°5 hepaticque, natusor hepavesical soria natural, cinarepa cristalfarma	[86-88]
Red sage, Danshen	<i>Salvia miltiorrhiza</i> Bunge.	Root	Tanshinones (tanshinone I, tanshinone, cryptotanshinone) miltirone and salvianolic acid a, b	Bupleurum complex mediherb	[18,89,90]
Common mallow	<i>Malva sylvestris</i> L.	Leaf	Amino acids/protein derivatives, flavonoids, mucilages, terpenoids, phenol derivatives, coumarin	Tisane mediflor N°5 hepaticque	[91-93]
Tinospora, Guduchi, Giloya	<i>Tinospora cordifolia</i> (Willd.) Hook. f. and Thoms. (Guduchi)	Root, stem	Flavonoids, alkaloids, sesquiterpenes, diterpenes arabinogalactan, syringine, cordiol, cordioside, cordifoliosides (a and b), berberine, tinosporine, giloin, giloinin	Dipana promed	[22,33,94-96]

Boldo, Boldu, Boldus	<i>Peumus boldus</i> Molina	Leaf	Alkaloids (isoquinoline-boldine, isoboldine, 6a,7-dehydroboldine, isocorydine, isocorydine-n-oxide, norisocorydine, lauroitsine, laurotetanine, n-methylaurotetanine, reticuline, (-)-pronuciferine, sinoacutine), flavonoids, volatile oil, coumarin, resin, tannin)	Tisane mediflor N°5 hepaticque, natrossil natiris, natusor hepavesical soria natural, prinachol zurita, farmasa, alcalofol luper, berberis complex blackmores, boldina plata, boldopeptan neo quimica, colachofra ems, cynarzym N altana, eparema nycomed, figatil catarinense, gotas digestivas bunker, jurubileno ibefar Livstim mediherb, livton complex mediherb, natusor hepavesical soria natural, schwohepan S schworer, berberis complex blackmores, chelicur hasco-lek, cynarzym N altana, hepatofalk falk, falk, darya-varia	[97-99]
Greater celandine	<i>Chelidonium majus</i> L.	Aerial parts	Isoquinoline alkaloids, such as sanguinarine, chelidonine, chelerythrine, berberine and coptisine, (-)-turkiyenine)	Natrossil natiris, meprofarm, dipana promed, gramuno graha, hepimun landson, imudator pyridam	[100-105]
Gale of the wind	<i>Phyllanthus niruri</i> L.	Whole plant	Flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins	Dipana promed	[106-108]
Kutki	<i>Picrorhiza kurroa</i> Royle ex Benth.	Rhizome, root	Iridoid glycoside (picrovil)		[109,110]
Rhubarb	<i>Rheum emodi</i>	Rhizome	Anthraquinone (rhein, chrysophanol, aloe-emodin, emodin, physcion, and their glycosides) and stilbene (picetannol, resveratrol and their glycosides), flavonoids, glycosides, tannins, volatile oils, saponins	Natrossil natiris, boldopeptan neo quimica, eparema nycomed, SIT, hepatofalk falk	[111]
Magnolia-vine, Schisandra	<i>Schisandra chinensis</i> (Turcz.) Baill.	Fruit	Dibenzocyclooctadiene derivative lignans (or schisandra lignans), organic acids (citric, malic, fumaric and tartaric acid), sugars, vitamic C, vitamin E, phenolic acids, tannins, phytosterols, essential oil	Curliv soho, soho capsule/syrup, hepacell medikon, hepamax dankos	[31,112,113]
European black nightshade	<i>Solanum nigrum</i> L.	Whole plant	Glycoalkaloids, glycoproteins, polysaccharides, polyphenolic compounds (gallic acid, catechin, protococatechuic acid (pca), caffeic acid, epicatechin, rutin, naringenin)	Liv-52 drops, dipana promed	[114-117]
French tamaris	<i>Tamarix gallica</i> L.	Aerial parts	Tannin, tamarixin, tamauxetin, troupin, 4-methylcoumarin and 3,3'-di-o-methylellagic acid, tannic acid, 4-methylcoumarin and 3,3'-di-o-methylellagic acid	Liv-52 drops	[118,119]
Turmeric	<i>Curcuma longa</i> L.	Rhizome	Curcuminoid	Meprofarm, tripid teguhsindo, turmerik knop, aptivium liver support cynergen, chelicur hasco-lek, galena, ivax, cinarepa cristalfarma, depatox progen, heparviton bode, tempo scan pacific, kalbe, hepatin lapi, falk Presselin dyspeptikum presselin, dipana promed, herbal cleanse vitaplex	[22,31,120]
Ginger	<i>Zingiber officinale</i> Roscoe	Rhizome	Volatile oils, pungent phenol compounds [sesquiterpenoids, beta-sitosterol palmitate, isovanillin, glycol monopalmitate, hexacosanoic acid 2,3-dihydroxypropyl ester, maleimide-5-oxime, p-hydroxybenzaldehyde adenine, 6-gingerol, 6-shogaol, 1-(omega-ferulyloxyeratyl) glycerols]		[121-127]

<sup>1</sup>Martindale W, Sweetman SC. Martindale: The complete drug reference. Pharmaceutical press, 2007.

derivatives such as silybin, silymarin, obtained from milk-thistle [*Silybum marianum* (L.) Gaertn.] decreased alkaline phosphatase (completely) and gamma-glutamyl transpeptidase (partially) in CCl<sub>4</sub> induced liver damage<sup>[17]</sup>. Moreover, it has been claimed that Silymarin containing preparations are the principal therapeutic of choice in liver diseases caused by oxidative stress. Many studies have proven that plant phyto compound Silymarin has medical applications to cure (alcoholic and non-alcoholic)

fatty liver, cirrhosis, ischaemic injury, drug and chemically-induced hepatic toxicity, radiation toxicity, viral and toxic hepatitis by means of its anti-oxidative, anti-lipidperoxidative, anti-fibrotic, anti-inflammatory, liver regenerating and immunomodulating effects. Several studies have identified that continuous usage of Silymarin has significantly proved to increase the survival period of patients with alcohol-caused liver cirrhosis and primary liver cancer<sup>[18]</sup> (Figure 1). Scientific studies also

**Table 3** Commonly used plants on liver disorders and their effect mechanisms

Scientific name	Effect/mechanism on liver	Effect/mechanism on immun system	Ref.
<i>Curcuma longa</i> L.	Acute liver damage by chemicals, <i>e.g.</i> , ethanol, CCl <sub>4</sub> , Dimethylnitrosamines	Immunostimulant, immunomodulatory The extract of the rhizome <i>C. longa</i> increased both Th1 (IL-2 and IFN gamma) and Th2 (IL-10) cytokines indicating its dual immune functions. NR-INF-02 significantly increased the IL-2 and IFN gamma levels in Con A stimulated splenic lymphocytes. The above results indicated that NR-INF-02 showed a specific immunity response by stimulating both Th1 and Th2 cells Polysaccharide fraction of the rhizome showed potent immunostimulatory activity towards proliferation of splenocytes cell number and IL-10 secretion. Polysaccharides might be contributing to this proliferative and cytokine release property in murine splenocytes Hot water extracts of the rhizome showed that the high polarity fraction exhibited stimulatory effects on PBMC. The cytokine productions (TGF-beta, TNF-alpha, GM-CSF, IL-1alpha, IL-5, IL-6, IL-8, IL-10, IL-13, <i>etc.</i> ) have been modulated by a polysaccharide-enriched fraction. The proportion of CD14 positive stained PBMC was increased by the fraction	[18,22-24]
<i>Foeniculum vulgare</i> Mill.	Oxidative stress of the liver bacterial and viral infections anti-inflammatory, acute hepatotoxicity	Anti-HIV-1 and HIV-2 Immunomodulatory Antimicrobial, antifungal	[18,25]
<i>Glycyrrhiza glabra</i> L.	Cirrhosis fibrosis chronic viral hepatitis B and C	Immunomodulatory Leukocyte count and phagocytic index (carbon clearance) was increased significantly with the treatment of water extract of <i>G. glabra</i> root. Zinc (45 mg/kg) in combination with ALE (0.75 g/kg) showed highly significant increase of leukocyte count and phagocytic index	[18,26,27]
<i>Silybum marianum</i> (L.) Gaertn.	Oxidative stress inflammation and fibrosis alcohol-induced cirrhosis mushroom poisoning viral hepatitis	Immunomodulatory Flavonoids from <i>S. marianum</i> normalize immunoregulatory defects <i>via</i> restoration of the cellular thiol status. T-cell activation (CD69), along with a significant decrease in TNF	[18,19]

IFN: Interferons; TNF: Tumor necrosis factor; IL: Interleukin; TGF: transforming growth factor; HIV: Human immunodeficiency virus; PBMC: Peripheral blood mononuclear cells; GM-CSF: Granulocyte-macrophage colony-stimulating factor; ALE: Aqueous liquorice extract.

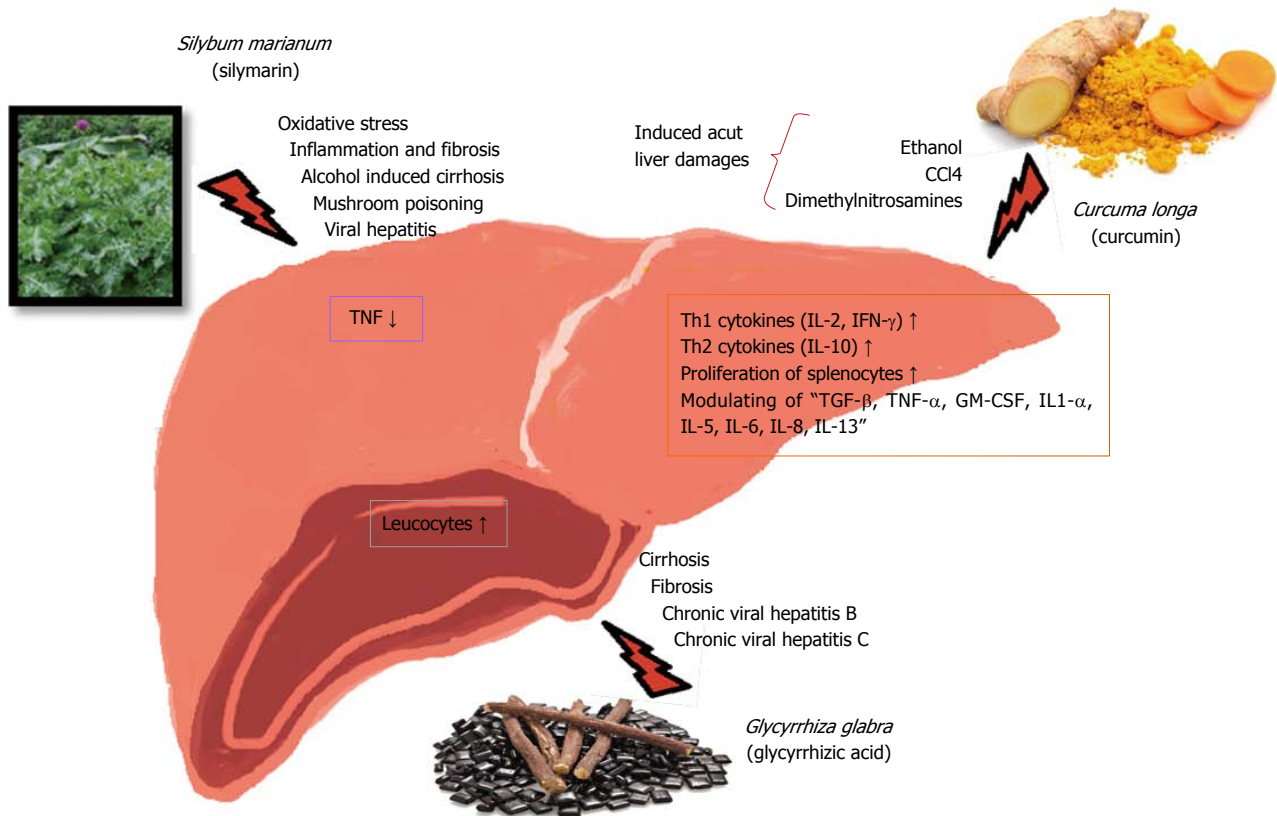
have shown that, both silybin and silymarin normalized immunoregulatory failures by restoration of the cellular thiol status, T-cell activation (CD69), together with a substantial decrease in TNF<sup>[18,19]</sup>. Effects of the selected herbal medicines on immune and liver were summarized in Figure 1. Another study demonstrated that an edible plant Artichoke (*Cynara scolymus* L.) prevented CCl<sub>4</sub> and oxidative stress-induced hepatotoxicity and it protected the liver<sup>[20]</sup>. Inulin, obtained from artichoke, stimulates components of the IS<sup>[21]</sup>. The extract of the rhizome Turmeric (*Curcuma longa* L.), which has hepatoprotective plant, amplified both Th1 (IL-2 and IFN gamma) and Th2 (IL-10) cytokines signifying its dual immune roles. Polysaccharide fraction of this rhizome showed potent immunostimulatory action in the direction of proliferation of splenocytes cell number and IL-10 secretion. Polysaccharides of the plant extract might be causative of these proliferative and cytokine release assets in murine splenocytes. In different studies have been shown that the cytokine productions (TGF-β, TNF-α, GM-CSF, IL-1α, IL-5, IL-6, IL-8, IL-10, IL-13, *etc.*) have been modulated by polysaccharide-enriched fractions<sup>[18,22-24]</sup>. Fennel (*Foeniculum vulgare* Mill.) and liquorice (*Glycyrrhiza glabra* L.) have also shown immunomodulatory and hepatoprotective effects<sup>[18,25-27]</sup>.

Herbal drugs are composed of complex mixtures of phytochemicals, unlike conventional and plant originated single compound drugs, which are composed of known

chemical constituents and are precisely quantified. For that reasons studying the clinical effects of individual chemical constituents separately will not be accurate, due to various reasons, such as the synergistic or inhibiting effects of phytochemicals on each other and neutralization of harmful chemicals in the mixture by other compounds, which provides a flawless combination for therapeutic purposes. Aspects such as, absorption, distribution intrinsic concentration and metabolism of the drug should be known precisely to determine the dosage, safety margin and length of treatment. Moreover, future research should include characterization of multifactorial mechanisms of action, elucidation of adverse effects and well-designed clinical trials in pediatrics and geriatrics as well.

## CONCLUSION

Scientists as well as immunologists, who study herbal treatments in hepatic diseases must be ready to face challenges and opportunities. *In vivo* and clinical molecular researches on immunomodulatory, immunoenhancing, immunostimulant effects of herbal treatments will offer novel perceptions into IS and immunotherapy. Not only single plant extract, but the interactions of the ingredients in a given herbal treatment formula determines the final clinical picture by finely tuning the balance between therapeutic effect and hepatotoxicity. Feature studies



**Figure 1** Immunological action mechanisms of some herbs on the liver. IFN: Interferons; TNF: Tumor necrosis factor; IL: Interleukin; TGF: Transforming growth factor.

must precisely define the interaction between the liver as an organ regulating local and systemic immune response and complex action mechanisms of herbal treatment.

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## Risk factors and outcomes associated with alcohol relapse after liver transplantation

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### Abstract

Alcoholic liver disease (ALD) is the second most common indication for liver transplantation (LT) in the United States and Europe. Unlike other indications for LT, transplantation for ALD may be controversial due to the concern for alcohol relapse and non-compliance after LT. However, the overall survival in patients transplanted for ALD is comparable or higher than in patients transplanted for other etiologies of liver disease. While the rate of alcohol use after liver transplantation does not differ among various etiologies of liver disease, alcohol relapse after transplantation for ALD has been associated with complications such as graft rejection, graft loss, recurrent alcoholic cirrhosis and reduced long-term patient survival. Given these potential complications, our review aimed to discuss risk factors associated with alcohol relapse and the efficacy of various interventions attempted to reduce the risk of alcohol relapse. We also describe the impact of alcohol relapse on post-transplant outcomes including graft and patient survival. Overall, alcohol liver disease remains an appropriate indication for liver transplantation, and long-term mortality in this group of patients is primarily attributed to cardiovascular disease or *de novo* malignancies rather than alcohol related hepatic complications, among those who relapse.

**Key words:** Cirrhosis; Relapse prevention; Recidivism

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**Core tip:** There are no established risk factors or scoring systems to predict alcohol relapse after transplantation for alcoholic liver disease. Studies regarding the "6-mo rule" demonstrated heterogeneous findings, suggesting that this rule is not a reliable predictor of relapse.



Comorbid psychiatric conditions, lack of social support, and tobacco use are consistently associated with alcohol relapse. Scoring systems have been proposed, but have not been validated. Alcohol relapse may be associated with graft rejection and graft loss, though reduction in long-term survival may be attributed to cardiovascular disease and *de-novo* malignancies rather than alcohol-related hepatic complications.

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## INTRODUCTION

Alcohol use disorder affects nearly 10% of the general population in both the United States and Europe and is one of the most frequent causes of liver cirrhosis in the Western world<sup>[1]</sup>. After hepatitis C virus (HCV) infection, alcoholic liver disease (ALD) is the second most common indication for liver transplantation (LT) in the United States and Europe<sup>[2,3]</sup>. According to the OPTN/SRTR 2015 annual report, 21% of liver transplantation was for alcoholic liver disease<sup>[4]</sup>.

Unlike other indications for LT, transplantation for ALD may be controversial because of the concern regarding relapse and medication non-compliance after transplantation<sup>[5]</sup>. The exact proportion of ALD patients who drink alcohol after LT is unclear and is reported to range anywhere between 7%-95%<sup>[6-8]</sup>. The broad range of percentages reported in the literature is because there are no standardized definitions for alcohol relapse<sup>[6-8]</sup>. Interestingly, the rate of alcohol use after LT does not differ between patients transplanted for other etiologies of liver disease, though recipients transplanted for ALD tend to drink in greater quantities<sup>[9,10]</sup>. In terms of patterns of alcohol use, there are varying frequencies given the different definitions and follow-up periods, but in general approximately 12%-33% of liver recipients for ALD relapse to abusive or harmful amounts of drinking<sup>[11-14]</sup> and 6%-26% relapse to occasional slips after transplantation<sup>[12,14,15]</sup>. Furthermore, the overall survival rate for patients transplanted for ALD is comparable or higher than those of patients transplanted for non-ALD<sup>[2,3,10,16]</sup>. Still, separate studies have identified harmful and excessive amounts of alcohol use to be associated with increased rates of graft rejection and failure<sup>[10,15,17-19]</sup>. Due to these potential adverse complications, our aim was to discuss risk factors associated with alcohol relapse after transplantation, the efficacy of interventions attempted to prevent relapse, and the post-transplant outcomes associated with alcohol relapse<sup>[5]</sup>.

## Definitions

There are no standardized definitions or classification

criteria to describe alcohol consumption after transplantation. Terms that have been used in the literature include recidivism or relapse<sup>[7,15-17,19]</sup>. Quantification of alcohol consumption after LT can also be described using terms such as abstinence, occasional slip, harmful drinking and excessive drinking, though the definitions of these terms are variable (Table 1)<sup>[8,20,21]</sup>. Lucey *et al*<sup>[22]</sup> defines harmful drinking as consumption of 4 or more drinks in one day or drinking for 4 or more days in succession, whereas a slip is defined as consumption of a limited amount of alcohol, followed by immediate measures to re-establish abstinence. De Gottardi *et al*<sup>[11]</sup> defined harmful drinking as alcohol consumption greater than 40 g/d that was associated with the presence of alcohol-related damage, such as histologic features of alcoholic liver injury on biopsy. The Diagnostic and Statistical Manual of Mental Disorders Version IV defined alcohol abuse as meeting one of the following criteria during a 12 mo period: Use which causes failure to fulfill major role obligations at work, school or home, use which causes a hazardous situation, use which causes legal problems or use continuing in the setting of recurrent social or interpersonal problems<sup>[23,24]</sup>. Faure's study used the World Health Organization definition where excessive alcohol consumption was > 20 g and > 30 g/d for women and men<sup>[10]</sup>.

## "The 6 mo rule"

Many centers require 6 mo of abstinence to be listed for liver transplantation. The 6 mo rule has two presumed purposes: To allow patients to recover from their liver disease and preclude the need for liver transplantation and to identify patients who are likely to remain abstinent after liver transplantation<sup>[1]</sup>. Nonetheless, there are conflicting findings as to whether this length of abstinence is needed to reduce the risk of relapse<sup>[11,25-27]</sup>. There have been several studies which have found that duration of abstinence less than 6 mo is associated with alcohol use and harmful drinking (Table 2)<sup>[11,28,29]</sup>. Additionally, Tandon *et al*<sup>[30]</sup> calculated that for every additional month of pre-LT abstinence there was a 5% decrease in the adjusted relapse rates. This is contrasted by other studies that have shown that the 6-mo rule is not a strong indicator of future drinking<sup>[26,27,31]</sup>. Based on the conflicting outcomes, the 6-mo rule may not reliably predict post-transplant relapse.

Furthermore, achieving 6 mo of abstinence is not always feasible, particularly for patients with severe alcoholic hepatitis that is refractory to treatment<sup>[32,33]</sup>. In fact, certain professional societies suggest that the 6-mo rule should not be required in patients where the expected mortality of the disease would not allow for a 6-mo waiting period<sup>[1,18,34]</sup>. Additionally, survival outcomes are superior among patients with severe alcoholic hepatitis that is refractory to corticosteroids and subsequently undergo OLT, as compared to those receiving standard of care<sup>[34-37]</sup>. As demonstrated by Mathurin *et al*<sup>[34]</sup> patients with severe alcoholic hepatitis who underwent OLT had a significantly greater cumulative 6 mo survival of 77% compared to

**Table 1** Definitions of alcohol use after liver transplantation

Study	Term	Definition
Lucey <i>et al</i> <sup>[21]</sup>	Harmful drinking	Consumption of 4 or more drinks in one day or drinking for 4 or more days in succession
	Occasional slip	Consumption of a limited amount of alcohol, followed by immediate procedures to re-establish abstinence
De Gottardi <i>et al</i> <sup>[11]</sup>	Harmful drinking	Consumption greater than 40 g/d that is associated with the presence of alcohol-related damage, such as histologic features of alcoholic liver injury on biopsy
Diagnostic and Statistical Manual of Mental Disorders Version IV	Alcohol abuse	Meeting one of the following criteria during a 12 mo period: Use which causes failure to fulfill major role obligations at work, school or home, use which causes a hazardous situation, use which causes legal problems or use continuing in the setting of recurrent social or interpersonal problems
World Health Organization	Occasional consumption	Men: < 20 g/d Women: < 30 g/d
	Excessive consumption	Men: > 20 g/d Women: > 30 g/d

23% for controls who did not receive transplantation ( $P < 0.001$ ).

## PATIENT FACTORS ASSOCIATED WITH RELAPSE

### Age

Like the 6-mo rule, age has an inconsistent association with alcohol relapse after LT. A few studies have found that younger age is associated with alcohol relapse after LT and that the category of patients that relapsed were significantly younger compared to those that did not<sup>[14,26,38]</sup>. One study found that age < 45 years was associated with increased risk of relapse and another found an association between relapse and age < 40 years<sup>[15,38]</sup>. These findings are contrasted by other studies that found no association between age and alcohol relapse<sup>[8,27]</sup>. Furthermore, two larger studies determined that age is not an independent risk factor associated with alcohol relapse<sup>[11,15]</sup>. Based on the heterogeneity of these findings, we believe that age is not a reliable predictor of risk of alcohol relapse.

### Social support

Lack of social support is an extrinsic factor that has consistently been associated with an increased risk of relapse for patients transplanted for ALD<sup>[13,15,31,39]</sup>. ALD patients who resumed alcohol use post-LT were more likely to be divorced or separated from their partners compared to those that remained abstinent, and multiple studies found that the lack of a spouse or life partner is a predictor of alcohol relapse<sup>[8,13,15,31]</sup>. One study also suggested that marriage is protective against binge drinking<sup>[13]</sup>. Therefore, it is important to ensure that patients with ALD have a strong support system during LT evaluation.

### Comorbid psychological conditions

The presence of psychiatric comorbidities or previous diagnosis of a mental illness has been found to be an important intrinsic risk factor for increased risk of relapse

after LT<sup>[11,13,31]</sup>. Multivariate analysis showed that a pre-LT diagnosis of a psychiatric disorder (anxiety or depressive disorder) at the time of listing was independently associated with a significantly increased risk of harmful levels of alcohol relapse, which is defined as consumption of greater than 40 g/d<sup>[11]</sup>. Another study also determined that a prior diagnosis of a mental illness was significantly associated with harmful drinking, which was defined in the study as consumption of greater than 140 g of ethanol per week<sup>[31]</sup>. Furthermore, prior treatment for co-morbid psychiatric disorders is a potential risk factor for alcohol relapse<sup>[40]</sup>. Evaluation for comorbid psychiatric conditions during the LT evaluation period may potentially help identify ALD patients that are at higher risk of both alcohol relapse and harmful drinking after transplantation.

### Employment

In a cross-sectional study of organ transplant patients, only 37.5% of liver transplant patients were employed post-transplant<sup>[41]</sup>. Furthermore, among liver transplant recipients, those transplanted for ALD are significantly less likely to be employed both before and after transplant compared to transplant recipients for non-ALD<sup>[9]</sup>. A total of 29% of transplant recipients with ALD and 59% of those with non-ALD worked pre-transplantation, vs 33% of those with ALD vs 80% of non-ALD at 3 years post-transplantation ( $P < 0.00001$ )<sup>[9]</sup>. Furthermore, ALD patients that were previously employed were less likely to return to work compared to patients transplanted for non-ALD<sup>[8]</sup>. Despite the low proportion of ALD patients that work pre and post-transplant, employment status does not appear to be significantly associated with the risk of alcohol relapse after transplantation<sup>[8,26,27,31]</sup>.

### Cigarette smoking

Studies have found cigarette smoking to be associated with alcohol relapse after transplant for alcoholic cirrhosis<sup>[17,31,40,42]</sup>. Kelly *et al*<sup>[31]</sup> demonstrated in univariate analysis that pre-transplant tobacco use was a predictor of harmful alcohol drinking in the post-transplant period. This was not a significant finding when subjects were

**Table 2 Risk factors associated with alcohol relapse**

Risk Factor	Ref.	Study design	Sample size	Results
Abstinence less than 6 mo pre-LT	Perney <i>et al</i> <sup>[26]</sup> (2005)	Retrospective	<i>n</i> = 61	Associated with severe relapse to heavy drinking <sup>1</sup>
	De Gottardi <i>et al</i> <sup>[11]</sup> (2007)	Retrospective	<i>n</i> = 387	Associated with relapse
	Pfizzmann <i>et al</i> <sup>[13]</sup> (2007)	Retrospective	<i>n</i> = 300	Associated with relapse
	Tandon <i>et al</i> <sup>[30]</sup> (2009)	Retrospective	<i>n</i> = 171	For every 1-mo increment increase in pre-transplant abstinence, there was a 5% decrease in the adjusted relapse rate
	Karim <i>et al</i> <sup>[29]</sup> (2010)	Retrospective	<i>n</i> = 80	Associated with relapse and is an independent risk factor for relapse
	Satapathy <i>et al</i> <sup>[42]</sup> (2015)	Retrospective	<i>n</i> = 148	Associated with alcohol relapse
	Osorio <i>et al</i> <sup>[28]</sup> (1994)	Retrospective	<i>n</i> = 43	No association
	Jauhar <i>et al</i> <sup>[27]</sup> (2004)	Retrospective	<i>n</i> = 112	No association
	Björnsson <i>et al</i> <sup>[8]</sup> (2005)	Retrospective	<i>n</i> = 103	No association
	Addolorato <i>et al</i> <sup>[25]</sup> (2013)	Retrospective	<i>n</i> = 55	No association
Abstinence < 1 yr pre-LT	Egawa <i>et al</i> <sup>[40]</sup> (2014)	Retrospective	<i>n</i> = 140	No association
	Kelly <i>et al</i> <sup>[31]</sup> (2006)	Retrospective	<i>n</i> = 100	No association with harmful relapse <sup>2</sup>
Age	Gedaly <i>et al</i> <sup>[79]</sup> (2008)	Retrospective	<i>n</i> = 142	Independent predictor of relapse
	Perney <i>et al</i> <sup>[26]</sup> (2005)	Retrospective	<i>n</i> = 61	Alcohol relapse group was younger compared to the non-relapse group
	Pfizzmann <i>et al</i> <sup>[13]</sup> (2007)	Retrospective	<i>n</i> = 300	Age < 40 yr of age was associated with relapse, but was not an independent risk factor
	Karim <i>et al</i> <sup>[29]</sup> (2010)	Retrospective	<i>n</i> = 80	Age < 50 yr of age approached clinical significance for alcohol relapse
	Rice <i>et al</i> <sup>[14]</sup> (2013)	Retrospective	<i>n</i> = 300	Alcohol relapse group was younger compared to the non-relapse group
	Grat <i>et al</i> <sup>[38]</sup> (2014)	Retrospective	<i>n</i> = 97	Younger age < 45 associated with relapse
	Satapathy <i>et al</i> <sup>[42]</sup> (2015)	Retrospective	<i>n</i> = 148	Older patients had lower likelihood of alcohol relapse
	De Gottardi <i>et al</i> <sup>[11]</sup> (2007)	Retrospective	<i>n</i> = 387	Age > 50 yr associated with relapse
	Jauhar <i>et al</i> <sup>[27]</sup> (2004)	Retrospective	<i>n</i> = 112	No association
	Björnsson <i>et al</i> <sup>[8]</sup> (2005)	Retrospective	<i>n</i> = 103	No association
Social support	Kelly <i>et al</i> <sup>[31]</sup> (2006)	Retrospective	<i>n</i> = 100	Lack of partner associated with harmful alcohol relapse <sup>2</sup>
	Pfizzmann <i>et al</i> <sup>[13]</sup> (2007)	Retrospective	<i>n</i> = 300	Absence of life companion associated with increased risk of alcohol relapse
	DiMartini <i>et al</i> <sup>[13]</sup> (2006)	Prospective	<i>n</i> = 167	Marriage is protective against binge use
	Rodrigue <i>et al</i> <sup>[39]</sup> (2013)	Retrospective	<i>n</i> = 118	Limited social support associated with alcohol relapse
	Egawa <i>et al</i> <sup>[40]</sup> (2014)	Retrospective	<i>n</i> = 140	Marital status associated with alcohol relapse and harmful relapse <sup>3</sup>
	Satapathy <i>et al</i> <sup>[42]</sup> (2015)	Retrospective	<i>n</i> = 148	Support from immediate family (spouse, parent or child) was highly correlated with reduced risk of alcohol relapse
	Björnsson <i>et al</i> <sup>[8]</sup> (2005)	Retrospective	<i>n</i> = 103	No association
	De Gottardi <i>et al</i> <sup>[11]</sup> (2007)	Retrospective	<i>n</i> = 387	Associated with relapse
	Karim <i>et al</i> <sup>[29]</sup> (2010)	Retrospective	<i>n</i> = 80	Associated with relapse
	Kelly <i>et al</i> <sup>[31]</sup> (2006)	Retrospective	<i>n</i> = 100	Previous diagnosis of a mental illness associated with harmful drinking <sup>2</sup>
Marital status	DiMartini <i>et al</i> <sup>[13]</sup> (2006)	Prospective	<i>n</i> = 167	History of depressive disorder associated with alcohol relapse
	Egawa <i>et al</i> <sup>[40]</sup> (2014)	Retrospective	<i>n</i> = 140	A history of treatment for psychological diseases other than alcoholism before LT is associated with risk of alcohol relapse but not harmful drinking <sup>3</sup>
	Jauhar <i>et al</i> <sup>[27]</sup> (2004)	Retrospective	<i>n</i> = 112	Comorbid psychiatric condition had no association with relapse
	Jauhar <i>et al</i> <sup>[27]</sup> (2004)	Retrospective	<i>n</i> = 112	No association
	Perney <i>et al</i> <sup>[26]</sup> (2005)	Retrospective	<i>n</i> = 61	No association
	Kelly <i>et al</i> <sup>[31]</sup> (2006)	Retrospective	<i>n</i> = 100	Previous occupation not associated with harmful drinking
	Egawa <i>et al</i> <sup>[40]</sup> (2014)	Retrospective	<i>n</i> = 140	Post-LT occupational status not associated with alcohol relapse
	Satapathy <i>et al</i> <sup>[42]</sup> (2015)	Retrospective	<i>n</i> = 148	Employment status at time of transplant was not associated with alcohol relapse
	Pageaux <i>et al</i> <sup>[127]</sup> (2003)	Retrospective	<i>n</i> = 128	Occasional and heavy drinkers were more likely to be cigarette smokers compared to abstinent patients
	Kelly <i>et al</i> <sup>[31]</sup> (2006)	Retrospective	<i>n</i> = 100	Median cigarette use per day was higher in harmful alcohol relapse group
Cigarette smoking	Rodrigue <i>et al</i> <sup>[56]</sup> (2013)	Retrospective	<i>n</i> = 118	Associated with alcohol relapse
	Egawa <i>et al</i> <sup>[40]</sup> (2014)	Retrospective	<i>n</i> = 140	Cigarette smoking after LT associated with alcohol relapse
	Satapathy <i>et al</i> <sup>[42]</sup> (2015)	Retrospective	<i>n</i> = 148	Active cigarette smoking at time of LT associated with alcohol relapse
	Egawa <i>et al</i> <sup>[40]</sup> (2014)	Retrospective	<i>n</i> = 140	Associated with alcohol relapse and harmful relapse <sup>3</sup>
	DiMartini <i>et al</i> <sup>[13]</sup> (2006)	Prospective	<i>n</i> = 167	Prior alcohol rehabilitation was associated with relapse
	Gedaly <i>et al</i> <sup>[79]</sup> (2008)	Retrospective	<i>n</i> = 142	Participation in rehabilitation was associated with relapse
	Jauhar <i>et al</i> <sup>[27]</sup> (2004)	Retrospective	<i>n</i> = 112	Substance abuse treatment before LT had no association with relapse
	Björnsson <i>et al</i> <sup>[8]</sup> (2005)	Retrospective	<i>n</i> = 103	No association
Non-compliance with clinic visits				
Pre-LT substance abuse or alcohol treatment				

<sup>1</sup>Alcohol consumption of more than 21 units per week for males and 14 units per week for females; <sup>2</sup>Alcohol consumption greater than 140 g of ethanol per week; <sup>3</sup>Alcohol consumption greater than 40 g per day that was associated with the presence of alcohol-related damage. LT: Liver transplantation.

divided into no smoking, prior smoking or active smoking categories<sup>[31]</sup>. Additionally, ALD patients who drank both occasionally and heavily after LT were more likely to be smokers compared to those who remained abstinent<sup>[17]</sup>. Independent of alcohol relapse, cigarette smoking is an important risk factor for recipient morbidity and mortality<sup>[20,31,43,44]</sup>. Long-term consequences of cigarette smoking include hepatic artery thrombosis, cardiovascular disease and new onset malignancy of the aerodigestive tract<sup>[43,44]</sup>. History of tobacco use was also found to be associated with poorer survival after LT from cardiovascular disease or *de novo* non-hepatic cancer<sup>[20,31,43,44]</sup>.

### **Noncompliance with clinic visits**

Egawa *et al.*<sup>[40]</sup> found noncompliance with clinic visits after LT, defined as 3 absences without notice, to be associated with both alcohol relapse and harmful drinking. In the study population, most patients underwent living donor liver transplantation, due to scarcity of deceased donors in Japan<sup>[40]</sup>. Furthermore, a cross-sectional study found that those who missed clinic appointments had lower adherence to immunosuppressive medications after liver transplant for any etiology ( $P < 0.001$ ). In the study, non-adherence to immunosuppressive medications was liberally defined as any missed doses of transplant medications<sup>[45]</sup>. This finding is significant because strict adherence to immune suppressant agents is a very important factor in long-term outcome after liver transplant<sup>[46]</sup>. In multivariate analysis, missing physician appointments was the only independent factor associated with non-adherence to immune suppressants. Survey respondents who missed clinic visits were more than 4.7 times as likely to be non-adherent with immune suppressants compared to those who did not miss clinic visits (OR = 4.7, 95%CI: 1.5-14.7,  $P = 0.008$ )<sup>[45]</sup>.

### **HCV infection**

HCV infection and ALD often co-exist and approximately 8%-10% of liver transplantation performed was for mixed HCV and ALD cirrhosis<sup>[47]</sup>. Aguilera *et al.*<sup>[48]</sup> compared post-transplantation outcomes among patients transplanted for alcoholic cirrhosis, mixed alcoholic cirrhosis and HCV and HCV alone. Interestingly, there was no significant difference in rate of alcohol relapse between the mixed HCV and alcoholic cirrhosis group (8%) and the alcoholic cirrhosis group (18%). Alcohol relapse also does not affect liver histology or liver functions tests differently in recipients with concomitant HCV vs ALD alone. Additionally, rates of rejection and graft loss were not significantly different between the mixed HCV and ALD and ALD groups. While recurrence of HCV is a major cause of reduced survival in patients transplanted for HCV cirrhosis, 5-year survival was comparable between the mixed HCV and ALD group (73%) and alcoholic cirrhosis group (76%)<sup>[49,50]</sup>. Though further studies are warranted, based on these studies, presence of HCV does not appear to result in greater risk of alcohol relapse

or worse post-transplantation outcomes.

### **Scoring systems to predict alcohol relapse**

The two main scoring systems in the literature for alcohol relapse after LT are the High Risk Alcoholism Relapse (HRAR) Scale and the Alcohol Relapse Risk Assessment (ARRA). The High Risk Alcoholism Relapse Scale was designed and piloted in the male veteran population and consists of 3 variables: Duration of heavy drinking, number of drinks per day and number of prior alcoholism inpatient treatment experiences<sup>[51]</sup>. Each item is scored 0-2 and possible score ranges from 0 to 6. A HRAR score greater than 3 is associated with high risk of alcohol relapse<sup>[11]</sup>.

The HRAR Scale has yet to be validated and thus far two studies did not find the HRAR score to be associated with post-OLT alcohol use<sup>[40,52]</sup>. In terms of the ARRA, this tool found 9 domains to be significantly predictive of alcohol relapse. This scoring system includes both intrinsic and extrinsic risk factors of alcohol relapse. The intrinsic factors include low motivation for alcohol treatment and poor stress management skills. The extrinsic factors include limited social support, engagement in social activities with exposure to alcohol and lack of nonmedical behavioral consequences. The remaining factors are absence of hepatocellular carcinoma, dependence on tobacco and ongoing alcohol use after diagnosis of liver disease. Groups in ARRA III and IV (with 4-6 and 7-9 out the 9 factors) had significantly higher rates of alcohol relapse and were more likely to return to pre-transplant levels of drinking<sup>[39]</sup>. The ARRA scale has not been validated by other studies.

The Stanford Integrated Psychosocial Assessment for Transplant (SIPAT) was developed from a comprehensive literature review of psychosocial factors found to predict outcomes in liver, lung and heart transplant patients<sup>[53]</sup>. The SIPAT has been evaluated by one prospective study in liver, lung, kidney and heart transplant recipients. While mortality and organ failure was not associated with SIPAT scores, secondary medical and psychosocial outcomes such as rejection episodes, hospitalizations, infections and psychosocial decompensation were predicted by SIPAT<sup>[54]</sup>. The SIPAT has not yet been studied separately in liver transplant patients. In conclusion, there are no validated scoring systems to predict risk of alcohol relapse after LT at this time.

## **INTERVENTIONS TO PREVENT RELAPSE**

### **Relapse prevention and psychosocial therapy**

Studies have been conducted regarding relapse prevention before and after OLT. Erim *et al.*<sup>[55]</sup> conducted a study that demonstrated that patients who received 6 mo of pre-LT psycho-educational therapy had significantly less alcohol recidivism during the pre-transplant waiting period. Björnsson *et al.*<sup>[8]</sup> evaluated the effectiveness of active addiction treatment prior to transplant and demonstrated that active addiction treatment during the



pre-LT period may reduce the risk of relapse after LT by more than 50% (from 48% to 22%). In the study, 19 out of 40 (48%) patients transplanted before the start of structured management had resumed alcohol compared to 13 (22%) out of 58 after this intervention that did not ( $P = 0.002$ ). No treatment was offered in the post-operative period. In a retrospective study, Addolorato *et al.*<sup>[25]</sup> evaluated the use of an alcohol addiction unit (AAU) that was integrated within the transplant center. Post-LT patients either followed up with an addiction specialist at the transplant center or were offered addiction counseling by a provider outside the transplant unit. Patients who followed up in the AAU received multimodal treatment with counseling and pharmacologic treatment. Counseling involved 30-min sessions that emphasized craving evaluation and identification of risk factors for alcohol relapse. Out of 92 cirrhotic liver transplant recipients the alcohol relapse rate was remarkably lower in recipients managed by the alcohol addiction unit within the transplant center (16.45%) compared to patients managed by psychiatrists not affiliated to liver transplant units (35.1%).

Rodrigue *et al.*<sup>[56]</sup> found that patients who had received substance abuse treatment before LT did not differ in alcohol relapse compared to patients who did not (30% vs 39%,  $P = 0.20$ ). Interestingly, he discovered that patients who received substance abuse treatment both before and after transplant had significantly lower rates of alcohol relapse (16% vs 41%) compared to patients who received substance abuse treatment only before transplant (45%) or those who did not receive any substance abuse treatment (41%). While more studies are needed to evaluate relapse prevention strategies, follow-up with addiction specialists integrated with a transplant unit and a combination of pre and post-transplant interventions may be more efficacious<sup>[56]</sup>.

### Pharmacological interventions

Several medications are approved for alcohol dependence, but only baclofen has been studied in a randomized control trial (RCT) in patients with alcoholic cirrhosis<sup>[57,58]</sup>. Baclofen is a gamma amino butyric acid receptor agonist that works by reducing craving for alcohol. In a RCT, a total of 84 patients with both alcohol use dependence and liver cirrhosis were randomized to receive baclofen 10 mg three times daily or placebo for 12 wk. Baclofen demonstrated significant efficacy in promoting alcohol abstinence and reducing alcohol relapse. There were no serious side effects reported and no patients discontinued the medication during the study<sup>[58]</sup>. Furthermore, the baclofen study group displayed a significant decrease in alanine aminotransferase, gamma-glutamyl transferase, bilirubin and international normalized ratio values compared to placebo. It is theorized that the improvement in liver function tests was due to the significant reduction of alcohol intake in the baclofen group<sup>[58]</sup>. Baclofen has yet to be studied in the decompensated patient and post-LT population.

Other drugs that are currently approved for alcohol dependence include disulfiram, naltrexone and acamprosate, however these have not been studied in the post-transplant population. Additionally, both disulfiram and naltrexone are not ideal options for ALD patients due to their risk of hepatotoxicity<sup>[59-62]</sup>.

Disulfiram was one of the first drugs approved for alcohol dependence and is an irreversible inhibitor of aldehyde dehydrogenase (ALD)<sup>[60,63,64]</sup>. If alcohol is consumed while taking disulfiram, acetaldehyde levels will increase and result in a disulfiram reaction of hypotension, flushing, nausea and vomiting that may deter patients from drinking alcohol<sup>[63]</sup>. Naltrexone is an antagonist of  $\kappa$ - and  $\mu$ -opioid receptors and increases dopamine release in the mesolimbic system, which may help reduce alcohol craving<sup>[65]</sup>. The long acting intramuscular formulation of naltrexone may be less hepatotoxic because it does not undergo first pass metabolism by the liver, but both the oral and intramuscular formulations currently carry a black-box warning for liver damage<sup>[59,62]</sup>. Another anti-craving medication, acamprosate, is an N-methyl-D-aspartate glutamate receptor antagonist with an unclear mechanism of action. It is not metabolized by the liver and is not associated with liver toxicity<sup>[66]</sup>. Furthermore, a preliminary study suggested that 1 d of administration was well tolerated in patients with Child-Pugh class A and B cirrhosis<sup>[67]</sup>. More studies are needed to establish its efficacy in patients transplanted for alcohol liver disease and its safety profile with repeated administration.

Other promising pharmacologic agents to reduce alcohol relapse include topiramate and ondansetron<sup>[59]</sup>. Topiramate is only partially metabolized by the liver (22%) and is primarily excreted by the kidneys<sup>[68]</sup>. Ondansetron is a serotonin (5-HT<sub>3</sub>) receptor antagonist that is thought to downregulate dopaminergic neurons, reducing the reward pathway for alcohol<sup>[69]</sup>. It has been shown to be more effective than placebo in increasing total days of abstinence and percentage of abstinent days<sup>[70]</sup>. Its major side effect was QT prolongation, which was a dose related complication<sup>[71]</sup>. More studies are needed to evaluate the efficacy and safety profiles of topiramate and ondansetron in post-liver transplant patients<sup>[68,70]</sup>.

### Consequences of alcohol use on allograft outcomes

Graft rejection, graft loss and recurrent alcohol cirrhosis are feared complications of alcohol relapse after transplant for ALD patients. It has been suggested that alcohol relapse may lead to reduced compliance associated with a significantly increased graft rejection rate<sup>[14,17,72]</sup>. Pageaux *et al.*<sup>[17]</sup> demonstrated that while there was no significant difference in graft rejection rates between abstinent, occasional drinkers or heavy drinkers, the rejection episodes observed in the heavy drinker category were related to poor compliance to immunosuppressant medications. Therefore, alcohol consumption after LT may be a marker of medication non-adherence and can potentially predict risk of graft rejection. Overall, graft

loss from recurrence of ALD is uncommon, but multiple studies have shown that alcohol use after transplant is associated with an increased risk of graft loss and advanced allograft fibrosis<sup>[14,17,72-74]</sup>. In a study by Rice *et al.*<sup>[14]</sup> any alcohol relapse increased the risk of graft failure, but upon subdivision by drinking pattern, a single slip or intermittent relapse was not associated with graft failure, but continuous heavy drinking was significantly associated with decreased graft survival. In terms of histopathology, patients with alcohol relapse were more likely to have advanced fibrosis (stage 3 or higher) compared to those that remained abstinent<sup>[14]</sup>. In the study, 20.8% of patients had a single slip and 33.3% of patients relapsed to continuous heavy drinking<sup>[14]</sup>. Multiple studies have demonstrated that patients with heavy post-transplant drinking were more likely to have more fatty changes and severe fibrosis<sup>[17,48]</sup>. Still, these histologic findings may also be explained by nonalcoholic hepatitis, given the fact that metabolic syndrome is common among post-LT patients<sup>[75]</sup>.

### Survival

The overall survival rates of patients transplanted for ALD are comparable or higher than the survival rates of patients transplanted for other etiologies<sup>[2,3,10,16]</sup>. According to an article by Dumortier, survival after liver transplant for ALD is 92.6% at 1 year, 88.5% at 3 years, 84.3% at 5 years and 73.4% at 10 years, which is comparable to that of patient's transplanted for other etiologies of cirrhosis<sup>[20]</sup>. While occasional slips are not associated with reduced survival, relapse to abusive or harmful levels of drinking is associated with increased mortality in ALD patients<sup>[15]</sup>. Interestingly, mortality after LT for ALD is rarely due to recurrent alcoholic cirrhosis. According to DuMortier *et al.*<sup>[20]</sup>, only 3% of deaths were related to alcohol cirrhosis after transplant and only 0.7% of the patients transplanted for alcoholic cirrhosis died from recurrent alcoholic cirrhosis. This finding was consistent with another study where only 1 (1%) death was related to alcohol relapse whereas the majority of deaths were attributed to cancer<sup>[27]</sup>. Björnsson *et al.*<sup>[8]</sup> also found that deaths in the group of patients that resumed alcohol use were not directly related to alcohol use. While alcohol use itself does not reduce post-transplant survival, recurrent alcoholic cirrhosis does significantly reduce post-transplant survival. One-, 5-, 10- and 15-year survival was 100%, 87.6%, 49.7% and 21.0%, respectively, for patients with recurrent alcoholic cirrhosis vs 100%, 89.4%, 69.9% and 41.1%, respectively, for the patients without recurrent alcoholic cirrhosis ( $P < 0.001$ )<sup>[76]</sup>. Furthermore, Cuadrado *et al.*<sup>[72]</sup> found no difference in 1 or 5 year survival in those who were abstinent vs those with alcohol relapse, but the study did find a remarkably worse 10 year survival in patients with alcohol use of more than 30 g/d (45.1% vs 85.5%). This difference in long-term mortality did not appear to be related to liver failure, graft rejection, infection rate or metabolic disturbances, but was attributed to a higher frequency of deaths from *de novo* malignancy

and cardiovascular events<sup>[72]</sup>. Therefore, the major long-term causes of mortality in patients transplanted for ALD appear to be due to cardiovascular disease and *de novo* malignancy rather than related to alcohol use<sup>[10,20,38,72,76]</sup>.

## CONCLUSION

Overall, ALD is a good indication for liver transplantation. Patients transplanted for ALD have comparable survival rates to patients transplanted for other etiologies of liver disease<sup>[2,3,10,16]</sup>.

Based on this review article, consistent predictors of alcohol relapse include comorbid psychiatric conditions, social support and tobacco use<sup>[11,13,15,29,31,40,77,78]</sup>. While the 6-mo rule is a common prerequisite for LT listing, it is not a reliable predictor of alcohol relapse<sup>[8,27,28]</sup>. It is also not feasible for some patients, particularly those with severe alcoholic hepatitis that is refractory to medical management<sup>[34]</sup>. Furthermore, scoring systems to predict relapse such as the HRAR and ARRA have been proposed but have yet to be validated by other studies.

Additionally, participation in an addiction unit integrated within a transplant center was found to be efficacious in reducing alcohol relapse after LT, but further studies are still needed to reproduce this finding<sup>[25]</sup>. Rodrigue *et al.*<sup>[56]</sup> did not find pre-LT treatment of substance abuse disorders to significantly impact relapse post-LT, but patients who received both pre-and post-transplant substance abuse treatment were significantly less likely to drink post-transplant. Therefore, continuous addiction treatment may play an important role in this population.

Multiple drugs have been approved for alcohol dependence, but the majority has not yet been studied in patients transplanted for ALD<sup>[57,58]</sup>. Baclofen appears to be the most promising pharmacologic agent in promoting abstinence post-transplant and was shown to have a good safety profile in patients with advanced liver disease. Further research is needed to determine whether baclofen can reduce alcohol relapse in ALD patients in the post-transplant period. Acamprosate, topiramate and ondansetron are also promising agents because of their lower risk of hepatotoxicity, but further research is needed<sup>[59,66,67]</sup>.

Lastly, alcohol relapse is associated with increased rates of graft rejection<sup>[14,17,72]</sup>. This is thought to be due to the association between alcohol use and non-adherence to immunosuppressive agents<sup>[14,17,72]</sup>. While occasional slips do not impact graft loss, a harmful or excessive amount of alcohol use post-LT has been found to be associated with an increased rate of graft loss and advanced fibrosis<sup>[14,17,48]</sup>. Heavy drinkers were also noted to have more fatty changes and steatohepatitis compared to those who remained abstinent, though this finding may be confounded by nonalcoholic steatohepatitis<sup>[14,17,72,73,75]</sup>. Overall, survival in ALD patients is comparable or higher compared to those transplanted for other etiologies of liver disease<sup>[2,3,10,16]</sup>. Long-term survival at 10 years was found to be significantly lower in those

that resumed alcohol use, but this was attributed to mortality from *de novo* malignancies and cardiovascular events rather than due to liver failure<sup>[72,75]</sup>.

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Basic Study

# Angiotensin II or epinephrine hemodynamic and metabolic responses in the liver of L-NAME induced hypertension and spontaneous hypertensive rats

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## Abstract

### AIM

To study hepatic vasoconstriction and glucose release induced by angiotensin (Ang) II or Epi in rats with pharmacological hypertension and spontaneously hypertensive rat (SHR).

### METHODS

Isolated liver perfusion was performed following portal vein and vena cava cannulation; Ang II or epinephrine (Epi) was injected *in bolus* and portal pressure monitored; glucose release was measured in perfusate aliquots.

### RESULTS

The portal hypertensive response (PHR) and the glucose release induced by Ang II of L-NAME were similar to normal rats (WIS). On the other hand, the PHR induced

by Epi in L-NAME was higher whereas the glucose release was lower compared to WIS. Despite the similar glycogen content, glucose release induced by Ang II was lower in SHR compared to Wistar-Kyoto rats although both PHR and glucose release induced by Epi in were similar.

### CONCLUSION

Ang II and Epi responses are altered in different ways in these hypertension models. Our results suggest that inhibition of NO production seems to be involved in the hepatic effects induced by Epi but not by Ang II; the diminished glucose release induced by Ang II in SHR is not related to glycogen content.

**Key words:** Epinephrine; Liver perfusion; Spontaneously hypertensive rat; Glucose; Angiotensin II; L-NAME

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**Core tip:** Angiotensin (Ang) II and epinephrine (Epi) induce hemodynamic and metabolic responses in a normal liver. These responses are altered in different ways in two models of hypertension. We observed that inhibition of NO production seems to be involved in the hepatic hemodynamic and metabolic effects induced by Epi but not by Ang II. Furthermore, diminished glucose release induced by Ang II in spontaneously hypertensive rat is not related to glycogen content, but might be due to the glycogen phosphorylase activation by Ang II.

Kimura DC, Nagaoka MR, Borges DR, Kouyoumdjian M. Angiotensin II or epinephrine hemodynamic and metabolic responses in the liver of L-NAME induced hypertension and spontaneous hypertensive rats. *World J Hepatol* 2017; 9(17): 781-790 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i17/781.htm> DOI: <http://dx.doi.org/10.4254/wjgh.v9.i17.781>

### INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) regulates blood pressure homeostasis and vascular injury and repair responses. This system has been associated with diverse physiological functions, but also with inflammation, fibrosis, and target-organ damage. Local forms of the RAAS have been described in many tissues<sup>[1-5]</sup>. The importance of RAAS in the pathophysiology of hypertension has been observed in brain, heart, adrenal glands, vasculature, and kidney<sup>[6-9]</sup>.

Several components of RAAS are present in the liver, which synthesizes angiotensinogen, a glycoprotein that contains the sequence of angiotensin in its amino-terminal portion. Angiotensin converting enzyme (ACE) is a carboxypeptidase present primarily in the perivenous region. Besides converting angiotensin (Ang) I in Ang II, it is the major kininase involved in bradykinin degradation in the liver<sup>[10]</sup>. In 1976, Borges *et al.*<sup>[11]</sup> showed that both Ang I and Ang II infused into the portal vein of a rat

induced hypertensive effect, and they also demonstrated for the first time the conversion of Ang I into Ang II by the rat liver. This hypertensive response induced by Ang II is mediated by AT1 receptor because when losartan was co-infused with Ang II into the liver portal vein it abolished the hypertension response<sup>[12]</sup>. Captopril infusion prevented pressor action of Ang I, thus the PHR previously attributed to Ang I is actually a result of its conversion to Ang II by hepatic ACE. This conversion is rapid, but the portal hypertensive action after Ang I *in bolus* injection is significantly delayed compared to Ang II injection<sup>[13]</sup>. Metabolic effects induced by Ang II, such as glucose release and O<sub>2</sub> consumption, are only diminished in the presence of losartan, which demonstrates that these effects are partially dissociated on bivascular liver perfusion. Therefore, another receptor besides AT1R might also be involved on these Ang II hepatic effects<sup>[12,14]</sup>.

ACE inhibition or blockade of angiotensin receptors are widely used in clinical medicine in the treatment of hypertension. The role of the hepatic RAAS has been associated with fibrosis and cirrhosis, and its resulting portal hypertension. Up-regulation of hepatic ACE, ACE2 and AT1R was observed in animal models of fibrosis and cirrhosis by bile duct ligation or carbon tetrachloride induction<sup>[15-17]</sup>. Ang II, *via* AT1R, stimulates activation of quiescent stellate cells, activates myofibroblasts proliferation, and promotes the release of inflammatory cytokines, as well as the excessive deposition of extracellular matrix components<sup>[18]</sup>.

The catecholaminergic sympathetic nervous system is another common system with metabolic (glucose and lactate release as well as oxygen consumption increase) and hemodynamic (vasoconstriction) effects. This system plays a key role in blood pressure homeostasis and normal metabolism and participates in the pathophysiology of many diseases. The liver contains abundant sympathetic innervation derived from the hepatic nerve plexus, and circulating catecholamines regulate liver tone<sup>[19]</sup>. The presence of the  $\alpha$ 1- and  $\beta$ -adrenergic receptors on hepatocytes was demonstrated in various species like catfish, goldfish, and rats<sup>[20-22]</sup>. In fed state, epinephrine (Epi) promotes hepatic glucose production by activation of glycogenolysis and, in fasted state, Epi accelerates gluconeogenesis<sup>[23]</sup>.

In patients with essential hypertension, plasma levels of norepinephrine are significantly elevated and the increased sympathetic activity is accompanied by diastolic and systolic pressure increases. Neuroadrenergic factors may contribute to the maintenance and progression of hypertensive state as well as its development<sup>[24]</sup>. A correlation between the RAAS and the sympathetic nervous system has also been described. The latter is activated by Ang II and plays a fundamental role in the homeostasis of blood pressure control<sup>[25]</sup>. The multifactorial etiology of hypertension has led researchers to postulate, over time, various experimental models, each one involving one or more mechanisms, contributing to the assembly of a human essential hypertension "mosaic". A pharmacological hypertension model is the blockade

of nitric oxide synthesis. Biancardi *et al.*<sup>[26]</sup> showed that vasoconstriction in response to L-NAME by the sympathetic tone plays an important role in the initiation and maintenance of hypertension. The RAAS also contributes to high blood pressure in animals chronically treated with L-NAME. Chronic treatment with ACE inhibitors or AT1 blockers is able to prevent the onset of, or reverse, a hypertension and renal injury already established, indicating a involvement of RAAS in the genesis and maintenance of this hypertension<sup>[27]</sup>. A spontaneously hypertensive rat (SHR) is the widely used genetic hypertension model that presents elevated sympathetic activity<sup>[28]</sup>. Although these animals are generally considered to be characterized by a low activity of circulating RAAS<sup>[29]</sup>, some studies indicate that treatment with ACE inhibitors or AT1 receptor blockers or both reduces cardiac or renal dysfunction or both of these dysfunctions in SHRs<sup>[30-32]</sup>.

Although the liver is not a target organ in physiopathology of hypertension, the presence of AT1 receptor and ACE may still indicate unknown specific roles. Sympathetic hyperactivity was described in most models of hypertension<sup>[28]</sup> but little is known about the consequences of this hyperactivity in the liver. Therefore, the aim of this work was to evaluate the hepatic response to Ang II and Epi in hypertension models. Using the isolated rat liver perfusion, we studied the vasoconstrictor hepatic effect as well as metabolic (glucose release) effect of Ang II and Epi in two different hypertension experimental models: One genetic (SHR) and one pharmacological (systemic inhibition of NO synthase).

## MATERIALS AND METHODS

### Animals

Adult male Wistar EPM-1 rats (WIS), SHRs (bred by the Central Animal House of the Federal University of São Paulo - UNIFESP), and Wistar Kyoto (WKY) rats (bred by Central House of the University of de São Paulo - USP) aged 12-16 wk were used. The animals were housed in a conditioned environment and were fed a standard laboratory diet (Purina) and water *ad libitum*. This study was conducted according to the International Guiding Principles for Biomedical Research Involving Animals<sup>[33]</sup> and was approved by the Ethics in Research Committee of UNIFESP (CEP 1455/09).

### Experimental groups

After one week of acclimatization, two experimental groups were studied: (1) L-NAME, pharmacologic induced model of hypertension: Wistar EPM-1 rats received N<sup>G</sup>-nitro-L-arginine methylester (0.5 mg/mL) in drinking water for 10 d and were compared to healthy, Wistar EPM-1 rats; and (2) SHRs were compared to WKY rats.

### Indirect systolic blood pressure

Body weight and tail indirect systolic blood pressure (SBP) were recorded weekly. SBP was measured by tail-

cuff plethysmography (NIBP Controller, ADInstruments, Australia) in unanesthetized rats that were placed in a warm cupboard (45 °C) for 15 min. SBP values for individual rats were obtained from the average of 3-4 consecutive measurements and were considered valid only when these readings did not differ by more than 5 mmHg. Procedure was performed at least 48 h before the perfusion experiments to minimize the influence of animal stress on our results. Upon confirmation of animal hypertension, perfusion of rat liver *in situ* was conducted as previously described<sup>[34]</sup>.

### Glycemia and insulinemia

Blood samples were collected from the abdominal aorta before portal vein cannulation. They were centrifuged at 3000 rpm to remove red cells, and serum was stored at -20 °C. Glucose was determined by enzymatic method (Glucose PAP kit, Labtest Diagnóstica, São Paulo, Brazil) and the concentration of insulin was determined using a direct ELISA kit specific for rat and mouse analysis (Millipore, United States).

### In situ rat liver perfusion

Monovascular rat liver perfusion was performed as previously described<sup>[34]</sup>. Briefly, the rat was anesthetized with urethane, 1.3 g/kg, *i.p.* (Sigma Chemical Co., United States), and hemoglobin-free, nonrecirculating liver perfusion was performed. Abdominal and thoracic cavities were opened and the portal vein (entry *via*) and the vena cava (exit *via*) cannulated. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer, pH 7.4, containing 1 mg/mL BSA (Sigma Chemical Co., United States) saturated with an oxygen/carbon dioxide mixture (95/5%). Fluid was pumped in a constant flow (3-4 mL/min.g liver) through a temperature-regulated membrane oxygenator (37 °C) prior to entering the liver *via* the portal vein. The oxygen uptake in the outflowing perfusate was monitored continuously with a polarographic type of probe (Delta OHM HD2109.2, Italy) adequately positioned in a chamber at the exit of the perfusate. Liver viability was evaluated by bile production and oxygen consumption. The portal pressure was measured by using a vertically positioned, graduated fluid-filled column attached before the afferent cannula open to the atmospheric. After 20 min of stabilization previously determined (glucose release and portal pressure), 2 nmol Ang II (Sigma Chemical Co., United States) or 40 nmol Epi (Sigma Chemical Co., United States) was injected *in bolus* into the portal vein cannula. Aliquots of perfusate were collected (0 and every 30 s until 5 min and 6, 8 and 10 min) for glucose determination.

### Portal pressure

Portal pressure was recorded during all experiments (0, 15, 30 and 45 s and 1-10 min). The portal pressure increase was determined over the basal pressure and the maximum increase measured. The portal hypertensive response (PHR; the area under the curve) was calculated



**Table 1** Serum parameters and glycogen content

Group	Glycemia (mg/dL)	<i>n</i>	Insulinemia (ng/mL)	<i>n</i>	Glycogen content (mg/100 mg liver)	<i>n</i>
WIS	75.4 ± 4.2	9	2.1 ± 0.4	12	2.9 ± 0.2	10
L-NAME	80.7 ± 7.5	8	2.0 ± 0.4	12	2.3 ± 0.2	10
WKY	76.9 ± 4.0	9	3.8 ± 0.6	12	2.8 ± 0.2	10
SHR	86.2 ± 4.0	8	2.7 ± 0.4	13	2.8 ± 0.2	10

Serum and liver fragment for glycogen content measurement were collected before the liver perfusion experiment. Values are expressed as mean ± SEM. Student's *t*-test; L-NAME *vs* WIS and SHR *vs* WKY. WIS: Similar to normal rats; SHR: Spontaneously hypertensive rat; WKY: Wistar Kyoto.

from the graphic: Portal pressure increase *vs* time after agonist injection and expressed as cmH<sub>2</sub>O.min.

### Metabolic effects

Metabolic effects were evaluated on the basis of oxygen consumption and glucose release by perfused liver. Oxygen consumption was calculated from input-output differences expressed as μmol O<sub>2</sub> consumed/min.g liver. Glucose released was determined in perfusate aliquots using an enzymatic method (Glucose PAP kit, Labtest Diagnóstica, Sao Paulo, Brazil) and expressed as μmol glucose released/min.g liver. This parameter was also used to assure the liver viability. The amount of glucose released was calculated (area under the curve) from the graphic: Glucose increase *vs* time after agonist injection and expressed as μmol/min.g liver.

### Glycogen

In order to avoid loss of the liver glycogen content during the 30 min of perfusion, a fragment of caudate lobe was removed after a rapid exsanguination at the beginning of the perfusion procedure. Quantification of the glycogen was based on the extraction of the polysaccharide with an alkaline solution (30% KOH) and its conversion into glucose during the reaction of the exergonic homogenized with a solution of sulfuric acid and anthrone<sup>[35]</sup>. The concentration of glycogen (expressed as mg/100 mg liver) was determined from a glucose standard curve. Furthermore, liver fragments were removed at the end of the experiment and processed by the company Histotech Teaching Blades (<http://www.histotech.com.br/site/>). The histological analysis of liver glycogen was performed using the periodic acid-Schiff (PAS) staining.

### Statistical analysis

The results are expressed as mean ± SEM. Comparisons were performed by using Student's *t*-test and a value of *P* < 0.05 was adopted as the level of significance. Analysis was performed using Graph Pad Prism 5.0 program.

## RESULTS

### Hypertension animal model characterization

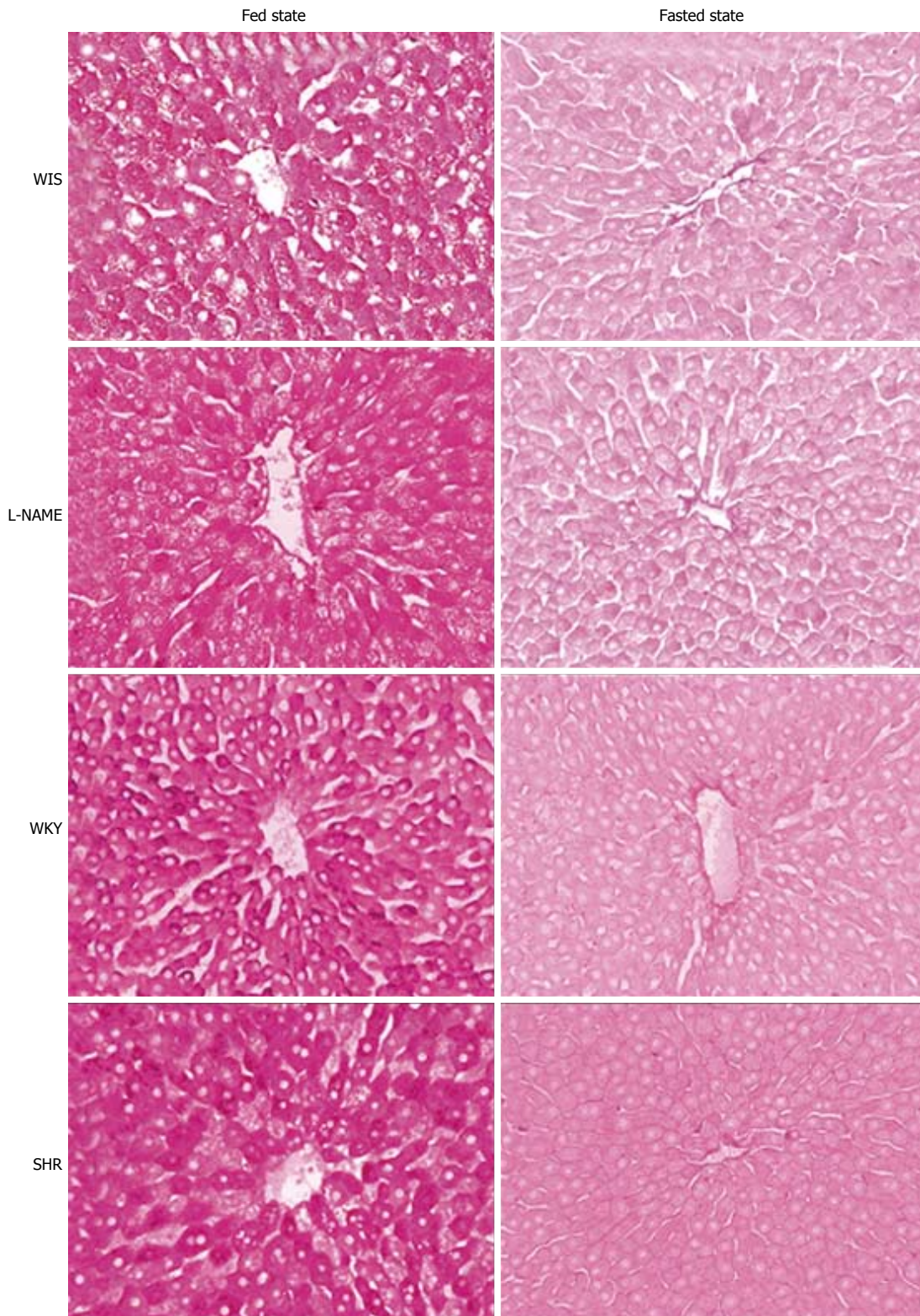
Arterial blood pressure of SHR and rats submitted to drug hypertension (L-NAME) was evaluated before the perfusion experiments. The tail systolic blood pressure (mmHg) of L-NAME (169.1 ± 4.8; *n* = 12) and SHR groups (180.2 ± 5.9; *n* = 10) were higher (*t*-test, *P* <

0.001) when compared to WIS (126.4 ± 2.9; *n* = 9) and Wistar Kyoto (127.0 ± 2.0; *n* = 15), respectively. The glycemia and insulinemia of the rats used in the experiments are shown in Table 1; values of glycemia of normotensive animals were taken as the reference value. The glycemia of both the L-NAME and SHR groups was similar when compared to their respective control groups. The insulinemia of all groups were within normal range (0-118 pmol/L)<sup>[36]</sup> without difference between groups.

The perfusion experiments were performed in the morning when the animals, which have nocturnal habits, were in a well-fed state confirmed by hepatic glycogen content. No difference in liver glycogen content among groups (Table 1) was found. At the end of perfusion another fragment of the liver was removed for histological analysis for glycogen content (PAS staining) and compared to the perfused livers of animals left for 24 h of fasting. We observed that even after 30 min of perfusion, the hepatic glycogen of all groups was noticeably higher than in fasted animals (Figure 1).

### Liver viability

To ensure liver viability during the period of liver perfusion experiment (approximately 30 min), bile production and oxygen consumption were monitored. The bile was collected before and after injection of Epi or Ang II. As the bile production before and after agonist injection were similar, the arithmetic average was used for statistical analysis. The bile production (mL/min.g liver) was similar among groups (WIS: 1.2 ± 0.1, *n* = 16; L-NAME: 1.2 ± 0.1, *n* = 15; WKY: 1.1 ± 0.1, *n* = 14; SHR: 1.1 ± 0.1, *n* = 13). The oxygen consumption was observed throughout the perfusion period ensuring the functioning of the organ. The basal oxygen consumption (μmol/min.g liver) of SHR (2.5 ± 0.1, *n* = 14) was lower (*t*-test, *P* = 0.0151) when compared to WKY (3.2 ± 0.2, *n* = 16). This parameter on L-NAME (3.1 ± 0.2, *n* = 15) was similar to WIS (3.2 ± 0.1; *n* = 17). After agonist injection, oxygen consumption was maintained but no standard response was observed: It remained the same in some experiments and increased in others. As the perfusion fluid did not contain glucose, its release was observed from the beginning of the experiment. Basal glucose release was similar in all groups (Figure 2A and B); after agonists injection its release continued throughout the entire experiment, ensuring hepatic viability.



**Figure 1 Hepatic glycogen.** Periodic acid Schiff's staining of cross-section of perfused livers from fed or 12 h fasted rats. Fragments taken after 30 min of perfusion. Increase 200  $\times$ . WIS: Similar to normal rats; SHR: Spontaneously hypertensive rat; WKY: Wistar Kyoto.

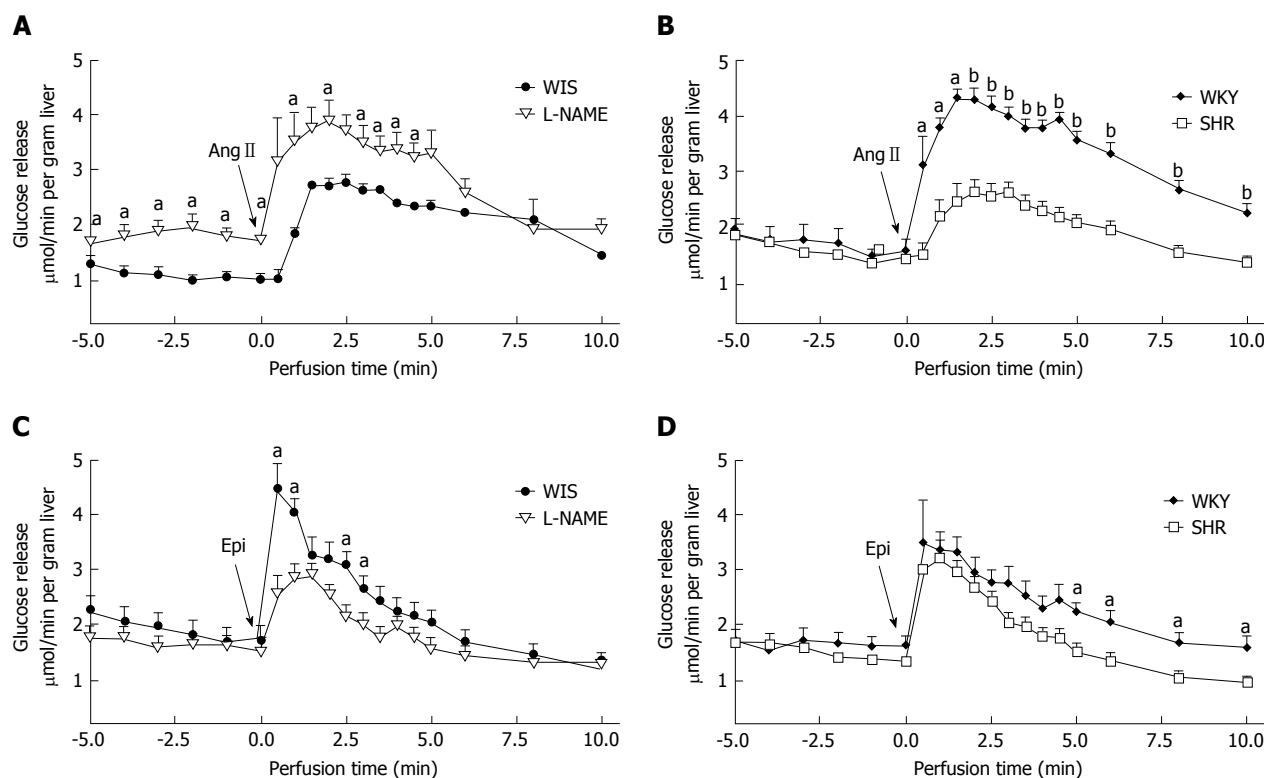
#### Glucose release induced by Ang II or Epi

Following Ang II injection, the amount of glucose released (Figure 2A and B) from the L-NAME group was similar compared to the WIS group, whereas the amount released from SHR livers was lower than its WKY control group

(Table 2).

The glucose release induced by epinephrine is shown in Figure 2C and D; the amount released (AUC) from the L-NAME group ( $4.2 \pm 0.4$ ) was lower when compared to its WIS control group ( $7.5 \pm 0.9$ ), whereas the SHR





**Figure 2** Glucose release induced by angiotensin II and epinephrine. Livers were perfused with Krebs-Henseleit-bicarbonate buffer and after stabilization 2 nmol Ang II (A, B) or 40 nmol Epi (C, D) was injected *in bolus* into afferent cannula and this moment was considered as time 0 min. Glucose release was determined in perfusate aliquots collected during all experiments. Student's *t*-test; <sup>a</sup>*P* < 0.05 and <sup>b</sup>*P* < 0.0001 compared with respective controls for each time point. WIS: Similar to normal rats; SHR: Spontaneously hypertensive rat; WKY: Wistar Kyoto; Ang: Angiotensin; Epi: Epinephrine.

group was similar to the WKY group (Table 2).

### PHR to Ang II or Epi

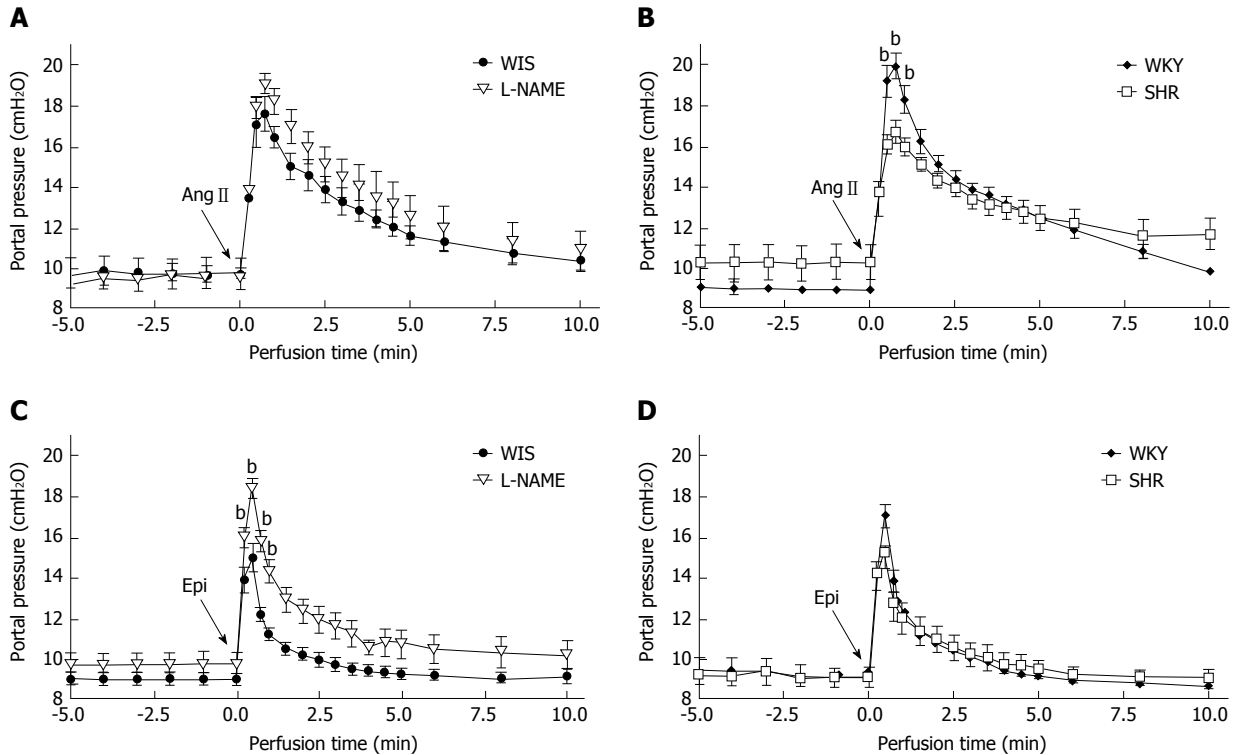
Basal portal pressure (before agonist injection) was similar in all groups. Ang II (2 nmol) or Epi (40 nmol) was injected in portal vein and both agonists promoted portal vasoconstriction. Despite a 20-fold difference in agonists doses, the maximum portal pressure increase (cmH<sub>2</sub>O) induced by Ang II and Epi was similar in among groups (Ang II: WIS:  $7.9 \pm 1.2$ , *n* = 7; L-NAME:  $7.6 \pm 1.1$ , *n* = 7; WKY:  $10.5 \pm 0.3$ , *n* = 7; SHR:  $6.5 \pm 1.2$ , *n* = 10; Epi: WIS:  $6.1 \pm 0.7$ , *n* = 10; L-NAME:  $8.9 \pm 0.7$ , *n* = 8; WKY:  $7.9 \pm 0.7$ , *n* = 8; and SHR:  $6.2 \pm 0.5$ , *n* = 6).

The hepatic portal pressure increase after bolus injection of Ang II was normalized after about 10 min of perfusion (Figure 3A and B). The curve profile of portal pressure of L-NAME and SHR groups was similar to their control groups (WIS and WKY, respectively). The PHR induced by Ang II in both L-NAME and SHR was similar when compared to their WIS and WKY control groups, respectively (Table 3). The effect of Epi in portal pressure was more transient than Ang II. Following Epi injection, the portal pressure increase was normalized after about 5 min (Figure 1). The PHR induced by Epi in the L-NAME group was higher when compared to the WIS group. On the other hand, no difference in PHR of SHRs existed compared to the control WKY group (Table 3).

## DISCUSSION

All key components of the RAAS are present in the normal liver and are up-regulated in response to chronic liver injury, with growing evidence that the intrahepatic RAAS plays important roles in both the pathophysiology of portal hypertension and liver fibrosis<sup>[18]</sup>. The use of ACE/Ang II/AT1R axis inhibitors associated with ACE2/Ang (1-7)/Mas axis activation is a promising strategy-serving regimen to prevent and treat chronic liver diseases as well as acute liver injury<sup>[37]</sup>. Hepatic glucose metabolism can be modulated by NO directly inhibiting glycogen synthesis and gluconeogenesis, and indirectly inhibiting glycogen breakdown *via* the secretion of other intrahepatic mediators<sup>[38,39]</sup>.

In the liver, both Ang II and Epi cause vasoconstriction and glucose release. Although the liver is not considered the target organ in hypertension pathophysiology, it is an important metabolic regulator organ. To study hepatic effects of Ang II and Epi, we used two different experimental models of hypertension: Pharmacological (systemic inhibition of NO synthase) and genetic (SHR). Chronic oral administration of L-NAME promotes a rapid deployment of hypertension in the first days of treatment that is largely mediated by the RAAS. The rats treated with ACE inhibitors, such as captopril and enalapril, or with AT1 receptor antagonists, such as losartan, restore blood pressure to near normal levels<sup>[40,41]</sup>. In our study,



**Figure 3 Portal pressure induced by angiotensin II or epinephrine.** Livers were perfused with Krebs-Henseleit-bicarbonate buffer and after 20 min stabilization, 2 nmol Ang II (A, B) or 40 nmol epinephrine (C, D) was injected *in bolus* into afferent cannula and this moment was considered as time 0 min. The portal pressure was continuously monitored by water manometer attached to the circuit before the cannula. Student's *t*-test; <sup>a</sup>*P* < 0.0001 compared with respective controls for each time point. WIS: Similar to normal rats; SHR: Spontaneously hypertensive rat; WKY: Wistar Kyoto; Ang: Angiotensin; Epi: Epinephrine.

**Table 2 Glucose release induced by angiotensin II or epinephrine**

Group	Glucose released $\mu\text{mol}/\text{min}\cdot\text{g liver}$			
	Angiotensin II	<i>n</i>	Epinephrine	<i>n</i>
WIS	11.3 $\pm$ 0.9	7	7.5 $\pm$ 0.9	10
L-NAME	11.2 $\pm$ 1.5	7	4.2 $\pm$ 0.4 <sup>d</sup>	8
WKY	16.4 $\pm$ 1.5	7	8.0 $\pm$ 0.9	8
SHR	5.42 $\pm$ 0.6 <sup>b</sup>	10	5.9 $\pm$ 0.7	6

The amount of glucose (area under the curve) was calculated from the curve glucose release increase *vs* time after agonist injection. Student's *t*-test; <sup>b</sup>*P* < 0.0001 and <sup>d</sup>*P* = 0.002 compared with respective control (L-NAME *vs* WIS and SHR *vs* WKY). WIS: Similar to normal rats; SHR: Spontaneously hypertensive rat; WKY: Wistar Kyoto.

**Table 3 Portal hypertensive response to angiotensin II or epinephrine**

Group	Portal hypertensive response $\text{cmH}_2\text{O}\cdot\text{min}$			
	Angiotensin II	<i>n</i>	Epinephrine	<i>n</i>
WIS	26.4 $\pm$ 3.2	7	8.2 $\pm$ 0.8	10
L-NAME	38.1 $\pm$ 4.8	7	18.5 $\pm$ 1.9 <sup>b</sup>	8
WKY	29.0 $\pm$ 1.1	7	10.0 $\pm$ 1.1	8
SHR	25.9 $\pm$ 3.7	10	10.5 $\pm$ 1.1	6

The portal hypertensive response (PHR; area under the curve) was calculated from portal pressure increase curve *vs* time after agonist injection and expressed as  $\text{cmH}_2\text{O}\cdot\text{min}$ . Student's *t*-test; <sup>b</sup>*P* < 0.0001 compared with respective control (L-NAME *vs* WIS and SHR *vs* WKY). WIS: Similar to normal rats; SHR: Spontaneously hypertensive rat; WKY: Wistar Kyoto.

10 d of L-NAME treatment were sufficient to induce a high level systolic blood pressure. On the other hand, the SHR strain is the most widely used phenotypic experimental model in hypertension research with specific potential in the study of polygenic hypertension, being associated with cardiac hypertrophy, heart failure, and renal dysfunction. Hepatic functions are also altered at the molecular level in this model of primary hypertension<sup>[42]</sup>.

Treatment with L-NAME did not affect fasting glucose levels but reduced significantly insulin levels in blood and increased insulin sensitivity of rats<sup>[43]</sup>. Gouveia *et al.*<sup>[44]</sup> described increased glycemia and insulinemia values for fasted or fed SHRs. We observed normal glycemia and insulinemia in both hypertension models in fed state, which contrasts with the studies that show changes in

these metabolic parameters. The discrepancy may be due to the metabolic states of the animals in the studies.

Tarsitano *et al.*<sup>[43]</sup> described how prolonged treatment (2-8 wk) with NO synthase inhibitor enhanced hepatic glycogen levels. In our study, as the treatment with L-NAME was only for 10 d, the amount of liver glycogen was similar to the WIS group. This short period of treatment might not have been enough to observe possible changes in the glycogen content. Chronic or acute administration of an inhibitor of NO synthesis (L-NAME or L-NNMA) was shown to alter systemic RAAS, decreasing plasma level Ang II as well as renin activity<sup>[45]</sup>. Nevertheless, hepatic glucose release profile induced by Ang II in chronically treated L-NAME animals was similar to the control, which suggests that NO is not involved in the glucose release



after induction.

Interestingly, in the L-NAME group, the glucose release induced by Epi was lower than in the control group, suggesting that this effect may be related to the inhibition of NO synthesis. In cultured rat hepatocytes, Hodis *et al.*<sup>[46]</sup> observed that glycogenolysis occurs *via*  $\alpha$ -adrenergic stimulation and signaling cascade that involves the production of NO. Similarly, our results suggest that the chronic inhibition of NO synthase might inhibit hepatic glycogenolysis, which in turn decreases the release of glucose in the perfusate during the experiment. Therefore, the differences in glucose release following the L-NAME treatment evidenced that the increase in hepatic glycogenolysis was probably mediated by NO when activated by Epi but not by Ang II.

In the SHR group, it was described that muscle glycogen content was lower, but livers presented similar levels of glycogen in the fed and fasted states<sup>[44]</sup>. Likewise, we found similar amounts of liver glycogen in the SHR and WKY groups. Despite this similarity, after Ang II *in bolus* injection, glucose released was lower in the SHR group compared to the control group. This result suggests that glucose release is not necessarily related to glycogen content, but may be due to a possible difference in glycogen phosphorylase activation by increased  $[Ca^{2+}]_i$  induced by Ang II<sup>[47]</sup>. On the contrary, in this hypertension model, glucose release induced by Epi was similar when compared to the control.

Both Ang II and Epi are potent physiological vasoconstrictors. We observed that although these agonists led to similar maximum increases of the portal pressure, Ang II promoted a higher PHR, even using doses 20-fold lower. These response differences may be related to the prolonged responses induced by Ang II in the liver or with the amount of Ang II receptor vs Epi receptor. An enhanced Ang II-mediated vasoconstriction was observed in healthy elderly individuals and this apparent increase is due, at least in part, to the potentiation of  $\alpha$ -adrenergic vasoconstriction. These findings suggest that cross-talk between RAAS and adrenergic systems may be an important regulator of resting vascular tone and muscle blood flow with advancing age<sup>[48]</sup>. Cross-talk between the  $\alpha$ 1-adrenergic receptor ( $\alpha$ 1R) and AT1R potentially exists on two levels: Receptor heterodimerization between  $\alpha$ 1R and AT1R and second messenger level<sup>[49]</sup>.

No difference in the PHR of Ang II in the pharmacologic hypertensive model was found, which suggests no changes in the expression of hepatic AT1 receptor. Our result contrasts with AT1R up-regulation described in the L-NAME model in other tissues such as the aorta<sup>[50]</sup>, adrenals<sup>[51]</sup> and heart<sup>[52]</sup>.

On the other hand, in L-NAME-treated animals, Epi induced increased PHR. It was shown that in rats, chronic inhibition of NO synthase produces endothelial dysfunction, increased vascular response to adrenergic stimulation, and perivascular inflammation<sup>[53]</sup>. NO is also involved in regulation of sympathetic nerve activity in human skin and muscle cells<sup>[54]</sup>. Therefore, this increased

hypertensive effect in the liver of L-NAME-treated rats may be related to increased sympathetic vascular activity. The disparity between the effects of portal vasoconstriction (higher) and glucose released (lower) in the L-NAME group is a further indication that these effects might be dissociated in two components: One with direct action in the hepatocyte and the other as a presinusoidal response.

We also observed similar vasoconstrictor effect of Ang II in the SHR group. Although in this strain, higher levels of AT1R gene expression was described in brain regions involved in arterial blood pressure control<sup>[55]</sup>. Despite widely described sympathetic hyperactivity in this model<sup>[56-58]</sup>, in this work, PHR to Epi on SHRs was similar to the control group.

In conclusion, Ang II and Epi responses are altered in different ways in these two models of hypertension. Our results suggest that inhibition of NO production seems to be involved in the hepatic hemodynamic and metabolic effects induced by Epi but not by Ang II. Furthermore, diminished glucose release induced by Ang II in SHR is not related to glycogen content, but to the glycogen phosphorylase activation by Ang II, that is under investigation.

## COMMENTS

### Background

In a normal liver, angiotensin (Ang) I is rapidly converted in Ang II by hepatic angiotensin converting enzyme, and Ang II promotes hypertensive response mediated by the AT1 receptor. Besides this hemodynamic effect, Ang II induces metabolic effects (glucose release and O<sub>2</sub> consumption). Epinephrine promotes hepatic metabolic (glucose and lactate release and O<sub>2</sub> consumption increase) as well as hemodynamic (vasoconstriction) effects. It has also been described as a correlation between the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system; the latter is activated by Ang II and plays a fundamental role in the homeostasis of blood pressure control. In hypertension, sympathetic hyperactivity is described but little is known about this hyperactivity in the liver. The hepatic response to Ang II and Epinephrine in hypertension has not been studied yet. Therefore, the relevance of this study is to understand the hepatic effects of these hormones in two different hypertensive models.

### Research frontiers

The RAAS and the catecholaminergic system are present in the normal liver. The interaction of RAAS with the catecholaminergic sympathetic nervous system in the liver of hypertensive animals might bring to light relevant aspects of the relationship among metabolic disorders such as hypertension, type II diabetes, obesity, and hypertriglyceridemia.

### Innovations and breakthroughs

No description of hemodynamic and metabolic effects of the two hormones Ang II and Epi exists in the literature on RAAS and the catecholaminergic system in the livers of hypertensive rats. This is the first study evaluating hemodynamic and metabolic effects of the two hormones Ang II and Epi. Inhibition of NO production in the L-NAME model increased hepatic hemodynamic and metabolic effects induced by Epi but not by Ang II. Furthermore, diminished glucose release induced by Ang II in SHRs is not related to glycogen content. Therefore, the hepatic effect of Ang II or Epi is different depending on the pathophysiology of systemic arterial hypertension.

### Applications

Although not target organs in hypertension, RAAS and sympathetic nervous system are overexpressed, elucidating the hepatic role of these systems, which

can bring knowledge about metabolic-related comorbidities and therapeutics.

## Terminology

The portal hypertensive response represents the area under the curve and was calculated from the graphic: Portal pressure increase (cmH<sub>2</sub>O) vs time after agonist injection (min) and expressed as cmH<sub>2</sub>O.min. It considers not only the perfusion pressure increase but the effect of the agonist over time.

## Peer-review

In this paper, authors give some new information about the effects of Epi and Ang II on glucose release, finding that inhibition of NO production seems to be involved in the hepatic hemodynamic and metabolic effects induced by Epi but not by Ang II.

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Retrospective Study

# Use of aspartate aminotransferase to platelet ratio to reduce the need for FibroScan in the evaluation of liver fibrosis

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## Abstract

### AIM

To evaluate the performance of aspartate aminotransferase to platelet ratio (APRI) score against FibroScan in predicting the presence of fibrosis.

### METHODS

Data of patients who concurrently had APRI score, FibroScan and liver biopsy to assess their hepatitis C virus (HCV) and hepatitis B virus (HBV) over 6 years were retrospectively reviewed and details of their disease characteristics and demographics were recorded. Advanced fibrosis was defined as  $\geq F3$ .

### RESULTS

Of the 3619 patients ( $47.5 \pm 11.3$  years, 97M:36F) who had FibroScans and APRI for HCV and HBV, 133 had concurrent liver biopsy. Advanced liver fibrosis was found in 27/133 (20%,  $F3 = 21$  and  $F4 = 6$ ) patients. Although APRI score ( $P < 0.001$ , AUC = 0.83) and FibroScan ( $P < 0.001$ , AUC = 0.84) predicted the presence of advanced fibrosis, the sensitivities and specificities were only modest (APRI score: 51.9% sensitivity, 84.9% specificity; FibroScan: 63% sensitivity, 84% specificity). Whilst 13/27 (48%) patients with advanced fibrosis had  $APRI \leq 1.0$ , no patients with  $APRI \leq 0.5$  had advanced fibrosis, with



100% sensitivity. The use of APRI  $\leq 0.5$  would avoid the need for FibroScan in 43% of patients.

## CONCLUSION

APRI score and FibroScan performed equally well in predicting advanced fibrosis. A proposed APRI cut-off score of 0.5 could be used as a screening tool for FibroScan, as cut-off score of 1.0 will miss up to 48% of patients with advanced fibrosis. Further prospective validation studies are required to confirm this finding.

**Key words:** Liver fibrosis; Aspartate aminotransferase to platelet ratio; Utilization; FibroScan

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**Core tip:** This is the first study to show that an aspartate aminotransferase to platelet ratio (APRI) score of 0.5 could potentially be used as a screening tool to predict the need for FibroScan in patients with hepatitis C or hepatitis B. Our study showed that an APRI score of 0.5 could reduce the need for FibroScan in 43% of the study cohort with high sensitivity.

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## INTRODUCTION

Chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) are among the most common causes of liver fibrosis<sup>[1]</sup>. A determination of the degree of liver fibrosis in these patients is essential to guide management as well as for prognostication<sup>[2-6]</sup>. Liver biopsy has long been considered the gold standard for assessment of liver fibrosis<sup>[2,7,8]</sup>. However, liver biopsy is an invasive procedure that carries a 0.3%-0.6% overall risk for complications and a 0.05% mortality rate<sup>[8,9]</sup>. Several contraindications also exist which may preclude patients from having a liver biopsy, namely coagulopathy<sup>[4]</sup>. As a liver biopsy only samples approximately 1/50000 of the liver, there have been concerns with sampling errors despite an adequate number of portal tracts and sample size<sup>[7,10,11]</sup>. Intra- and inter-observer variation in histological interpretation has also been reported<sup>[12,13]</sup>. Given these limitations, much research has been dedicated to evaluating non-invasive methods to determine liver fibrosis<sup>[5,9]</sup>. Of these, the FibroScan and aspartate aminotransferase (AST) to platelet ratio (APRI) are commonly used in our hospital.

FibroScan is a novel non-invasive method that measures liver stiffness using both ultrasound and low-frequency elastic waves<sup>[14]</sup>. A recent meta-analysis showed that

FibroScan had a good sensitivity, specificity and high accuracy for detecting liver cirrhosis<sup>[15]</sup>. However, invalid assessments rates have been quoted to range between 2.4% and 9.4%, mainly due to high body mass index<sup>[13]</sup>.

In 2003, Wai *et al*<sup>[2]</sup> proposed a novel index APRI with an area under the receiver operating curve (AUROC) for predicting significant fibrosis and cirrhosis 0.80 and 0.89 respectively. A recent meta-analysis showed that an APRI score greater than 1.0 is able to predict cirrhosis with a sensitivity of 76% and a specificity of 72%<sup>[16]</sup>. This suggests that an APRI score of 1.0 or more would not be an ideal screening tool given it could miss a proportion of patients with cirrhosis. The aim of this study was thus, to evaluate and compare the performance of APRI score against FibroScan in predicting the presence of liver fibrosis and to determine the best APRI cut-off score which can predict the likelihood of fibrosis and the need for further assessment with FibroScan.

## MATERIALS AND METHODS

### Study population

A retrospective analysis was performed of all the patients with HCV or HBV, who had been referred for FibroScan to the Department of Gastroenterology and Hepatology in the Royal Adelaide Hospital, the largest tertiary referral hospital in South Australia, between January 2010 and June 2016. Inclusion criteria were infection with either HCV or HBV, a valid FibroScan assessment, a liver biopsy within 12 mo of the FibroScan and an APRI score within 6 mo of the liver biopsy. HCV was defined as a positive HCV RNA and HBV was defined as a positive hepatitis B surface antigen and HBV DNA. Exclusion criteria were the use of the XL probe, current interferon-based treatment, co-infection with human immunodeficiency virus, other causes of chronic liver disease, hepatocellular carcinoma, prior liver transplantation, blood results more than 6 mo before or after the liver biopsy, incomplete FibroScan reports and invalid FibroScan assessments. An invalid FibroScan was defined as an interquartile range of more than 30% and a success rate of less than 60%. The project was approved by The Royal Adelaide Hospital Research Ethics Committee, and all patient data were de-identified (RAH protocol approval number: R20160616).

### Data collection

Detailed data was collected from FibroScan reports and electronic medical records which included age, gender, HCV or HBV, FibroScan results, FibroScan success rate, FibroScan interquartile range, AST level, platelet count and Scheuer fibrosis scores on liver biopsy reports.

The APRI score was calculated using the proposed formula:

$$\text{APRI} = [(\text{AST level/ULN})/\text{platelet count (10}^9\text{/L)}] \times 100 \text{ (2)}$$

The reference value for AST used was 45 IU, which is the upper limit of normal in our laboratory. The FibroScan cut-offs used to define cirrhosis were a median of 14kPa

**Table 1** Baseline characteristics of the 133 patients

Characteristic	Value
Gender	97M/36F
Age (yr)	47.5 ± 11.3
Indication for FibroScan	
HCV	79
HBV	54
Mean FibroScan score (kPa)	11.5
Mean IQR (kPa)	2.17
Mean success rate	95.6%
Mean APRI score	0.75
Mean AST level (U/L)	65.5
Mean platelet count (× 10 <sup>9</sup> /L)	214

HCV: Hepatitis C virus; HBV: Hepatitis B virus; IQR: Interquartile range; AST: Aspartate aminotransferase; APRI: AST to platelet ratio.

and 12.9 kPa for HCV and HBV respectively. Liver fibrosis based on the Scheuer fibrosis system was either no fibrosis (F0), enlarged, fibrotic portal tracts (F1), periportal or portal-portal septa but intact architecture (F2), fibrosis with architectural distortion but no obvious cirrhosis (F3) or probable or definite cirrhosis (F4)<sup>[17,18]</sup>. Advanced fibrosis was defined as F3 and F4.

### Statistical analysis

Patient characteristics were expressed as mean ± SD or *n* (%). Diagnostic performances for FibroScan and APRI score were analysed separately according to sensitivity (Se), specificity (Sp), negative predictive values (NPV), positive predictive values (PPV) and AUROC.

## RESULTS

Of the 3619 patients (47.5 ± 11.3 years, 97M:36F) who had FibroScans performed, 133 (3.7%) had either HCV or HBV with concurrent APRI score and liver biopsy assessment. The mean FibroScan score was 11.5 kPa and the mean APRI score was 0.75. The baseline characteristics of the 133 patients are summarized in Table 1. Histological analysis revealed that 25 (18.8%) patients were F0, 42 (31.6%) were F1, 39 (29.3%) were F2, 21 (15.8%) were F3 and 6 (4.5%) were F4. Therefore, advanced fibrosis was found in 27/133 (20%) patients.

### Performance of standard FibroScan cut-offs and an APRI score of 1.0 in predicting advanced fibrosis

Although both APRI ( $P < 0.001$ , AUC = 0.83) and FibroScan ( $P < 0.001$ , AUC = 0.84) assessments were able to predict the presence advanced fibrosis (Figure 1), the Se, Sp, NPV and PPV of both APRI and FibroScan were only modest (Table 2). Overall, there was good correlation between the APRI score and FibroScan score (Figure 2).

### Optimal APRI cut-off scores to predict the presence of liver fibrosis

Based on liver biopsy 9/39 (23%) patients with F2, 12/21 (57%) patients with F3 and 2/6 (33%) patients with F4 had an APRI score of 1.0 or more. Thus, the use of APRI

**Table 2** Performance indicators of aspartate aminotransferase to platelet ratio score 1.0 and FibroScan for advanced fibrosis

	APRI	FibroScan
Sensitivity	51.9%	63.0%
Specificity	84.9%	84.0%
PPV	46.7%	50.0%
NPV	87.4%	89.9%
Accuracy	78.2%	79.7%

NPV: Negative predictive values; PPV: Positive predictive values; APRI: Aspartate aminotransferase to platelet ratio.

score of 1.0 or more to screen for the need for FibroScan would have missed 13/27 (48%) patients with advanced fibrosis (F3 and F4).

In contrast, based on our plot chart (Figure 3), none of the patients with APRI score of 0.5 or less had F3 or F4 on liver biopsy. Using a lower cut-off APRI score of 0.5 would increase the sensitivity to 100%, but reduce the specificity to 59%. More importantly, the use of APRI score of 0.5 or less would avoid the need of FibroScan assessment in 43% of patients with HCV or HBV who were referred for the procedure.

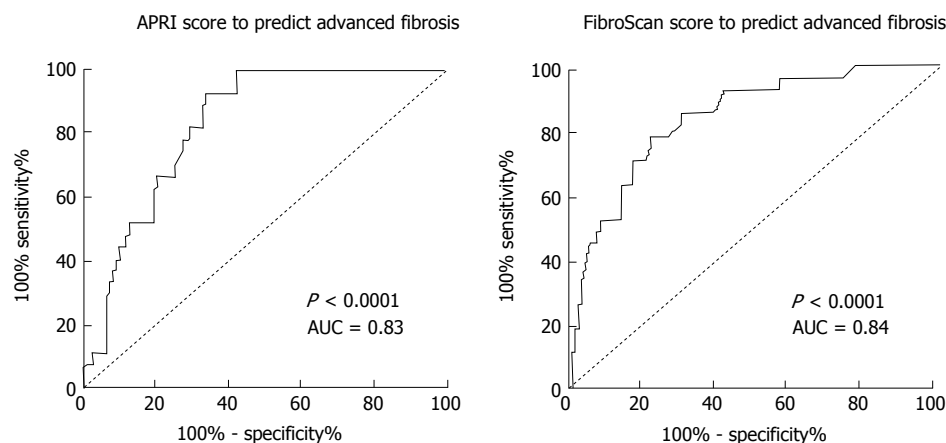
## DISCUSSION

Early and accurate assessment of the degree of liver fibrosis is essential in the management and prognosis of patients with HCV and HBV<sup>[2-6]</sup>. Given the issues associated with liver biopsy, much research has been dedicated to evaluating non-invasive methods to determine liver fibrosis<sup>[5,9]</sup>. This study focused on the performance of FibroScan as well as APRI to detect liver fibrosis as these are commonly used in our hospital.

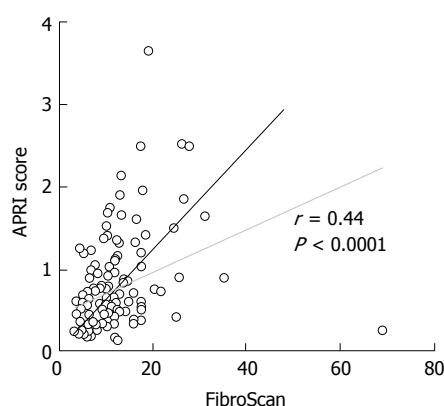
In regards to FibroScan, the AUROC for advanced fibrosis in our study was 0.84. This is comparable to previous studies where the AUROC has ranged between 0.85 to 0.91<sup>[3,5,8]</sup>. Similarly, the AUROC of 0.83 obtained in the study for APRI was in concordance with previous reports of approximately 0.83 to 0.89<sup>[2,3,16]</sup>. Overall, our study showed that there was good correlation between FibroScan and APRI in predicting the presence of fibrosis and this is in keeping with results from previous studies<sup>[3,8]</sup>.

There has been an increasing use of FibroScan in our hospital as evident by the growing number done over the past few years; 472 FibroScans in 2013, 612 in 2014 and 761 in 2015. FibroScan is painless, easy to perform and has good patient acceptance<sup>[13]</sup>. The diagnostic performance is however, influenced by high body mass index<sup>[8,13,19]</sup>. Thus, the study design excluded patients who required the use of the XL probe.

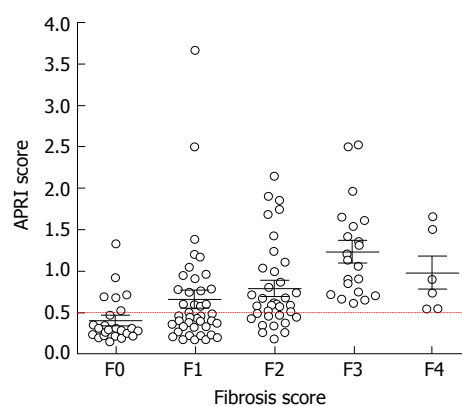
A recent systematic review looking at the cost-effectiveness of FibroScan compared to liver biopsy showed that FibroScan is economically attractive, but does incur added cost of approximately \$1250 to \$2922<sup>[1]</sup>. Apart from the cost, the accessibility of FibroScan may be an issue in the primary health care and resource limited setting. Thus, it would be ideal to have a less expensive,



**Figure 1** Area under the receiver operating curves depicting the performance of aspartate aminotransferase to platelet ratio score and FibroScan in the prediction of advanced fibrosis on liver biopsy. APRI: Aspartate aminotransferase to platelet ratio; AUC: Area under curve.



**Figure 2** Correlation between aspartate aminotransferase to platelet ratio score and Z-score of FibroScan. APRI: Aspartate aminotransferase to platelet ratio.



**Figure 3** Plot diagram depicting the aspartate aminotransferase to platelet ratio scores in each category fibrosis scores rated on liver biopsy. APRI: Aspartate aminotransferase to platelet ratio.

non-invasive method to screen for patients who would need a FibroScan. The APRI score is an appealing tool, particularly in rural areas, given its ease of use and routine availability of the components of the score in all laboratories.

We evaluated the use of APRI score for this purpose and found that: (1) the use of the currently suggested APRI cut-off score of 1.0 or more to screen for FibroScan would have missed 13/27 (48%) patients with advanced fibrosis; and (2) the use of a lower APRI cut-off score of 0.5 will prevent this problem and avoid the need for FibroScan assessment in 43% of patients with HCV or HBV. A recent study detected the F4 cut-off value for APRI to be 0.7<sup>[20]</sup>. This cut-off would have missed 2/6 (33.3%) F4 patients in our study.

Although the proposed APRI cut-off of 0.5 would miss approximately one-third of patients with significant fibrosis (F2), this proportion would be even higher if the cut-off value of 1.0 is used. With the recent evolution in the treatment of HCV, sustained virological response is achievable in the vast majority of patients. Current guidelines recommend anti-viral therapy for all patients except those with limited life expectancy or clear

contraindications<sup>[21]</sup>. The decision for treatment initiation is no longer guided by fibrosis stage except in situations where there are limitations to universal treatment of all patients, and for guiding the duration of treatment in patients with established cirrhosis. Fibrosis staging, however, remains relevant for prognostication. While the new suggested cut-off may miss patients with F2 fibrosis, it is more critical to identify patients with F3 and F4 patients who require ongoing hepatocellular cancer surveillance and screening/surveillance for varices<sup>[21]</sup>. Current guidelines do not recommend routine follow-up of patients with F0-F2 fibrosis following successful treatment of HCV, although this decision would be dependent on clinical judgement especially in patients with confounding risk factors for fibrosis progression (obesity, alcohol, etc).

In regards to HBV, the decision to initiate treatment is based on the disease phase (immune tolerant, immune active, immune control or immune escape) and risk of disease progression or liver related complications. This is mostly guided by ALT and HBV DNA level<sup>[22]</sup>. Liver biopsy or FibroScan is not required for make treatment decision but may be useful in patients who have elevated

DNA levels but normal ALT levels<sup>[22]</sup>. As the nature of chronic hepatitis B is dynamic, it is recommended that all patients undergo serial monitoring. Given the indices for the APRI score are routine laboratory test and will change with disease progression, this should prompt recalculation of the APRI score and re-staging of the disease by FibroScan or liver biopsy if deemed necessary.

The weakness of this study is the relatively small sample size of patients with liver biopsies. While liver biopsy has historically been considered the “gold standard” for assessment of liver fibrosis, it is imperfect with concerns with of sampling error due to patchy distribution of fibrosis, risk of complications and expense. It has now largely been replaced by non-invasive measures of fibrosis as first line/standard of care for fibrosis assessment. Consequently, the volume of liver biopsies performed in our centre and across most centres has fallen dramatically and it would no longer be considered to perform routine liver biopsies in patients with viral hepatitis. In this study, we only included patient with hepatitis B and C and the finding cannot be generalised to patients with other aetiology for their liver disease. Furthermore, differences exist between patients with hepatitis B and hepatitis C which may impact on their APRI score or FibroScan readings. High ALT levels in hepatitis B may lead to overestimation of fibrosis by FibroScan, whilst HCV-associated immune thrombocytopenia may falsely elevate the APRI score<sup>[23]</sup>. We also acknowledge that this is a retrospective study from a single centre. Intra- and inter-observer variation in histological interpretation was avoided with the use of a single pathologist who specializes in gastrointestinal pathology.

We, therefore, propose that the use of a new cut-off APRI score of 0.5 could potentially be used to predict the need for FibroScan in the evaluation of patients with viral hepatitis, which would result in significant reduction in health care cost and resources.

In the evaluation of patients with HCV or HBV, APRI score and FibroScan performed equally well in predicting advanced fibrosis. The use of APRI  $\geq 1.0$  to predict the need for FibroScan would miss 48% of patients with advanced fibrosis. In the current study, we found that an APRI cut off score of 0.5 is more reliable than 1.0, and able to predict the presence of advanced fibrosis in 100%. More importantly, the use of APRI score of 0.5 or more as a screening tool for advanced fibrosis can reduce the need for FibroScan in 43%. Larger prospective validation studies are warranted to confirm this finding.

## COMMENTS

### Background

FibroScan is a novel non-invasive method that identifies significant liver fibrosis and cirrhosis. Consequently, its use has greatly increased, posing a demand to the health care system. Aspartate aminotransferase (AST) to platelet ratio index (APRI) is a cheap, blood-test based scoring system that can predict liver fibrosis. Previous study suggested that a score of 1.0 has modest sensitivity and specificity in predicting cirrhosis. This study examined the relationship between the APRI and F-score in predicting advanced fibrosis related to viral hepatitis, and whether it can be used to predict the need of FibroScan.

### Research frontiers

The focus of this study is to examine the use of APRI score to predict the need for FibroScan assessment, thus, allowing a better stratification of need and demand of FibroScan in a busy hepatology centres.

### Innovations and breakthroughs

Using liver biopsy as gold standard, APRI score and FibroScan performed equally well in predicting advanced fibrosis. More important, the current study found that an APRI cut-off score of 0.5 can be used as a screening tool for FibroScan, as the previously proposed cut-off score of 1.0 missed up to 48% of patients with advanced fibrosis. The use of the newer APRI cut-off score of 0.5 resulted in the avoidance of needs for FibroScan assessment in 43% of referred patients.

### Applications

APRI, therefore, should be routinely used in clinical practice and can be used a guide to perform FibroScan. This practice is likely to be cost-effective and improve the work flow of the FibroScan service.

### Terminology

FibroScan is a novel non-invasive method that measures liver stiffness using both ultrasound and low-frequency elastic waves. AST to platelet ratio index (APRI) {calculated by [(AST level/ULN)/Platelet count ( $10^9/L$ )]  $\times 100$ } is a scoring system that can predict the presence of advanced fibrosis with good sensitivity and specificity.

### Peer-review

The manuscript is a retrospective study evaluated the performance of APRI score against FibroScan in predicting the presence of fibrosis and proposed a new-cut off score of APRI as a screening tool. This study provides a good concept and enhances utilization of APRI score.

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## Systemic treatment of hepatocellular carcinoma: Past, present and future

Esther Una Cidon

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### Abstract

Hepatocellular carcinoma (HCC) is a common neoplasia which represents the second leading cause of cancer related death. Most cases occur in developing countries, but its incidence is rising in Western countries due to

hepatitis C. Although hepatitis therapies have evolved and the HCC screening has increased in several areas, 40% present with advanced disease which is only amenable for palliative systemic treatment. HCC continues posing a challenge, in part due to the inherent chemoresistance of this neoplasia, the pharmacologic challenges due to an ill liver, difficulty in assessing radiological responses accurately, *etc.* Traditional chemotherapy have shown some responses without clear survival benefit, however, sorafenib demonstrated advantages in survival in advanced HCC when liver function is kept and recently immunotherapy seems to be a promising approach for some patients. This article will briefly expose the most relevant systemic treatment modalities to offer a general view from the past to the future.

**Key words:** Hepatocellular carcinoma; Alphafetoprotein; Sorafenib; Nivolumab; MEK

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**Core tip:** The incidence of hepatocellular carcinoma (HCC) is rising in Western countries due to hepatitis C. Unfortunately, 40% of patients present with advanced disease which is only amenable for palliative systemic treatment. The development of effective therapies for HCC is a challenge, due partly to its inherent chemoresistance, the pharmacologic challenges due to an ill liver, *etc.* Although some responses to traditional chemotherapy have been reported, the multikinase inhibitor sorafenib has shown survival benefit in advanced HCC with preserved liver function. Recently immunotherapy seems to be a promising approach for some patients.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is a hepatic neoplasia that occupies the second place as cause of cancer related deaths<sup>[1]</sup>. It appears most frequently in a liver with chronic injury and cirrhosis<sup>[2]</sup> and it is usually diagnosed as an advanced stage with a poor median survival rate (6-20 mo)<sup>[3]</sup>.

Its incidence varies depending on geographical zones and races. This is mainly related to differences in incidences of hepatitis B and C. The highest rates are seen in Asia (where hepatitis B incidence is very high) and Africa, though increasing in developed areas due to hepatitis C<sup>[4]</sup>. Other risk factors include steatohepatitis, alcoholic liver disease, aflatoxins and hemochromatosis.

Unfortunately 40% of diagnosis will present with an advanced disease with the only options of systemic therapy in most of them<sup>[5]</sup>. HCC nowadays continues to pose a significant challenge to the therapy, in part due to poor chemosensitivity (expression of drug resistance genes) and the liver dysfunction which hinders the delivery of these drugs. Moreover, cirrhosis will have an impact on the drug distribution volumes<sup>[6]</sup>.

Although newer treatments have appeared, the survival rates of advanced HCC patients have not yet significantly improved.

HCC is an aggressive tumour whose treatment possibilities will depend on the phase of the tumour, the liver functionality and patient's performance status. There are several staging systems available<sup>[7-9]</sup> but no consensus on which to use. The Child-Pugh system will assess the patient's hepatic reserve and liver function. Other staging systems, such as Barcelona Clinic Liver Cancer, will consider tumour phase, performance status, hepatic status, symptoms, etc. This system may provide the link between disease and treatment strategies. In very early/early stages, curative treatment (liver surgery or hepatic transplantation) and locoregional treatments (such as radiofrequency ablation), have better survival benefits.

Intermediate stage is very heterogeneous and transarterial chemoembolization/radioembolization are the main options if preserved hepatic function (Child-Pugh A) and performance status 0.

Advanced cases have got a short prognosis. For these patients, systemic palliative therapies might be considered.

This article will briefly expose the most relevant systemic treatment modalities to offer a general view from the past to the future.

## CYTOTOXIC CHEMOTHERAPY: MONOTHERAPY

HCC is poorly chemosensitive due to the expression of drug resistance genes, and the liver dysfunction which hinders the delivery of drugs. In the past years, no single treatment or regimen have shown superiority to another<sup>[10]</sup>.

Glutathione-S-transferase, topoisomerase II  $\alpha$ , p-glycoprotein, heat shock proteins, and p53<sup>[11-17]</sup> are related to chemotherapy sensitivity. Most published studies with chemotherapy have shown RRs of less than 25% and there is no evidence of improvement in OS<sup>[18-20]</sup>. However, chemotherapy may still be an option after progression on sorafenib if good performance status and preserved liver function.

Nagahama *et al*<sup>[21]</sup> carried out a study in 147 HCC patients in first line. Results showed that those cases affected by severe cirrhosis, tumour involving > 50% of the liver, ECOG performance 2-3 and tumour thrombus in the portal vein do not respond to chemotherapy.

Doxorubicin has been used since the 1970s. A study carried out in Africa enrolled 14 patients and found a 79% of responses<sup>[22]</sup>. However, posterior trials showed much less RR (10% to 20%)<sup>[23,24]</sup>.

It is not clear whether doxorubicin prolongs survival. A single study with 60 cases randomised to doxorubicin vs no treatment and it demonstrated a significant extension in survival (10.6 wk vs 7.5 wk,  $P = 0.036$ ) favouring doxorubicin<sup>[25]</sup>. Later a meta-analysis comparing doxorubicin to no treatment or other treatments did not find a survival benefit<sup>[26]</sup>. Another randomized study comparing doxorubicin against nolatrexed, found better survival with doxorubicin (32.3 wk vs 22.3 wk,  $P = 0.007$ ) but the authors concluded that results could be biased due to more patients failed to continue treatment with nolatrexed due to side-effects<sup>[27]</sup>.

Several phase II trials with other anthracyclines did not show any significant benefits over doxorubicin in outcomes or toxicity<sup>[28-31]</sup> (Table 1).

5-fluorouracil (5-FU) and other fluoropyrimidines have been used in HCC. 5-FU has undergone extensive evaluation in HCC and shown RRs in the range of 10%<sup>[32,33]</sup>. 5-FU bolus with leucovorin showed higher gastrointestinal adverse effects, and responses of 0%-28%<sup>[33,34]</sup>.

Capecitabine is a prodrug that is converted at the site of the tumour to 5-FU. Its toxicity profile appears to be more manageable<sup>[35]</sup>, but RRs remain relatively low<sup>[36]</sup>. A retrospective study by Patt *et al*<sup>[35]</sup> investigated the role capecitabine in 63 patients (37 HCC). Capecitabine in HCC showed a RR of 1% with an OS of around 10 mo. Most frequent adverse events included hand-foot syndrome and thrombocytopenia<sup>[35]</sup>. Jiang *et al*<sup>[37]</sup> have reported a high activity of dihydropyrimidine dehydrogenase in liver cancer. This could impact on the chemoresistance to these chemotherapy agents. In the adjuvant setting, Xia *et al*<sup>[38]</sup> carried out a randomized, controlled trial with capecitabine after HCC operation. Sixty patients were randomized to capecitabine or control. Results favoured the capecitabine arm with a lower recurrence rate (53.3% vs 76.7%), longer median time to recurrence (40 mo vs 20 mo,  $P = 0.046$ ) and higher 5-year OS (62.5% vs 39.8%,  $P = 0.216$ ) with tolerable side effects<sup>[38]</sup>.

Gemcitabine is another chemotherapy drug which appears to be very active *in vitro* (HCC cell lines). However, several clinical studies have shown limited activity<sup>[39]</sup>.

**Table 1 Doxorubicin as first line treatment in hepatocellular carcinoma**

Ref.	n	Line/treatment	Relevant data
Nagahama <i>et al</i> <sup>[21]</sup>	147	First line doxorubicin	Severe cirrhosis, PS 2-3, tumour occupying > 50% liver do not respond to chemo
Olweny <i>et al</i> <sup>[22]</sup>	14	First line doxorubicin	RR 79%
Sciarrino <i>et al</i> <sup>[23]</sup>		First line doxorubicin	RR 10%-20%
Chlebowski <i>et al</i> <sup>[24]</sup>			

RR: Response rate.

Only one small study (28 patients) reported by Yang *et al*<sup>[40]</sup> showed a RR of 17%. The subsequent trials have only shown RRs of 0%-2%<sup>[41,42]</sup>. Cisplatin is a platinum analog that has demonstrated a 15% of responses as monotherapy<sup>[43]</sup>.

## CYTOTOXIC CHEMOTHERAPY: COMBINATION

In an attempt to increase the rate of clinical benefits, several combinations of chemotherapy have been studied but to date none has proven superiority when compared with single agents. This is very important as combinations are more toxic and thus clinicians should weigh the toxicity against any added palliative benefit they hope to get.

The EACH is a phase III, open-label study comparing FOLFOX4 (infusional FU, leucovorin, oxaliplatin) vs doxorubicin in 371 patients with advanced HCC. FOLFOX4 showed a higher RR (8.15% vs 2.67%,  $P = 0.02$ ), disease control rate (DCR) (52.17% vs 31.55%,  $P < 0.001$ ), longer PFS (2.93 mo vs 1.7 mo,  $P = 0.001$ ; HR = 0.62) and OS (6.40 mo vs 4.97 mo, HR = 0.80;  $P = 0.07$ )<sup>[44]</sup>.

Shin *et al*<sup>[45]</sup> reported a trial of cisplatin combined with capecitabine and doxorubicin in 25 patients. They found a RR of 26% and around 1/3 of patients showed a significant reduction in alfa-fetoprotein (AFP) levels, though this reduction is not a reliable marker for clinical benefit. This study mentioned toxicity only briefly with one treatment-related death. Lee *et al*<sup>[46]</sup> carried out a study with the combination of cisplatin and doxorubicin. This phase II trial showed responses in the line of 19%, with around 1/3 of the patients having a significant reduction of AFP. Significant neutropenia was reported in 14.3%.

Combinations of platinum derivatives and gemcitabine seem to be more effective with tolerable adverse events if hepatic function is acceptable. Gemcitabine and oxaliplatin have shown responses of 15%-20% and stabilizations of 48%-58% in small studies<sup>[47,48]</sup>.

A retrospective study in 204 patients with advanced HCC treated with a combination of gemcitabine and oxaliplatin (GEMOX) was reported in 2011 ASCO meeting. Fifty-one percent had Child Pugh A, 20.6% Child Pugh B, and 4.4% Child Pugh C. The results showed a RR of 22% and DCR of 66%. PFS, TTP and OS of 4.5, 8 and 11 mo. Authors found that if an objective response was seen, OS was higher (19.9 mo vs 8.5 mo). Grade 3/4 toxicity occurred in 44.1% and most frequent adverse events were diarrhoea, neutropenia, thrombocytopenia

and neuropathy<sup>[48]</sup>. In addition, 8.5% became candidates for curative treatments thanks to responses. Moreover, the response to GEMOX, among other factors, was independently associated to OS.

Patrikidou *et al*<sup>[49]</sup> carried out a retrospective study of GEMOX as second line. Forty patients were included after failure of one anti-angiogenic treatment minimum. Severe adverse events were found 25% of the cases. Partial response was observed in 20% of patients, while 46% had stable disease.

Median OS was 8.3 mo and survival rate at 6 mo was 59%. Median PFS was 3.1 mo. Performance status, baseline AFP levels and BCLC score were independently associated with OS. Another study has demonstrated RR of 21% with cisplatin and gemcitabine but with 1/3 of the patients suffering from severe neutropenia and 1/4 significant thrombocytopenia<sup>[50]</sup>. Another trial with cisplatin, 5-FU and mitoxantrone found RR of 27% with 71% patients with severe neutropenia<sup>[51]</sup>.

Docetaxel plus gemcitabine showed a 10% RR and unacceptable hematologic toxicity<sup>[52]</sup>. Irinotecan has shown minimal effectiveness with significant adverse events, so its use is not advisable<sup>[53,54]</sup> (Table 2).

## HORMONAL THERAPY

As there is a significant male predominance in morbidity and mortality in HCC, it has long been considered that sex hormones play a role in its development. Some HCCs express estrogen receptors (ER) and estrogens have shown some protective effects against HCC.

Tamoxifen, a competitive antagonist of the estrogen receptors, have been studied in several clinical trials to assess its activity against HCC but only a little benefit in response or survival has been found<sup>[55,56]</sup>.

Megestrol acetate blocks wildtype and variant forms of ERs and it has been assessed in HCC with variant ER. Benefits varied according to trials. Whereas some of them showed some benefits, a study of megestrol acetate vs placebo as first line of advanced HCC did not prolong OS<sup>[57-60]</sup>.

Octreotide is a somatostatin analogue and around 40% of hepatic carcinomas express these receptors. Octreotide has shown direct antitumor effect in HCC<sup>[61,62]</sup>. Several studies have shown different benefits but a metaanalysis showed survival rates at 6 and 12 mo higher than those seen in the other arms, though only in Eastern studies<sup>[63]</sup>. However, these results are still controversial.



**Table 2 Clinical trials with chemotherapy agents in hepatocellular carcinoma**

Ref.	n	Treatment	Results
Lai <i>et al</i> <sup>[25]</sup>	60	Doxorubin <i>vs</i> placebo	OS 10.6 wk <i>vs</i> 7.5 wk in favour of chemo
Gish <i>et al</i> <sup>[27]</sup>		Doxorubicin <i>vs</i> nolatrexed	OS 32.3 wk <i>vs</i> 22.3 wk in favour of doxorubicin
Patt <i>et al</i> <sup>[35]</sup>	37	Capecitabine	RR 1%, OS 10.1 mo
Qin <i>et al</i> <sup>[44]</sup>	371	FOLFOX 4 <i>vs</i> doxorubicin	RR 8.15% <i>vs</i> 2.67% DCR 52.17% <i>vs</i> 31.55% PFS 2.93 m <i>vs</i> 1.7 m OS 6.4 m <i>vs</i> 4.97 m All in favour of FOLFOX 4
Shin <i>et al</i> <sup>[45]</sup>		Cisplatin, Capecitabine and Doxorubicin	RR 26%
Lee <i>et al</i> <sup>[46]</sup>		Cisplatin/doxorubicin	RR 19%
Zaanan <i>et al</i> <sup>[48]</sup>	204	GEMOX	RR 22% DCR 66% PFS 4.5 m OS 11 m
Patrikidou <i>et al</i> <sup>[49]</sup>	40	GEMOX after antiangiogenics failed	Partial responses 20% Stable disease 46% OS 8.3 m
Yang <i>et al</i> <sup>[50]</sup>		Cisplatin/gemcitabine	RR 21%
Kim <i>et al</i> <sup>[52]</sup>		Cisplatin/infusional FU/mitoxantrone	RR 27% but 71% severe neutropenia

RR: Response rate; DCR: Disease control rate; PFS: Progression free survival; OS: Overall survival.

## MOLECULARLY TARGETED THERAPY

Carcinogenesis is a complex process involving multiple signalling cascades. Sorafenib is a small inhibitor of several tyrosine protein kinases (TKI), such as VEGFR, platelet derived growth factor receptor (PDGFR) and Raf family kinases. It will inhibit growth of multiple kinases related to angiogenesis, cell proliferation and differentiation<sup>[64,65]</sup>. In preclinical studies, sorafenib has shown antiproliferative effects in HCC cell lines. It also decreased tumour angiogenesis and tumour-cell signalling, increasing apoptosis in a mouse model<sup>[65]</sup>.

Abou-Alfa *et al*<sup>[66]</sup> carried out an uncontrolled phase II study with sorafenib in advanced HCC and Child-Pugh A or B. Results favoured sorafenib with OS of 9.2 mo and a TTP 5.5 mo.

A large phase III, multicenter, randomized, double-blind, placebo controlled trial (SHARP trial) was undertaken in advanced HCC. Six hundred and two patients naïve for treatment, were randomized to sorafenib or placebo. This study showed an OS of 10.7 mo *vs* 7.9 mo in favour of sorafenib, with a hazard ratio of 0.69; 95%CI: 0.55 to 0.87;  $P < 0.001$ ). Both groups were similar in the median time to symptomatic progression (4.1 mo *vs* 4.9 mo,  $P = 0.77$ ).

Two percent of partial responses were seen in patients with sorafenib and 1% in the placebo; overall toxicity was similar between the treatment and placebo arm (52% *vs* 54%), though diarrhoea, hand-foot syndrome, weight loss and hypophosphatemia were more prominent with sorafenib.

Another phase III placebo controlled trial was carried out in Asian patients (Oriental study). Two hundred and twenty-six patients with Child-Pugh A cirrhosis and no prior systemic treatment were randomized to sorafenib or placebo. Sorafenib showed significantly longer median OS (6.5 mo *vs* 4.2 mo) and median TTP (2.8 mo *vs* 1.4 mo)<sup>[67]</sup>.

Sorafenib in combination with chemotherapy has been examined. A study compared doxorubicin with sorafenib

*vs* doxorubicin alone<sup>[68]</sup>. The combination prolonged median TTP (6.4 mo *vs* 2.8 mo,  $P = 0.02$ ), PFS (6.0 mo *vs* 2.7 mo,  $P = 0.006$ ) and median OS (13.7 mo *vs* 6.5 mo,  $P = 0.006$ )<sup>[68]</sup>. CALGB80802 study<sup>[69]</sup> recruited patients with advanced HCC, naïve for palliative treatment and Child-Pugh A. The patients received either doxorubicin 60 mg/m<sup>2</sup> every three weeks plus sorafenib or sorafenib monotherapy. After 346 patients the study was halted. An interim analysis reported that the combination arm produced higher toxicity and did not improve OS<sup>[69]</sup>. Other studies were designed to evaluate the combination of GEMOX regimen and sorafenib. A randomized, controlled, phase II trial (GOTEXT), compared sorafenib and GEMOX combined with sorafenib as first-line treatment. Ninety-four patients were randomized. The results showed that RRs, DCRs, PFS and median OS were 9% *vs* 70%, 16% *vs* 77%, 54% *vs* 61%, and 13 mo *vs* 13.5 mo, respectively, favouring the combination<sup>[70]</sup>.

Sorafenib combined with oxaliplatin has shown good activity in phase II trials but requires further investigation in larger randomized clinical trials. Regorafenib is a multi-kinase inhibitor which has shown activity against HCC. Bruix *et al*<sup>[71]</sup> carried out a study, open-label, phase II, multicenter, to assess safety and efficacy of regorafenib in patients diagnosed with advanced HCC after failure with sorafenib. Thirty-six patients were included and disease control was achieved in 26 with one partial response. TTP and OS of 4.3 and 13.8 mo respectively and a tolerable safety profile. Most frequent side effects were fatigue, hand-foot syndrome and diarrhoea.

The phase III trial (RESOURCE, NCT01774344) showed a benefit for regorafenib with longer median progression-free survival (3.1 mo *vs* 1.5 mo) compared to placebo. OS (primary end point) was 10.6 mo *vs* 7.8 mo in favour of regorafenib. Overall, authors found that 65.2% of patients on regorafenib showed complete/partial response or stable disease, compared to 36.1% in the placebo group. Side effects were similar to those reported with sorafenib namely hypertension, hand-foot skin reaction, fatigue and

diarrhea<sup>[72]</sup>.

Cabozantinib is a multiple receptor tyrosine kinases inhibitor, including HGF receptor [mesenchymal-epithelial transition (MET)], Ret, and the VEGF receptor. A phase II trial which included 41 patients with HCC has shown promising results<sup>[73]</sup>. These patients had Child-Pugh A and had progressed to a previous systemic therapy. Patients on cabozantinib showed 5% of partial responses, 78% stable disease, and 7% progressive disease, with a median OS of 15.1 mo and median PFS of 4.4 mo, regardless of previous treatment with sorafenib. Most frequent side-effects grade 3 or higher were diarrhea, palmar-plantar erythrodysesthesia, and thrombocytopenia.

A multinational phase III clinical trial, CELESTIAL, has been planned to recruit 760 patients with advanced HCC after progression on sorafenib. Patients will receive cabozantinib daily or placebo (randomization 2:1). The trial is expected to show data in 2017<sup>[74,75]</sup>. The endpoints are OS (primary), RR and PFS.

Lenvatinib is a multitargeted (VEGFR, PDGFR, RET, FGFR and KIT) tyrosine kinase inhibitor. The recommended dose was 12 mg daily in Child-Pugh A (5-6 score) and 8 mg in Child-Pugh B (7-8 score)<sup>[76]</sup>.

A phase II clinical trial, multicenter, evaluated lenvatinib in advanced HCC. Patients receive 12 mg once daily in 28-d cycles. The primary endpoint was TTP. Forty-six patients were included in Japan and South Korea showing TTP of 7.4 mo (95 %CI: 5.5-9.4).

Thirty-seven percent had partial response and 41% stable disease (DCR 78%). Median OS was 18.7 mo (95%CI: 12.7-25.1). Frequent adverse events such as hypertension (> 75%), palmo-plantar syndrome (> 60%), reduced appetite (> 60%) and proteinuria (> 60%). Dose reductions in 74% and treatment was stopped in 22%, due to adverse effects. Authors found that median body weight was lower in patients with an early (< 30 d) dose withdrawal or reduction.

This study concluded that lenvatinib shows clinical activity with acceptable toxicity but early dose modification is needed if low body weight. Further studies should consider this<sup>[77]</sup>.

The pivotal Phase III REFLECT trial comparing lenvatinib to sorafenib has been completed, and its results will determine whether lenvatinib represents another potential option. A clinical trial of lenvatinib vs sorafenib in naïve patients will recruit 1000 patients with unresectable HCC and its completion is estimated for later this year<sup>[78]</sup>.

Tivantinib is a selective small MET tyrosine kinase inhibitor with antitumor activity, especially in MET-high patients. Its activity is due to a disruption of microtubules<sup>[79]</sup>. An initial study in 20 patients with Child-Pugh A or B<sup>[80]</sup> found that most relevant side-effects were fatigue (> 1/2), anorexia, alopecia and diarrhoea (15% each). Serious neutropenia (38%) and anaemia (24%) were seen, which implies that a careful haematological monitoring is needed during the treatment.

A phase II randomised trial in second line has been carried out. Patients were stratified by circulating levels of MET, hepatocyte growth factor and levels of alpha-

fetoprotein. Circulating levels of MET were related to prognosis as OS was 4.6 mo in high levels vs 8.9 mo if low (HR = 0.61;  $P = 0.023$ ). If low MET tumours, TTP, OS or DCR did not show differences.

This trial found relevant toxicities such as grade 3 anemia (9%), neutropenia (6%) and thrombocytopenia (6%). This led to a dose recommendation of 240 mg BID for second-line.

MET expression was also correlated with sorafenib as 40% of biopsies taken prior to sorafenib therapy were MET-high compared with 82% after sorafenib. A significant interaction in OS between tivantinib and MET expression was reported ( $P = 0.039$ ). The other biomarkers examined were not predictive of tivantinib response<sup>[81]</sup>.

A phase III, randomized, double-blind trial in second line, after progression on sorafenib is ongoing in HCC patients with high-expression of MET. The endpoints include OS (primary), PFS and safety. The anticipated study completion date is mid-2017<sup>[81-83]</sup>.

Ramucirumab is a fully human monoclonal anti-VEGFR-2 antibody. It binds to the receptor with high affinity and prevents ligand activation. HCC has got high expression levels of VEGF which entails worse results<sup>[84]</sup>. REACH is a randomized, double-blind trial, in HCC patients refractory or not amenable to locoregional treatments who had failed to sorafenib. OS, which was the primary endpoint, was not significantly different with ramucirumab or placebo (9.2 mo vs 7.6 mo; HR = 0.87; 95%CI: 0.72-1.05;  $P = 0.14$ ). On the contrary PFS was improved as objective RR. Regarding toxicity, most common side effects grade 3 or above were ascites, hypertension, asthenia, and increased aspartate aminotransferase<sup>[85]</sup>. When patients were stratified by AFP, OS benefited ramucirumab if AFP > 400 ng/mL (7.8 mo vs 4.2 mo; HR = 0.67; 95%CI: 0.51-0.90;  $P = 0.006$ ). These results suggested that patients with elevated AFP might be more likely to benefit from ramucirumab. A prospective phase III trial, REACH 2, whose completion is estimated for late 2017, will assess the safety and efficacy of ramucirumab as second-line in patients with elevated baseline AFP<sup>[85]</sup>.

Apatinib is a small-molecule multi-kinase inhibitor of VEGFR-2. Qin *et al*<sup>[86]</sup> carried out a phase II dose-finding study in naïve patients with HCC Child-Pugh A. These patients were randomised to apatinib 850 mg/qd or 750 mg/qd. Endpoints TTP (primary), OS, RR, DCR, level of AFP and safety. One hundred and twenty-one patients were recruited. The results showed a median TTP of 4.2 and 3.3 mo for the two different dosages respectively. DCR was 48.57% and 37.25% respectively. Median OS was 9.7 and 9.8 mo respectively. The authors concluded that apatinib produced a survival benefit and both doses were recommended for further study<sup>[86]</sup>.

Most frequent adverse effects were elevated levels of bilirubin, aminotransferase, blood pressure, thrombocytopenia, leukocytopenia, palmo-plantar erythrodysesthesia, fatigue, but most of them were easily managed by dose interruptions or reductions.

A phase 1/phase 2 trial of apatinib for advanced HCC

**Table 3** Clinical trials with tyrosine kinase inhibitors in hepatocellular carcinoma

Ref.	n	Treatment	Results
Abou-Alfa <i>et al</i> <sup>[66]</sup>		Sorafenib	OS 9.2 m TTP 5.5 m
Cheng <i>et al</i> <sup>[67]</sup>	602	Sorafenib <i>vs</i> placebo	OS 6.5 m <i>vs</i> 4.2 m
	226	Sorafenib <i>vs</i> placebo	TTP 2.8 m <i>vs</i> 1.4 m
Abou-Alfa <i>et al</i> <sup>[68]</sup>		Sorafenib <i>vs</i> doxorubicin	TTP 6.4 m <i>vs</i> 2.8 m PFS 6 m <i>vs</i> 2.7 m OS 13.7 m <i>vs</i> 6.5 m
Assenat <i>et al</i> <sup>[70]</sup>	94	Sorafenib <i>vs</i> sorafenib/GEMOX	RR 9% <i>vs</i> 70% DCR 16% <i>vs</i> 77% PFS 54% <i>vs</i> 61% OS 13 m <i>vs</i> 13.5 m In favour of the combination
Bruix <i>et al</i> <sup>[71]</sup>	36	Regorafenib second line	DCR in 26/36 patients Partial response 1/36 TTP 4.3 m OS 13.8 m
LBA-03 <sup>[72]</sup>		Regorafenib <i>vs</i> placebo	DCR 65.2% <i>vs</i> 36.1% PFS 3.1 m <i>vs</i> 1.5 m OS 10.6 m <i>vs</i> 7.8 m
Verslype <i>et al</i> <sup>[73]</sup>	41	Cabozantinib	Partial response 5% Stable disease 78% PFS 4.4 m OS 15.1 m
Exelixis <sup>[74,75]</sup>	760	Cabozantinib second line (after sorafenib)	Primary end point OS Expected data in 2017
Koyama <i>et al</i> <sup>[76]</sup>	46	Lenvatinib	DCR 78% TTP 7.4 m OS 18.7 m
Eli Lilly and Company <sup>[85]</sup>		Ramucirumab <i>vs</i> placebo	OS 9.2 m <i>vs</i> 7.6 m
Qin <i>et al</i> <sup>[86]</sup>	121	Apatinib <i>vs</i> placebo	TTP 4.2 m <i>vs</i> 3.3 m DCR 48.57% <i>vs</i> 37.25% OS 9.7 m <i>vs</i> 9.8 m

RR: Response rate; DCR: Disease control rate; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression.

after first-line treatment failure (NCT02772029) will be soon recruiting patients. A multicenter, randomised, double blind phase III trial (NCT02329860) was started in December 2014, aiming to assess its activity and toxicity profile after progression on sorafenib and/or chemotherapy. It has planned to recruit 360 patients (randomized 2:1). Primary endpoint is OS. This trial is still ongoing. See all the results in Table 3.

## IMMUNOTHERAPY

Recently tumor immunotherapy has evolved rapidly. As most HCC are driven by inflammation, there is a strong rationale to evaluate immunotherapy in these patients.

### Pembrolizumab

The single-arm, multisite, phase 2 KEYNOTE-224 study (ClinicalTrials.gov, NCT02702414) was designed to assess the activity and toxicity pembrolizumab in patients with previously treated advanced HCC. This trial plans to recruit 100 patients. The primary end point will be objective RR.

Another single-arm phase II trial of Pembrolizumab in patients with advanced, unresectable HCC is ongoing. Endpoints are DCR (primary), PFS, OS, RR, duration of response and toxicity. Researchers will assess the

expression levels of programmed death-ligand 1 (PD-L1) in tumor tissue, and serum titers of hepatitis B or C in patients with hepatitis B or C, respectively, for whom specimens are available.

### Nivolumab

Several tumours express PD-1, among them HCC and this is related with poor prognosis. The union PD-1/PD-L1 block the T cell receptor signal transduction, inhibit proliferation and induce depletion of T cells achieving tumour immune escape. Blocking the PD-1 pathway will promote an antitumoral immune response<sup>[87]</sup>. Nivolumab is an anti-PD-1 antibody<sup>[88]</sup>.

A phase I / II study (Interim analysis of the Check-Mate-040 dose escalation study) in advanced HCC was reported at the 2015 ASCO annual meeting.

Patients with advanced HCC, Child-Pugh  $\leq 7$ , who had failed, declined, or did not tolerate sorafenib were included. Patients had nivolumab 0.1-10 mg/kg every two weeks for a maximum of 2 years. Three parallel cohorts were made depending on hepatitis: No active infection, hepatitis B, hepatitis C. Endpoints were safety (primary), efficacy and RR. Biomarkers assessment was included as an exploratory endpoint.

Fifty-one patients were included. Seventy-three percent of them had prior sorafenib. Twenty-nine percent

**Table 4 Clinical trials with immunotherapy in hepatocellular carcinoma**

Authors	n	Phase	Treatment	Primary end-point
Keynote-224	100	II	Pembrolizumab	RR
ongoing		II	Pembrolizumab	DCR
CheckMate-040		I / II	Nivolumab	Safety
CheckMate-459	726	III	Nivolumab <i>vs</i> Sorafenib	OS TTP

RR: Response rate; DCR: Disease control rate; OS: Overall survival; TTP: Time to progression.

had response or stable disease and most common adverse effects were rash and AST increase. Responses were seen regardless PD-L1 status evaluated by IHC.

Authors concluded that nivolumab showed manageable toxicity with long duration responses or stabilizations regardless dosage or cohorts<sup>[89-91]</sup>. CheckMate-040 shows that nivolumab is effective with acceptable toxicity in HCC, regardless hepatitis status.

Another phase III study, CheckMate-459, (NCT02576509) has planned to recruit 726 patients to assess nivolumab compared to sorafenib as first line. Endpoints will be OS, TTP (as primary), RR, PFS, expression of PD-L1 and efficacy. The stratification will observe geographical area, etiology, vascular invasion and extrahepatic dissemination. It is planned to be finished by May 2017.

### Tremelimumab

It is a humanized anti T-lymphocyte-associated antigen-4 (CTLA-4) IgG2 antibody which has shown good results in the treatment of 21 patients with hepatitis C<sup>[92]</sup>. RR of 18% and DCR of 76%, with TTP of 6.48 mo<sup>[93]</sup> were seen.

Transarterial chemoembolization and radiofrequency ablation can also trigger immune activity against HCC and potentiate the anti-CTLA-4 activity<sup>[94]</sup>.

Twenty patients were included and Duffy *et al*<sup>[94]</sup> presented the results in ASCO 2015. Disease free survival was 16 mo and median PFS 7.4 mo. Forty percent of patients treated with transarterial chemoembolization/radiofrequency ablation showed partial response and 5 out of 7 patients with hepatitis C had a significant reduction in viral load. Most frequent side effect was itching and only 1 patient stopped due to pneumonitis. These authors found evidence of immune cells infiltration in tumour biopsies taken at 6 mo. As clinical activity was encouraging, tremelimumab combined with transarterial chemoembolization/radiofrequency ablation has been considered for further investigation<sup>[94]</sup> (Table 4).

### MEK inhibitors

A relevant signalling pathway in hepatocarcinogenesis is the MEK cascade. This is involved in cellular adaptation and survival. A key role is played by MEK, with MEK 1/2 as interesting targets for new drugs.

Refametinib is an oral MEK inhibitor which has been combined with sorafenib in a phase II trial<sup>[95]</sup>. The RR

6.2% and DCR 43%, with a median OS of 9.6 mo. The best response was seen in RAS mutated group. Unfortunately, the rate of grades 3 and 4 side-effects was 80% and 4 patients died due to liver failure, hepatic encephalopathy, tumour lysis syndrome and unknown reason.

Another phase II<sup>[96]</sup> of refametinib alone or combined with sorafenib in HCC with mutant RAS was carried out. Patients with HCC, unresectable, Child-Pugh A, no prior systemic therapy for HCC (except prior sorafenib in monotherapy study) were eligible. Patients in the monotherapy trial were treated with refametinib 50 mg bid, while in the combination they were treated with refametinib 50 mg bid and sorafenib 400 mg bid.

Four hundred and ninety-eight patients in the monotherapy and 820 patients in the combination were enrolled. Median PFS was 58 d, median time to radiological progression 84 d, and median OS 177 d. In the combination study no patients achieved a confirmed partial response, median PFS was 46 d, TTP 84 d, and median OS 427 d<sup>[96]</sup>. Authors concluded that either monotherapy or combination did not show sufficient efficacy to warrant further development in this group of patients.

Some other some small molecule c-MET inhibitors, such as foretinib<sup>[97]</sup> as first line or tepotinib<sup>[98]</sup> particularly in C-MET positive tumours, have shown promising activity with high safety profile. The most common side effects were hypertension, fever and anorexia. Capmatinib<sup>[99]</sup>, golvatinib<sup>[100]</sup>, and others are also under study<sup>[101]</sup>.

## CONCLUSION

HCC is one of the most frequent worldwide neoplasias and although many efforts have been made to get a prompt detection, many cases are still diagnosed in an advanced stage no amenable to radical treatments. The treatment of an advanced HCC is still challenging and although there are many trials under way to evaluate new drugs targeting different molecular pathways relevant in hepatocarcinogenesis, much knowledge remains still in early stages. Sorafenib improved survival but sorafenib resistance is still a significant issue and several clinical trials assessing other new molecular targeted agents have failed. Regorafenib and lenvatinib showed promising activity in phase II clinical trials and are undergoing evaluation in phase III. Immunotherapy has recently emerged as a promising therapy for many cancers including HCC. Nivolumab has shown benefits and awaits trials to confirm these positive results. Tremelimumab open the door to combination with locoregional treatments and it has also shown a reduction in tumour viral load in hepatitis C<sup>[100]</sup>.

The efforts will continue and hopefully will soon pay off.

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## Conventional *vs* drug-eluting beads transarterial chemoembolization for hepatocellular carcinoma

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### Abstract

Transarterial chemoembolization (TACE) is the current standard of therapy for patients with intermediate-stage hepatocellular carcinoma (HCC) according to the Barcelona Clinic Liver Cancer classification. The concept of conventional TACE (cTACE) is the selective obstruction

of tumor-feeding artery by injection of chemotherapeutic agents, leading to ischemic necrosis of the target tumor *via* cytotoxic and ischemic effects. Drug-eluting beads (DEBs) have been imposed as novel drug-delivering agents for TACE, which allows for higher concentrations of drugs within the target tumor and lower systemic concentrations compared with cTACE. Despite the theoretical advantages of DEB-TACE, it is still controversial in clinical practice as to whether DEB-TACE is superior to cTACE in regard to overall survival and treatment response. In this review article, we summarize the clinical efficacy and safety of DEB-TACE for patients with intermediate or advanced stage HCC in comparison with cTACE.

**Key words:** Hepatocellular carcinoma; Drug-eluting beads transarterial chemoembolization; Transarterial chemoembolization

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**Core tip:** Transarterial chemoembolization (TACE) is the current standard of therapy for patients with intermediate-stage hepatocellular carcinoma. Drug-eluting beads (DEBs) have been introduced as novel drug-delivery agents for TACE, allowing for higher concentrations of drugs to the target tumor and lower systemic concentrations, compared with conventional TACE (cTACE). Despite the theoretical advantages of DEB-TACE, whether DEB-TACE shows superior efficacy to cTACE remains controversial. Reviewing the literature, we found that DEB-TACE shows similar clinical outcomes to and fewer adverse events than cTACE.

Song JE, Kim DY. Conventional *vs* drug-eluting beads transarterial chemoembolization for hepatocellular carcinoma. *World J Hepatol* 2017; 9(18): 808-814 Available from: URL: <http://www.wjnet.com/1948-5182/full/v9/i18/808.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i18.808>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is listed as the sixth most common cancer worldwide and the third most frequent cause of cancer-related mortality<sup>[1,2]</sup>. The majority of HCC cases occur stem from chronic liver disease and cirrhosis. Therefore, the selection of treatment modalities for HCC is determined by tumor size, multiplicity, and liver function status<sup>[3]</sup>.

Transarterial chemoembolization (TACE) has been frequently performed and has become the first-line treatment option for large or multinodular HCC with preserved liver function, no evidence of vascular invasion or extrahepatic spread, and the absence of cancer-related symptoms, which is defined as intermediate stage according to the Barcelona Clinic Liver Cancer (BCLC) classification<sup>[4,5]</sup>. Moreover, in clinical settings, TACE is considered the standard treatment for patients with early stage HCC who are not appropriate candidates for curative therapy<sup>[4]</sup>.

The mechanism of action for conventional TACE (cTACE) is the selective obstruction of tumor-feeding arteries by injection of chemotherapeutic agents (doxorubicin or cisplatin) mixed with lipiodol<sup>[6]</sup>. This leads to ischemic necrosis of target tumors by cytotoxic and ischemic effects. There are several drawbacks of cTACE that are associated with ineffective treatment responses in many cases: (1) the liquid motility of lipiodol to reduce the effective concentrations of chemotherapeutic agents; (2) the inability to release drugs in a controlled and sustained manner; and (3) heterogeneity in the technique and treatment schedules. To reduce these drawbacks, drug-eluting beads (DEBs) have been introduced as drug-delivering agents for TACE. After delivery of the beads to target tumors, the beads release chemotherapeutic drugs (doxorubicin or epirubicin) in a sustained fashion over a prolonged period of time<sup>[7,8]</sup>. Figure 1 shows the mechanism of action of DEB-TACE. Treatment with DEB-TACE allows higher concentrations of drugs within the target tumor and lower systemic concentrations compared with cTACE<sup>[9,10]</sup>. Thus, the use of DEBs can reduce drug-related adverse events such as post-embolization syndrome. As DEB-TACE is widely used interchangeably with cTACE in many hospitals globally, it is necessary to assess the current status of DEB-TACE in comparison with cTACE. Thus, this article aims to evaluate the characteristics of each modality and to compare the clinical outcomes of DEB-TACE with those for cTACE.

## cTACE VS DEB-TACE: CHARACTERISTICS

### cTACE

Typically, cTACE involves the infusion of chemotherapeutic drugs blended with lipiodol and embolic agents into the cancer-feeding artery<sup>[6]</sup>. Both single chemotherapeutic agents and combination chemotherapy have been used as part of the drug regimen in TACE. However, there is no agreement on the optimal anticancer drug(s)

to be used in cTACE. Globally, the most widely used chemotherapeutic agent for TACE of HCC is doxorubicin. The dose of doxorubicin generally ranges from 30 to 75 mg/m<sup>2</sup>, at a maximum of 150 mg emulsified in 5 to 20 mL of lipiodol<sup>[11]</sup>.

Lipiodol is a key element in TACE due to its distinctive combination of features as a drug-carrying, tumor-seeking, and embolizing agent<sup>[12]</sup>. Even though the principle is not concretely comprehended, it seems to be absorbed by a pump in the cancer cell wall and transported to the intracellular space. Then, upon hypoxia within cancer cells, this pump is disabled, such that lipiodol is retained within the cell. Lipiodol is confined to tumors when injected *via* the hepatic artery, and it is generally trapped in HCC for months, even up to a year, whereas it is washed out from non-tumor portions of the liver within 4 wk.

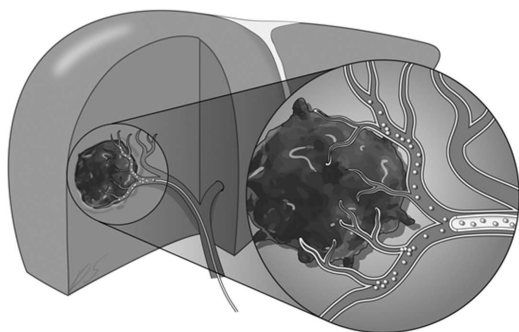
Several embolic agents, such as gelfoam, polyvinyl alcohol (PVA) particles, and tris-acryl gelatin microspheres, have been used over the past three decades in chemoembolization<sup>[12]</sup>. Among these embolic agents, gelfoam has recently emerged the most commonly used substance worldwide. The intended aim of embolization is as follows: To assist lipiodol to be sustained selectively in the tumor, to inhibit chemotherapeutic agent washout from HCC, and to cause ischemic necrosis.

A significant problem of cTACE is the great inhomogeneity of the technique and treatment schedules used in clinical centers worldwide. Two randomized controlled trials on cTACE used quite different technical approaches. Furthermore, some HCCs do not show lipiodol uptake which may result in lower effectiveness of the treatment<sup>[13]</sup>.

### DEB-TACE

The most commonly used DEB, DC Beads (BTG, United Kingdom) are nonbiodegradable PVA microspheres, loaded with calibrated doxorubicin. They can release doxorubicin in a controlled and maintained mode<sup>[14]</sup>. Through an ion-exchange mechanism, DC Beads actively sequester oppositely charged drugs. In initial *in vitro* studies, doxorubicin could be efficiently loaded into the DC beads up to 45 mg/mL, regardless of the size of beads<sup>[15]</sup>. Currently, a loading of 37.5 mg doxorubicin/mL beads is recommended, in consideration of a practical therapeutic dose and optimum handling characteristics. According to an animal pharmacokinetic study comparing two sizes of doxorubicin-eluting beads (100-300  $\mu$ m and 700-900  $\mu$ m) loaded with same amount of doxorubicin, treatment with the smaller beads (100-300  $\mu$ m) elicited higher doxorubicin plasma levels<sup>[16]</sup>. This finding was caused by the increased surface area of the smaller beads, leading to a profuse release of doxorubicin.

In DEB-TACE, the extent of the liver cancer burden should be considered in planning the dose of doxorubicin. As a general rule, for patients within the Milan criteria (defined as single tumor  $\leq$  5 cm, or multiple tumors of up to three and  $<$  3 cm each), a planned dose should be up to 75 mg doxorubicin loaded into one vial, including



**Figure 1** Action mechanism of drug-eluting bead-transarterial chemoembolization in hepatocellular carcinoma. Sustained release of chemotherapeutic agents from microbeads of uniform size, which embolize supplying vessels more distally, enables local concentration of cytotoxic agents to be higher within tumor.

2 mL of DC beads in each single treatment. Meanwhile, for patients beyond the Milan criteria, each treatment should involve a dose up to 150 mg loaded into two vials of DC beads<sup>[17]</sup>. Generally, the recommended size of beads is 100–300  $\mu\text{m}$  for standard DEB-TACE procedures. This choice is based on the fact that small particles can be transported inside the tumor or in nearness to the tumor margin, and thus they are ideal for drug delivery or accurate embolization.

## cTACE VS DEB-TACE: OVERALL EFFICACY AND SAFETY IN INTERMEDIATE AND ADVANCED STAGE HCC

The survival benefit of cTACE has been the issue of a finite number of randomized controlled trials (RCTs) that have provided controversial results<sup>[18]</sup>. Among seven RCTs<sup>[19–25]</sup> all published between 1988 and 2002, only two trials showed favorable results in respect of overall survival<sup>[19,20]</sup>. However, a systematic review based on these seven RCTs showed that cTACE has been found to improve 2-year survival (OR = 0.53; 95%CI: 0.32–0.89;  $P = 0.017$ ) of patients with unresectable HCC, compared with best supportive care<sup>[26]</sup>. Subsequent sensitivity analysis in this study showed a significant survival benefit for chemoembolization with cisplatin or doxorubicin by analyzing 323 patients in four studies (OR = 0.42; 95%CI: 0.20–0.88), but not for embolization alone by assessing 215 patients in three studies (OR = 0.59; 95%CI: 0.29–1.20)<sup>[26]</sup>. In a current Cochrane review, the evidence based survival benefits of cTACE was challenged<sup>[27]</sup>. This meta-analysis involved RCTs published after 2002 and showed no solid evidence to support TACE or transarterial embolization (TAE) compared with conservative management, in patients with unresectable HCC. However, some experts have doubted such conclusions, because this review involved RCTs with inappropriate selection of patients and control arms, which likely biased the results of the analysis.

Primarily, DEB-TACE has been introduced to enhance the ability of drug-delivery to target tumor while reducing systemic toxicity and to provide a standardized embolic effect. The role of doxorubicin in embolic microspheres was evaluated in a randomized, cancer-size adjusted trial assessing DEB-TACE vs TAE with similar characteristics (BeadBlock-TAE)<sup>[28]</sup>. Although no survival benefit was reported in the study, the value of doxorubicin was favorable in the setting of TACE with microspheres, because DEB-TACE showed higher local response, less recurrence at 12 mo, and a longer time-to-progression than BeadBloc-TAE. Another trial assessed the rate of tumor necrosis after chemoembolization with epirubicin-loaded beads vs TAE with unloaded microspheres (Embosphere particles), which was pathologically proved in explanted livers of HCC patients undergoing liver transplantation: Epirubicin-loaded beads TACE showed complete necrosis in 77% of lesions, while TAE showed complete necrosis in only 27% of lesions ( $P = 0.043$ )<sup>[29]</sup>. A recently reported prospective clinical trial of DEB-TACE in a large Korean HCC population showed an overall 6-mo survival rate was 97.4%, although more than half of patients had early stage HCC (BCLC-A,  $n = 77$ , 50.7%)<sup>[30]</sup>. Varela *et al.*<sup>[7]</sup> firstly reported that systemic concentrations of doxorubicin were significantly lower in patients treated with DEB-TACE than patients treated with cTACE. This result was verified by Poon *et al.*<sup>[14]</sup>, who performed DEB-TACE with possibly the highest dose of doxorubicin (150 mg). Both studies showed that none of treated patients exhibited doxorubicin-related systemic toxicity (alopecia, bone marrow suppression, or dyspnea)<sup>[7,14]</sup>.

Despite the aforementioned theoretical advantages of DEB-TACE, previous studies comparing DEB-TACE with cTACE in HCC of intermediate stage have shown rather conflicting results. Recently reported meta-analysis showed that the two modalities represent comparable results, suggesting an absence of difference in tumor response between DEB-TACE and cTACE<sup>[31]</sup>. On the contrary, three other meta-analyses, assessing the efficacy of DEB-TACE vs cTACE in HCC patients, showed different results<sup>[32–34]</sup>. Huang *et al.*<sup>[32]</sup> (seven studies,  $n = 700$ ) and Xie *et al.*<sup>[33]</sup> (six studies,  $n = 652$ ) demonstrated that significantly better objective tumor response was found for DEB-TACE than for cTACE. In another meta-analysis of nine studies (866 patients) conducted in 2016, DEB-TACE presented significantly higher complete response rate and better overall survival, although similar objective tumor responses compared with cTACE. Regarding adverse events in these meta-analyses<sup>[32–34]</sup>, overall and severe adverse events were similar or slightly lower in patients receiving DEB-TACE than patients receiving cTACE. Tables 1 and 2 summarize the clinical outcomes and adverse events of the studies that were included in these meta-analyses comparing DEB-TACE and cTACE.

Among randomized controlled trials reported until recently, the largest trial is the PRECISION V phase-2 trial assessing DEB-TACE vs cTACE in 212 patients with mostly HCC of intermediate stage<sup>[10]</sup>. The primary

**Table 1 Clinical outcomes from studies comparing drug-eluting bead-transarterial chemoembolization and conventional transarterial chemoembolization in patients with intermediate-stage hepatocellular carcinoma**

Ref.	Study design	Arm	BCLC stage <i>n</i> (A/B/C)	Clinical outcomes in intermediate-stage (BCLC-B) (DEB-TACE/cTACE)					
				OS rate	<i>P</i> value	TTP	<i>P</i> value	Response rate	<i>P</i> value
Lammer <i>et al</i> <sup>[10]</sup>	RCT	DEB-TACE	24/69/0	NR		NR		OR 52.4%/34.7% <sup>2</sup>	0.038
		cTACE	29/79/0					DC 63.5%/44.4% <sup>2</sup>	0.026
Wiggenermann <i>et al</i> <sup>[46]</sup>	Retrospective	DEB-TACE	1/17/3	70%/55% (1-yr survival rate)	0.01	NR		OR 22.7%/22.7% <sup>3</sup>	
		cTACE	4/15/2					DC 90.9%/68.2% <sup>3</sup>	0.066
Song <i>et al</i> <sup>[9]</sup>	Retrospective	DEB-TACE	27/33/0	DEB > cTACE (log-rank test)	0.020	DEB > cTACE (log-rank test)	0.038	OR 75.6%/34.1% <sup>4</sup>	< 0.001
		cTACE	28/41/0						
Golfieri <i>et al</i> <sup>[35]</sup>	RCT	DEB-TACE	41/26/22	NR		NR		CR 19.2%/26.1% <sup>5</sup>	0.734
		cTACE	41/23/24					CR 42.1%/22.2% <sup>6</sup>	0.295

<sup>1</sup>In this study, subgroup analysis according to BCLC stage was not performed. However, majority of patients was BCLC-B (DEB-TACE, 81%; cTACE, 71%);

<sup>2</sup>The 6-mo tumor response rate, according to the European Association for the Study of the Liver response criteria; <sup>3</sup>The average 8-mo tumor response rate, according to the EASL response criteria; <sup>4</sup>The 3-mo tumor response rate, according to the mRECIST; <sup>5</sup>The 1-mo; <sup>6</sup>The 6-mo tumor response rate, according to the EASL criteria and mRECIST. RCT: Randomized controlled trial; OS: Overall survival; TTP: Time to progression; NR: Not reported; OR: Objective response; DC: Disease control; CR: Complete response; mRECIST: Modified Response Evaluation Criteria in Solid Tumors; BCLC: Barcelona Clinic Liver Cancer; TACE: Transarterial chemoembolization; DEB: Drug-eluting bead.

**Table 2 The incidence of adverse events from studies comparing drug-eluting bead-transarterial chemoembolization and conventional transarterial chemoembolization in patients with unresectable hepatocellular carcinoma**

Adverse event	Lammer <i>et al</i> <sup>[10]</sup>	Wiggenermann <i>et al</i> <sup>[46]</sup>	Song <i>et al</i> <sup>[9]</sup>	Golfieri <i>et al</i> <sup>[35]</sup>
Nausea	Post-embolization syndrome	Post-embolization syndrome	Post-embolization syndrome	2.2%/3.4%, <i>P</i> = 0.682
Pain	24.7%/25.9%	21.7%/16.3%, <i>P</i> = 0.52	22.2%/20.6%, <i>P</i> = 0.850	24.7%/71.6%, <i>P</i> = 0.01
Fever				7.9%/11.4%, <i>P</i> = 0.457
Fatigue				0%/4.5%, <i>P</i> = 0.059
Marrow suppression	5.4%/5.6%	NR	NR	NR
Cholecystitis	NR	NR	4.7%/3.3%, <i>P</i> = 0.692	2.2%/1.1%, <i>P</i> = 0.999
Abscess	NR	<sup>2</sup>	NR	1.1%/1.1%, <i>P</i> = 0.999
Alopecia	1.1%/20.4%	NR	NR	NR
Liver function worsening	Significant reduction in DEB <sup>1</sup>	NR	AST, 36%/52%, <i>P</i> = 0.259 ALT, 31%/20%, <i>P</i> = 0.280	1.1%/5.7% <sup>3</sup> , <i>P</i> = 0.118
Hematoma	NR	NR	NR	1.1%/3.4%, <i>P</i> = 0.368
Infection	NR	NR	NR	0%/1.1%, <i>P</i> = 0.497

<sup>1</sup>The mean maximum ALT elevation in the DEB-TACE group was 50% less than in the cTACE group (95%CI: 39%-65%; *P* < 0.001) and 41% less with regard to AST (95%CI: 46%-76%; *P* ≤ 0.001); <sup>2</sup>Major complications was defined hospitalization > 24 h, greater therapy and unplanned added costs in treatment, permanent persisting sequelae and death of the patient. DEB-TACE *vs* cTACE, 13.0% (*n* = 6, including 2 liver abscesses) *vs* 2.3% (*n* = 1), *P* = 0.06; <sup>3</sup>Increase in Child-Pugh score of ≥ 2 points. DEB-TACE: Drug-eluting bead-transarterial chemoembolization; cTACE: Conventional transarterial chemoembolization; ALT: Alanine aminotransferase; AST: Aspartate transaminase; NR: Not reported.

efficacy endpoint (response at 6 mo, *P* = 0.11) and primary safety endpoint (incidence of severe adverse events within 30 d, *P* = 0.86) were comparable in both two groups. After performing a post hoc comparison, the DEB-TACE group indicated a significant decrease in chemotherapeutic agent-related systemic and liver toxicity compared to the cTACE group. Furthermore, in subgroup analysis, the objective response rate and disease control rate were significantly better (*P* = 0.038 and *P* = 0.026, respectively) with DEB-TACE than with cTACE in 67% of patients with more advanced disease (Child-Pugh B, bilobar or recurrent disease, ECOG 1). Another RCT for evaluating the potential effect of DEB-TACE on overall survival, compared to cTACE using epirubicin, showed no statistical differences between both modalities in terms of survival, treatment response, or adverse episodes<sup>[35]</sup>. However, it should be considered that the maximally used dose of doxorubicin/epirubicin was limited to only 75 mg for both procedures in this trial.

Furthermore, the trial mainly recruited patients with low tumor burden (46% of patients with early HCC, only 20% patients with bilobar disease). Thus, this restricted one of the significant advantages of DEB-TACE, which is the ability to use higher doxorubicin doses without rising drug-related systemic toxicity in patients with larger tumor burden as mentioned in the PRECISION V study. This trial indicated that DEB-TACE did not show better clinical outcomes, compared with cTACE in patients with relatively well preserved liver function and low tumor burden. A retrospective study by Song *et al*<sup>[9]</sup> reported that overall survival and treatment responses for DEB-TACE were significantly better than those for cTACE. Performing subgroup analysis in accordance with BCLC stage, treatment efficacy was shown in intermediate stage HCC (BCLC B) but not in early stage HCC (BCLC A). Regarding adverse events, there was no statistically significant difference between DEB-TACE and cTACE. On the contrary, a recently published retrospective study



showed that overall survival, time to progression, and disease control rate were not significantly different between DEB-TACE and cTACE groups, even when subgrouped by BCLC stage<sup>[36]</sup>. However, the incidence of adverse events was significantly lower, particularly in HCC larger than 5 cm in BCLC-B patients receiving DEB-TACE<sup>[36]</sup>. Considering the results from these studies, there is still controversy regarding clinical outcomes. However, it seems that DEB-TACE shows at least similar efficacy and less adverse events than cTACE. DEB-TACE might be favorable to cTACE for large HCC especially in patients with decreased liver function, even though there is lack of evidence that DEB-TACE is superior to cTACE in term of efficacy.

For advanced HCC (BCLC C), the role of chemoembolization has not been fully established. In accordance with the BCLC staging system, it recommends systemic treatment or palliative therapy to patients with advanced stage. In a small retrospective study comparing cTACE and sorafenib in patients with advanced HCC, overall survival in the cTACE group was higher than the sorafenib group (9.2 mo vs 7.4 mo,  $P = 0.377$ )<sup>[37]</sup>. Recently, two studies on DEB-TACE for patients with advanced HCC were reported: Kalva *et al.*<sup>[38]</sup> conducted a retrospective trial recruiting 80 patients with advanced HCC treated with DEB-TACE. This study reported median progression free survival of 5.1 mo (95%CI: 4.1-7.7) and overall survival of 13.3 mo (95%CI: 10.1-18.6). Subgroup analysis showed that median survival was better in patients with ECOG performance status (PS)  $\leq 1$  than ECOG PS  $> 1$  (17.7 mo vs 5.6 mo,  $P = 0.025$ , respectively). Another retrospective study by Prajapati *et al.*<sup>[39]</sup> reported median survival of 13.5 mo (range, 8.2-18.7 mo) without severe adverse episodes. Subgroup analyses showed that the median survival of Child-Pugh class A patients was 17.8 mo (range, 9.0-26.7 mo). In comparison with median survivals of 10.7 mo and 6.5 mo for sorafenib in the SHARP and Asia-Pacific trials<sup>[40,41]</sup>, it appears that cTACE as well as DEB-TACE shows better or at least comparable efficacy in patients with advanced stage HCC, Child-Pugh class A and good performance status.

A major limitation of TACE is a high rate of cancer recurrence. In two RCTs, a sustained response lasting for 3 to 6 mo was reported in only 28% to 35% of patients treated with cTACE<sup>[19,20]</sup>. Recently, several trials made an attempt to analyze the potential benefit of combined strategies with chemoembolization and other treatment options for overcoming this limitation. Several RCTs have sought to determine the benefit of an addition of sorafenib to cTACE or DEB-TACE in patients with more advanced HCC. The rationale for this concept is grounded in the demonstration that TACE causes hypoxia and induce angiogenesis by activating angiogenic factors and that the use of sorafenib could decrease angiogenesis. However, these RCTs have not proved definite improvement of clinical outcomes in combination therapy of sorafenib and chemoembolization, compared with chemoembolization alone<sup>[42,43]</sup>. Recently, trials have been conducted on combination of TACE with other

molecular target agents, such as brivanib, sunitinib, and thalidomide. It is hoped that these ongoing trials will contribute to the determination of optimal combinations.

## CONTROVERSIAL ISSUES ON cTACE VS DEB-TACE

Apart from the overall comparison of clinical outcomes between conventional and DEB-TACE, it is still controversial as to whether DEB-TACE is superior to cTACE in large HCC ( $\geq 5$  cm), which frequently suffers from incomplete response or recurrence after cTACE<sup>[44]</sup>. Considering that liver damage given by DEB-TACE is less than that by cTACE, it might be assumed that DEB-TACE offers more therapeutic advantages over cTACE in large HCC. However, regarding response to procedures, complete response rates at 1 and 6 mo were lower in HCC larger than 5 cm, compared with HCC less than 2 cm or 2-5 cm in size<sup>[30]</sup>. Moreover, in a Korean retrospective study, there was no significant difference in survival between cTACE and DEB-TACE in HCC larger than 5 cm (36.3 mo vs 33.4 mo,  $P = 0.702$ )<sup>[36]</sup>. Therefore, the notion that a big tumor is more appropriate for DEB-TACE than for cTACE is not currently accepted. Paradoxically, small HCC (less than 2 cm) is sometimes difficult to achieve complete response to both cTACE and DEB-TACE, because the tumor vascularity is fine. In particular, unlike lipiodol in cTACE, the diameter of microspheres in DEB-TACE is still too wide to block peripheral hepatic arteries. Accordingly, the outcomes of small HCC ( $< 2$  cm) treated with DEB-TACE, compared to cTACE are controversial. Indeed, the time to progression after DEB-TACE was shorter than after cTACE in HCC  $< 2$  cm (10.3 mo vs 13.8 mo,  $P = 0.023$ ), although there was no difference in overall survival between the two modalities<sup>[36]</sup>. Lastly, repeated sessions of a procedure could be another distinguishing advantage or disadvantage between cTACE and DEB-TACE. The severity of hepatic arterial damage has been compared between cTACE and DEB-TACE in a retrospective study. After a single session of cTACE or DEB-TACE, the incidence of hepatic arterial damage was significantly higher for DEB-TACE group than cTACE, with doxorubicin dose being a possible risk factor for such damage<sup>[45]</sup>.

## CONCLUSION

In comparison with cTACE, DEB-TACE facilitates higher concentrations of drugs within the target tumor and lower systemic concentrations. Despite the theoretical advantages of DEB-TACE, it is still controversial in several clinical studies as to whether DEB-TACE is superior to cTACE in terms of efficacy. However, it seems that DEB-TACE shows at least similar clinical outcomes and less adverse events than cTACE. In order to gain better results for these treatment modalities, selecting proper candidate patients for DEB-TACE or cTACE is needed. Moreover, further well-defined studies are required to identify combination strategies and to develop better

treatment approaches for patients with advanced HCC.

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Retrospective Cohort Study

# Risk factors for acute kidney injury after partial hepatectomy

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## Abstract

### AIM

To identify risk factors for the occurrence of acute kidney injury (AKI) in the postoperative period of partial hepatectomies.

### METHODS

Retrospective analysis of 446 consecutive resections in 405 patients, analyzing clinical characteristics, pre-operative laboratory data, intraoperative data, and postoperative laboratory data and clinical evolution. Adopting the International Club of Ascites criteria for the definition of AKI, potential predictors of AKI by logistic regression were identified.

### RESULTS

Of the total 446 partial liver resections, postoperative AKI occurred in 80 cases (17.9%). Identified predictors of AKI were: Non-dialytic chronic kidney injury (CKI), biliary obstruction, the Model for End-Stage Liver Disease (MELD) score, the extent of hepatic resection, the occurrence of intraoperative hemodynamic instability, post-hepatectomy haemorrhage, and postoperative sepsis.

### CONCLUSION

The MELD score, the presence of non-dialytic CKI



and biliary obstruction in the preoperative period, and perioperative hemodynamics instability, bleeding, and sepsis are risk factors for the occurrence of AKI in patients that underwent partial hepatectomy.

**Key words:** Kidney injury; Hepatectomy; Postoperative; Liver; Resection

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**Core tip:** Acute kidney injury (AKI) is a serious complication after partial hepatectomy. This research aims to identify risk factors for the occurrence of AKI in the postoperative period of partial hepatectomies. The Model for End-Stage Liver Disease score, the presence of non-dialytic chronic kidney injury and biliary obstruction in the preoperative period, and perioperative hemodynamics instability, bleeding, and sepsis are risk factors for the occurrence of AKI in patients that underwent partial hepatectomy.

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## INTRODUCTION

Despite of the limited data regarding the occurrence of acute kidney injury (AKI) after partial hepatectomy, the reported incidence ranges from 0.9% to 15.1%<sup>[1-4]</sup>. A comprehensive analysis of the scarce data<sup>[5]</sup> is also hampered by the lack of consensus in the exact definition of AKI after liver resection.

Candidates for liver resections often present with multiple potential risk factors regarding postoperative AKI, such as excessive bleeding during the hepatectomy, and the occurrence of post-hepatectomy liver failure (PLF)<sup>[2,3,5-7]</sup>. Eventually, patients can have a combination of insults, that can be aggravated by distributive circulatory derangements by sepsis<sup>[2,3,5-8]</sup> or exposure to nephrotoxic drugs<sup>[9]</sup>.

The hemodynamic changes in patients after major liver resections, mainly in patients with underlying chronic liver injury, may simulate those of patients with acute liver failure or cirrhosis<sup>[10]</sup>. Thus, the current criteria suggested by the International Club of Ascites (ICA) for definition of AKI would be the most appropriate criteria for these patients<sup>[11]</sup>, since urine output measurement and static serum creatinine (sCr) levels are not included in ICA criteria.

Assuming post-operative AKI as primary endpoint, the aim of the present report was to identify the risk factors for the occurrence of this serious complication after partial hepatectomies.

## MATERIALS AND METHODS

This report is based on a historical cohort study of patients

who underwent partial hepatectomy from January 2008 to July 2016 at the Hepatobiliary Surgery Department of Cancer Hospital-UOPECCAN. Patients with evidence of dialytic chronic renal dialysis at the time of surgery, the need of emergency hepatectomy or patients who died at the intraoperative or immediate postoperative period (within the first 24 h after the procedure) were excluded. The study was approved by the Research Ethics Board at West Parana University (No. 1.665.135; July 2016), and the need for informed written consent was waived. The study was conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

### Preoperative data

The data collected included: Patient demographic data, preoperative use of nonsteroidal anti-inflammatory drugs, angiotensin-converting enzyme and inhibitors, the presence of comorbidities including: Non-dialytic chronic kidney disease (CKI), defined as estimated glomerular filtration rate (eGFR) less than 60 mL/min per 1.73 m<sup>2</sup><sup>[12]</sup>, liver cirrhosis with Model End- Liver Disease (MELD) score calculation<sup>[13]</sup>, biliary obstruction and prior exposure to chemotherapy.

Preoperative baseline laboratory tests values were obtained from the patient electronic charts in the previous 3 mo, and in patients with more than one value, the value closest to the hospital admission date were selected. Laboratory tests included: Serum dosages of urea, creatinine, sodium, potassium, bilirubin, and albumin, International Normalized Ratio value, serum platelet count and eGFR value calculation according to the formula<sup>[14]</sup>:

$$\text{eGFR: mL/min per 1.73 m}^2 = k \times 186 \times (\text{sCr})^{-1.15} \times (\text{age})^{-0.203},$$

$$K = 1 \text{ (if male) or } 0.72 \text{ (if female)}$$

### Intraoperative and surgical data

The surgical and anesthetic covariates recorded were: Open or laparoscopic resection, extent of liver resection (major hepatectomy was defined as resection of at least three Couinaud liver segments), resection modalities according to Brisbane nomenclature<sup>[15]</sup>, type of vascular clamping of the liver (intermittent Pringle maneuver<sup>[16]</sup>, continuous Pringle maneuver<sup>[17]</sup> or total vascular exclusion<sup>[18]</sup>), segment I resection, two-stage resection<sup>[19]</sup>, associated extrahepatic resection, complex vascular reconstruction (portal vein, hepatic artery or hepatic veins, with or without prothesis), regional lymphadenectomy (hepatic pedicle lymph nodes<sup>[20]</sup>), intraoperative transfusions of red blood cells, and intraoperative hemodynamic instability, defined as a sustained systolic blood pressure less than 90 mmHg or more than 40 mmHg below the patient's usual systolic blood pressure during 30 min.

### Postoperative data and complications

Similarly to the preoperative laboratory blood tests, we retrieved its values in the postoperative period, including the most altered values in the first 30 postoperative days.

Postoperative complications the first 30 postoperative

**Table 1 Postoperative overall complications and acute kidney injury staging according to International Club of Ascites<sup>[11]</sup>, risk, injury, failure, loss, end-stage<sup>[27]</sup> and Acute Kidney Injury Network<sup>[28]</sup> criteria (*n* = 446) *n* (%)**

Overall complications	113 (25.3)
Overall complications (Clavien-Dindo classification)	
I	46 (10.3)
II	25 (5.6)
III a/b	18 (4.0)
IV a/b	7 (1.6)
V (death)	17 (3.8)
AKI (ICA)	80 (17.9)
I	26 (5.8)
II	21 (4.7)
III	33 (7.4)
AKI (RIFLE)	70 (15.7)
Risk	16 (3.6)
Injury	21 (4.7)
Failure	33 (7.4)
AKI (AKIN)	80 (17.9)
I	26 (5.8)
II	21 (4.7)
III	32 (7.2)
HRS	11 (2.5)
RRT (hemodialyses)	9 (2.0)

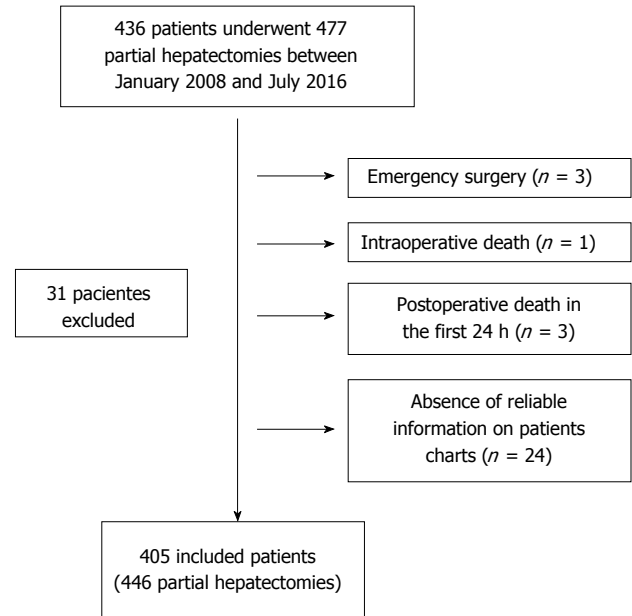
AKI: Acute kidney injury; ICA: International Club of Ascites; RIFLE: Risk, injury, failure, loss, end-stage; AKIN: Acute Kidney Injury Network; HRS: Hepatorenal syndrome; RRT: Renal replacement therapy.

days recorded were: Post-hepatectomy haemorrhage (PHH)<sup>[21]</sup>, post-hepatectomy liver failure (PHLF)<sup>[22]</sup>, biliary fistula<sup>[23]</sup>, postoperative ascites, wound infection<sup>[24]</sup>, pulmonary complications, including pulmonary infection<sup>[25]</sup>, acute respiratory distress syndrome and acute lung injury<sup>[26]</sup>, cardiovascular complications, including coronary insufficiency, cardiac arrhythmias, peripheral thrombosis, thromboembolism, and stroke<sup>[5]</sup>.

The occurrence and staging of AKI were defined according to the ICA<sup>[11]</sup> criteria, although the RIFLE<sup>[27]</sup> and AKIN<sup>[28]</sup> criteria were used for comparative purposes (Table 1). The use of aminoglycosides, renal replacement therapy (hemodialysis), the occurrence of hepatorenal syndrome (HRS)<sup>[11]</sup> and hospitalization time in days were recorded. The overall complications were classified according to Clavien-Dindo classification for postoperative complications<sup>[29]</sup>.

### Statistical analysis

To ensure the stability of our multivariate model, the sample size of the study was determined based on the results of a historical cohort not published in our Hepatobiliary Surgery Department, with an incidence of ARF after partial hepatectomies fixed at 18%, ensuring the adequate number of events per variable<sup>[30]</sup>. Categorical variables were expressed in absolute numbers and percentages were compared by the  $\chi^2$  test or Fisher's exact test when indicated. Continuous variables were expressed as absolute and mean  $\pm$  SD, and the comparison by the Student's *t*-test or non-parametric Mann-Whitney test after checking the normality assumptions by the Shapiro-

**Figure 1** Flow chart outlining the included and excluded patients in the study.

Wilk test. The variables selected in the univariate model ( $P < 0.05$ ) were tested in the multiple logistic regression model to identify independent binary predictors on the occurrence of postoperative AKI. The results of the model were expressed by means of the odds ratio, together with the corresponding 95% CIs and the *p* values of the Wald test. A value of  $P < 0.05$  (two-tailed) was considered significant. Statistical calculations were made with the software GPower 3.0.10 and SPSS 16.0 package for Windows.

## RESULTS

During the period from January 2008 to July 2016, 436 patients underwent liver resection surgery, of which 31 patients were excluded, with 405 included patients in the study for the final analysis (Figure 1).

Of the total of included patients, 271 underwent minor partial hepatectomies (60.7%) and 175 patients (39.3%) underwent major resections, and the most common resection modalities according to Brisbane nomenclature<sup>[14]</sup> were bisegmentectomy in 105 patients, segmentectomy in 103 patients, right hepatectomy in 85 patients, non-anatomical resections in 63 patients and left hepatectomy in 45 patients. The segment I were resected in 31 patients.

The most common indications for partial hepatectomy in patients with malignant tumors were colorectal cancer metastases 183 patients (41%) and hepatocellular carcinoma in 75 patients (16.8%), and patients with benign tumors were hepatic adenoma in 35 patients (7.8%) and hepatic hemangioma in 15 patients (3.4%).

Table 2 shows the clinical data of the patients prior the 466 partial hepatectomies according to the occurrence of AKI. It is observed that in the AKI group the prevalence

**Table 2** Preoperative patient characteristics according to the occurrence of postoperative acute kidney injury in 466 partial hepatectomies *n* (%)

	No AKI ( <i>n</i> = 366)	AKI ( <i>n</i> = 80)	<i>P</i>
Gender, male	180 (49.2)	43 (53.8)	0.269
Age (years), mean (SD)	54.6 (16.57)	57.4 (16.10)	0.842
ACE inhibitors	19 (5.2)	10 (12.5)	0.210
NSAIDs	26 (7.1)	10 (12.5)	0.082
Non-dialytic CKI	1 (0.27)	8 (10.0)	< 0.001
Diabetes mellitus	44 (12.0)	14 (17.5)	0.121
Systemic arterial hypertension	70 (19.1)	15 (18.8)	0.467
Preoperative chemotherapy	85 (23.2)	24 (30.0)	0.402
Cirrhosis	23 (6.3)	10 (12.5)	0.042
MELD score, mean (SD)	7.67 (1.15)	8.05 (1.05)	0.020
Biliary obstruction	6 (1.6)	13 (16.2)	< 0.001
Baseline laboratory tests			
Serum urea (mg/dL), mean ± SD	31.45 ± 10.71	35.63 ± 23.77	0.021
Serum creatinine (mg/dL), mean ± SD	0.90 ± 0.71	0.98 ± 0.62	0.229
eGFR (mL/min per square meter), mean ± SD	98.38 ± 51.32	89.86 ± 35.46	0.944
Sodium (mEq/L), mean ± SD	135.67 ± 3.25	134.25 ± 3.00	0.350
Potassium (mEq/L), mean ± SD	4.44 ± 0.63	4.34 ± 0.75	0.697
INR, mean ± SD	1.13 ± 0.45	1.14 ± 0.20	0.912
Bilirubin (mg/dL), mean ± SD	1.63 ± 3.05	2.84 ± 3.95	0.002
Albumin (g/dL), mean ± SD	3.61 ± 0.87	3.41 ± 0.92	0.505
Platelets (mm <sup>3</sup> ), mean ± SD	211869.55 ± 103744.67	215522.81 ± 115186.57	0.129

AKI: Acute kidney injury; ACE: Angiotensin conversion enzyme; NSAIDs: Non-steroidal anti-inflammatory drugs; CKI: Chronic kidney injury; MELD: Model for End-Stage Liver Disease; INR: International normalized ratio.

**Table 3** Intraoperative characteristics of the 446 liver resections according to the occurrence of postoperative acute kidney injury *n* (%)

	No AKI ( <i>n</i> = 366)	AKI ( <i>n</i> = 80)	<i>P</i>
Surgical approach			0.071
Open	343 (93.7)	79 (98.8)	
Laparoscopic	23 (6.3)	1 (1.2)	
Extention of resection			0.002
Major resection	128 (35.2)	45 (56.2)	
Minor resection	238 (64.8)	35 (43.8)	
Tumoral histology			0.134
Benign	70 (19.1)	6 (7.5)	
Malignant	296 (80.9)	74 (92.5)	
Segment I resection	23 (6.3)	8 (10.0)	0.098
Two-stage hepatectomy	26 (7.1)	6 (7.5)	0.439
Intermittent Pringle maneuver (15' \ 5')	124 (33.9)	31 (38.8)	0.438
Continuous Pringle maneuver	26 (7.1)	10 (12.5)	0.254
Total vascular exclusion	6 (1.6)	2 (2.5)	0.212
Complex vascular reconstruction	6 (1.6)	1 (1.2)	0.634
Regional lymphadenectomy	86 (23.5)	25 (31.2)	0.155
Associated extrahepatic resection	27 (7.4)	10 (12.5)	0.103
Intraoperative instability	26 (7.1)	25 (31.2)	< 0.001
Red blood cell transfusion	31 (8.5)	23 (28.8)	< 0.001

AKI: Acute kidney injury.

of non-dialytic CKI and cirrhosis were higher, as well as higher MELD scores and biliary obstruction prior to partial hepatectomy. Regarding preoperative laboratory tests, the AKI group had higher bilirubin levels than non-AKI group,  $2.84 \pm 3.95$  mg/dL vs  $1.63 \pm 3.05$  mg/dL, respectively.

Overall and renal postoperative complications rates are shown in Table 1. A total of 113 patients (25.3%) presented some type of complication, and according to

the Dindo-Clavien scale, the complications grade I were the most common, occurring in 46 patients (10.3%). According to ICA criteria, AKI occurred in 80 patients (17.9%), as well as by the AKIN criteria. A slight difference in the incidence of AKI was observed according to RIFLE criteria (15.7%).

Regarding surgical and intraoperative information, patients with AKI underwent more extensive surgical procedures (major hepatectomies), and especially, had significantly higher rates of hemodynamic instability and red blood cell transfusion during liver resections than non-AKI patients, 31.2% vs 7.1% and 28.8% vs 8.5%, respectively, with  $P < 0.001$  for both variables (Table 3).

According to the postoperative laboratory tests (Table 4), patients with AKI had significantly higher levels of urea and creatinine after surgery, with a significant lower eGFR,  $53.73 \pm 34.38$  mL/min per square meter vs  $83.24 \pm 60.04$  mL/min per square meter ( $P < 0.001$ ).

In the postoperative evolution, patients with AKI had higher rates of IHPH (25%), PHH (11.2%), sepsis (16.2%) and longer hospital stay ( $12.20 \pm 9.41$  d) (Table 4). According to the univariate model (Table 5), six covariates were statistically more frequent in the AKI group and the six were confirmed in the multiple logistic regression model as predictors: MELD score, the presence of biliary obstruction and non-dialytic CKI in the preoperative period, intraoperative hemodynamic instability, and finally PHH and sepsis in the postoperative period.

## DISCUSSION

This study aimed to identify the main risk factors for AKI in the postoperative period of partial hepatectomies.

**Table 4** Postoperative laboratory tests values and complications after 466 partial hepatectomies according to the occurrence of postoperative acute kidney injury *n* (%)

	No AKI ( <i>n</i> = 366)	AKI ( <i>n</i> = 80)	<i>P</i>
Laboratory tests			
Serum urea (mg/dL), mean ± SD	47.61 ± 49.36	82.19 ± 77.45	< 0.001
Serum creatinine (mg/dL), mean ± SD	1.29 ± 1.16	2.29 ± 2.21	< 0.001
eGFR (ml/min/m <sup>2</sup> ), mean ± SD	83.24 ± 60.04	53.73 ± 34.38	< 0.001
Sodium (mEq/L), mean ± SD	132.88 ± 4.27	132.29 ± 5.55	0.385
Potassium (mEq/L), mean ± SD	4.86 ± 0.81	5.16 ± 0.94	0.013
INR, mean ± SD	1.82 ± 2.46	2.08 ± 1.161	0.438
Bilirubin (mg/dL), mean ± SD	3.46 ± 4.54	4.54 ± 6.84	0.001
Albumin (g/dL), mean ± SD	2.58 ± 0.62	2.36 ± 0.59	0.069
Platelets (mm <sup>3</sup> ), mean ± SD	144101.93 ± 120446.829	132906.89 ± 113193.18	0.518
Aminoglycosides	7 (1.9)	3 (3.8)	0.341
PHLF	7 (1.9)	21 (26.3)	< 0.001
A	4 (1.1)	3 (3.8)	
B	3 (0.8)	10 (12.5)	
C	0 (0)	8 (10.0)	
PHH	1 (0.3)	9 (11.3)	< 0.001
A	0 (0)	2 (2.5)	
B	0 (0)	4 (5.0)	
C	1 (0.3)	3 (3.8)	
Biliary fistula	25 (6.8)	10 (12.5)	0.086
A	15 (4.1)	6 (7.5)	
B	7 (1.9)	3 (3.8)	
C	3 (0.8)	1 (1.2)	
Postoperative ascites	58 (15.9)	23 (28.8)	0.059
Wound infection	13 (3.6)	7 (8.8)	0.062
Pulmonary complications	15 (4.1)	6 (7.5)	0.177
Cardiovascular complications	7 (1.9)	2 (2.5)	0.501
Sepsis	2 (0.5)	13 (16.2)	< 0.001
Hospital stay (d), mean ± SD	6.68 ± 3.65	12.20 ± 9.41	0.008

AKI: Acute kidney injury; eGFR: Estimated glomerular filtration rate; PHLF: Post-hepatectomy liver failure; PHH: Post-hepatectomy haemorrhage.

**Table 5** Univariate and logistic regression analyses of risk factors for acute kidney injury

Univariate analyses	Multiple logistic regression				
	<i>P</i>	OR	95%CI	<i>P</i>	
Extent of resection	0.002	2.249	1.217	4.156	0.010
Biliary obstruction	< 0.001	10.240	3.094	33.891	< 0.001
Hemodynamics instability	< 0.001	5.244	1.337	20.568	0.017
Red blood cell transfusion	< 0.001				0.244
Cirrhosis	0.042				0.241
MELD score	0.020	4.342	1.347	15.654	0.046
Sepsis	< 0.001	11.609	3.185	39.911	< 0.001
Posthepatectomy haemorrhage	< 0.001	12.652	7.769	53.612	< 0.001
CKI	< 0.001	8.975	1.533	44.675	0.022

AKI: Acute kidney injury; OR: Odds ratio; MELD: Model for End-Stage Liver Disease; CKI: Chronic kidney injury.

There is a certain disparity of the available criteria for postoperative AKI definition in these situations, thus, we adopted the current criteria suggested by the ICA<sup>[11]</sup> for definition of AKI in cirrhotic patients. In patients eligible for partial hepatectomy with underlying liver diseases or who underwent major liver resections, often the both, the ICA criteria<sup>[11]</sup> do not include unreal measurements for these patients, such as static sCr measurements and urine output.

The incidence of AKI in the present study according to ICA and AKIN criteria was 17.9%, and according to RIFLE criteria was 15.7%. These AKI incidence were higher than other publications on the subject<sup>[1-5]</sup>. The

AKIN and RIFLE criteria were applied for comparison, and this slight underestimation of AKI by RIFLE criteria can be probably explained by the fact that the ICA and AKIN criteria consider as stage I AKI a small increase of 0.3 mg/dL in sCr.

Including AKI, the overall complication rate in this study was 25.3%, and the mortality rate was 3.8%, that is comparable to the results of two large retrospective studies evaluating morbidity and mortality of partial hepatectomies<sup>[31,32]</sup>.

The present study did not neglect the analysis of the two main AKI risk factors after partial hepatectomies, which would be perioperative bleeding and PHLF<sup>[6]</sup>. Peri-



operative haemorrhage with renal hypoperfusion<sup>[6]</sup>, with or without the deleterious effects of blood transfusion<sup>[3]</sup>, was a strong predictor of postoperative AKI in this study, reflected by intraoperative hemodynamic instability and posthepatectomy haemorrhage. An increased renal susceptibility to the perioperative renal ischemia<sup>[22-25]</sup>, such as in CKI, was a predictor in the authors' series.

Additionally, it is expected that major resections may have larger blood losses during operation and higher incidence of PHLF as well, it was corroborated by the significant influence of major resections on AKI occurrence, according to our logistic regression model. In a recent report of a large series of liver resections for hepatocellular carcinoma, major liver resection was a predictor for postoperative AKI<sup>[4]</sup>.

For prevention of intraoperative bleeding, there are intraoperative maneuvers that may be crucial, such as vascular control of the liver<sup>[2]</sup> and LCVP anesthesia<sup>[1,33,34]</sup>, preventing the back bleeding from hepatic veins. The Pringle maneuver (intermittent<sup>[16]</sup> or continuous<sup>[17]</sup>) is routinely applied in liver resections at the authors' Department, thus there was no difference between the groups, and LCVP anesthesia parameters were not evaluated.

Second factor relates to the occurrence of PLF with its distributive circulatory changes, which is a major cause of death after hepatic resection, and eventually can progress to HRS<sup>[11]</sup>. Similar to the results from a previous report<sup>[4]</sup>, the MELD score<sup>[13]</sup>, a usefully and extensively validated tool for predicting liver failure progression, was a predictor of postoperative AKI, and the most important, it can be applied in the preoperative period.

The presence of biliary obstruction was an independent predictor of postoperative AKI according to the authors' results, and the mechanism by which bilirubin may be toxic to the kidneys seems to be inflammatory as well as obstructive<sup>[35]</sup>, and hemodynamic changes may also play a role in biliary cast nephropathy<sup>[36]</sup>. In addition to the aforementioned effects, patients who are candidates for surgery in the presence of biliary obstruction with congestive cholestasis in the liver<sup>[37,38]</sup> may undergo major hepatic resections, with consequent decrease in the volume of a functionally deficient liver parenchyma, predisposing for PHLF.

Eventually, patients can have combinations of renal insults that can be aggravated by sepsis<sup>[2,3,5,6]</sup>, which was an independent predictor in the authors' analysis. The septicemia and its hemodynamic and systemic repercussions may eventually coexist with liver failure, often being the final event of PHLF<sup>[5]</sup>.

The shortcomings of the current study, besides its retrospective nature, were the non-inclusion of anesthetic maneuvers among covariates, such as LCVP anesthesia, and the non-inclusion of hepatic steatosis, since it is a determinant of the functional quality of the parenchyma<sup>[39,40]</sup>. As mentioned, the retrospective nature of the study did not allow the authors to include non-standardized non-reliable data.

In order to reduce the incidence of postoperative AKI after partial hepatectomy, a careful patient selection and preoperative resection planning are mandatory, specially

in the case of predisposing CKI, biliary obstruction and underlying cirrhosis, in which MELD score calculation can be extremely worthwhile<sup>[41-43]</sup>. Measures for preventing sustained intraoperative hypotension and postoperative bleeding must be undertaken, as well as prevention and prompt treatment of sepsis. In the case of high risk patients for postoperative AKI, the nephrologist must be promptly involved in multidisciplinary discussions.

## COMMENTS

### Background

Acute kidney injury (AKI) is a serious complication after partial hepatectomy, however, there are limited published data regarding this subject, in addition, there is no consensus about the definition of AKI in these patients.

### Research frontiers

The present study did not neglect the analysis of the two main AKI risk factors after partial hepatectomies, which would be perioperative bleeding, with or without the deleterious effects of blood transfusion, and post-hepatectomy liver failure.

### Innovations and breakthroughs

The hemodynamic changes in patients after major liver resections may simulate those of patients with acute liver failure or cirrhosis. Thus, the current criteria suggested by the International Club of Ascites (ICA) for definition of AKI would be the most appropriate criteria for these patients.

### Applications

In order to reduce the incidence of postoperative AKI after partial hepatectomy, a careful patient selection and preoperative resection planning are mandatory, specially in the case of predisposing CKI, biliary obstruction and underlying cirrhosis, in which Model for End-Stage Liver Disease score calculation can be extremely worthwhile.

### Terminology

Candidates for liver resections often present with multiple potential risk factors regarding postoperative AKI, such as excessive bleeding during the hepatectomy, and the occurrence of post-hepatectomy liver failure (PLF). The current criteria suggested by the ICA for definition of AKI would be the most appropriate criteria for these patients. For prevention of intraoperative bleeding, there are intraoperative maneuvers that may be crucial, such as vascular control of the liver and low central venous pressure anesthesia. Second factor relates to the occurrence of PLF with its distributive circulatory changes, that eventually can progress to hepatorenal syndrome.

### Peer-review

This paper was retrospectively analyzed the clinical data, and found some risk factors of acute kidney injury. The material was rich, the result was reasonable, and the discussion did have some valuable information.

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Retrospective Study

## Early acute kidney injury after liver transplantation: Predisposing factors and clinical implications

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### Abstract

#### AIM

To investigate the additional clinical impact of hepatic ischaemia reperfusion injury (HIRI) on patients sustaining acute kidney injury (AKI) following liver transplantation.

#### METHODS

This was a single-centre retrospective study of consecutive adult patients undergoing orthotopic liver transplantation (OLT) between January 2013 and June 2014. Early AKI was identified by measuring serum creatinine at 24 h post OLT ( $> 1.5 \times$  baseline) or by the use of continuous veno-venous haemofiltration (CVVHF) during the early post-operative period. Patients with and without AKI were compared to identify risk factors associated with this complication. Peak serum aspartate aminotransferase (AST) within 24 h post-OLT was used as a surrogate marker for HIRI and severity was classified as minor ( $< 1000$  IU/L), moderate (1000-5000 IU/L) or severe ( $> 5000$  IU/L). The impact on time to extubation, intensive care length of stay, incidence of chronic renal failure and 90-d mortality were examined firstly for each of the two complications (AKI and HIRI) alone and then as a combined outcome.

#### RESULTS

Out of the 116 patients included in the study, 50% developed AKI, 24% required CVVHF and 70% sustained



moderate or severe HIRI. Median peak AST levels were 1248 IU/L and 2059 IU/L in the No AKI and AKI groups respectively ( $P = 0.0003$ ). Furthermore, peak serum AST was the only consistent predictor of AKI on multivariate analysis  $P = 0.02$ . AKI and HIRI were individually associated with a longer time to extubation, increased length of intensive care unit stay and reduced survival. However, the patients who sustained both AKI and moderate or severe HIRI had a longer median time to extubation ( $P < 0.001$ ) and intensive care length of stay ( $P = 0.001$ ) than those with either complication alone. Ninety-day survival in the group sustaining both AKI and moderate or severe HIRI was 89%, compared to 100% in the groups with either or neither complication ( $P = 0.049$ ).

### CONCLUSION

HIRI has an important role in the development of AKI post-OLT and has a negative impact on patient outcomes, especially when occurring alongside AKI.

**Key words:** Hepatic ischaemia reperfusion injury; Liver transplantation; Perioperative care; Acute kidney injury; Marginal grafts

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**Core tip:** Acute kidney injury (AKI) is common after liver transplantation (LT), and has a significant impact on patient outcomes. It is multifactorial in aetiology and has been shown to correlate with the use of higher risk grafts, due to an increased risk of hepatic ischaemia reperfusion injury. In context of the growing use of marginal grafts to meet demands, this study has demonstrated that hepatic ischaemia reperfusion injury was the only variable that predicted early AKI post-LT and that the presence of both HIRI and AKI led to worse clinical outcomes and higher mortality than either complication alone.

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## INTRODUCTION

Acute kidney injury (AKI) developing immediately after orthotopic liver transplantation (OLT) is common, and is associated with increased morbidity, mortality and resource utilisation<sup>[1]</sup>. It affects between 25% and 60% of recipients<sup>[2-4]</sup>, the variation being largely related to the definition of AKI utilised<sup>[5,6]</sup>. The incidence of AKI following OLT is much higher than with other non-cardiac major surgery, in patients with previously normal renal function<sup>[7]</sup>. This reflects the additional risks facing the liver

transplant recipient during the intra and post-operative course.

Multiple factors predisposing liver transplant patients to post operative AKI have been identified. Pre-operatively, existing renal impairment, increasing Model for End stage Liver Disease Score, diabetes mellitus, hypertension<sup>[4]</sup> and obesity have all been associated with post OLT renal dysfunction. Intraoperative mean arterial pressure, vaso-pressor requirements, blood loss and transfusion of blood products are additional risk factors<sup>[8,9]</sup>. Post-operatively, graft dysfunction and immunosuppression therapy have been regarded as the main renal insults.

More recently, the growing demand for organs in LT has led to the use of increasingly higher risk grafts, in order to reduce the waiting list mortality<sup>[10-12]</sup>. These include grafts from older donors, prolonged preservation period, graft steatosis, split or partial liver allografts and donation after cardiac death (DCD). In the United Kingdom, DCD organs in particular have been increasingly used over the last decade, accounting for 20% of all liver transplants in 2014/15 (compared to 17% in 2012 and 5% in 2005)<sup>[13-15]</sup>. This group have been shown to be at a higher risk of both hepatic and extra-hepatic complications, including AKI, which is the most common complication encountered following the transplantation of marginal grafts. The post-operative systemic inflammatory response that occurs as a result of the hepatic ischaemia reperfusion injury (HIRI) following warm ischaemia at retrieval is thought to play a critical role in the pathogenesis of renal injury in these patients<sup>[16]</sup>. In keeping with this, peak peri-operative serum aspartate aminotransferase (AST), which is a surrogate marker for HIRI<sup>[17,18]</sup>, has been found to be a significant variable related to renal outcomes<sup>[19-21]</sup>.

The additional impact of HIRI on early post-operative renal dysfunction of liver transplant recipients has not been widely investigated. Previous studies, especially those based on large national databases do not usually have sufficient clinical information to analyse the predisposing factors. Early identification of those at risk, followed by prevention and management of HIRI may have important implications on the outcomes of these patients.

The aims of this study were firstly to identify the incidence, risk factors and clinical outcomes of early AKI in our cohort of patients, and secondly to investigate the incidence of HIRI, its correlation with AKI and its additional impact on patient outcomes.

## MATERIALS AND METHODS

Single-centre retrospective observational study of consecutive adults ( $\geq 18$  years of age) undergoing OLT between January 2013 and June 2014 at the Royal Free London NHS Foundation Trust, one of the 8 Liver Transplant centres in the United Kingdom and Ireland. Exclusion criteria were those requiring urgent transplantation for acute liver failure and those receiving a combined liver-kidney transplant.

To analyse possible factors associated with post OLT AKI we included recipient age, gender, weight, aetiology of liver disease, Model for End-Stage Liver Disease (MELD) score, presence of anaemia (haemoglobin) and the presence of diabetes mellitus, hypertension and pre-existing renal dysfunction [serum creatinine > 100 µmol/L on the day of admission prior to OLT (baseline)]. Donor data was taken from a prospectively compiled database and included donor age, donor status [DCD or donation after brain death (DBD)] and cold ischaemic time.

Intraoperative factors assessed included surgical technique (piggy back or caval replacement), blood products transfused during OLT (packed red cell units, fresh frozen plasma and platelets), transfused cell salvage blood (as a reflection of blood loss) and noradrenaline infusion rate on admission to intensive care (as a reflection of possible ischaemia reperfusion injury).

Post-operative AKI was determined from serum creatinine at midnight on Day 1 and the need for continuous veno-venous haemofiltration (CVVHF) during the post-operative stay on intensive care unit (ICU). Peak serum AST within the first 24 h post-OLT was used as a measure of ischaemia reperfusion injury.

Clinical outcomes measured aside from the presence of AKI included time to extubation, intensive care length of stay, the incidence of chronic renal failure (CRF), as demonstrated by an estimated glomerular filtration rate of < 60 mL/min per 1.73 m<sup>2</sup> at 6 mo post transplantation and 90-d patient survival.

Extent of AKI was assessed using the AKIN criteria<sup>[22]</sup>, with the multiple rise in creatinine at 24 h post OLT compared to baseline, categorising the post-operative renal status into: No AKI (< 1.5-fold rise in creatinine); Stage 1 (1.5-2 fold rise); Stage 2 (2-3 fold rise) and Stage 3 (> 3 fold rise or the commencement of renal replacement therapy). This time frame was chosen to ensure that the renal complication was not due to post-operative factors such as nephrotoxicity secondary to immunosuppression.

The incidence of AKI at 24 h post OLT was determined and patients with no AKI vs those with any grade of AKI were compared in terms of baseline recipient characteristics, donor graft characteristics and intraoperative variables. The two groups were then compared with respect to time taken until extubation, intensive care length of stay (ICU LOS), incidence of CRF and 90 d patient survival. Risk factors for early AKI were then determined including the variables outlined above.

Incidence and severity of HIRI were determined using peak serum AST within 24 h of OLT, putting recipients into the groups: Mild HIRI (AST < 1000 IU/L); moderate HIRI (AST 1000-5000 IU/L) and severe HIRI (AST > 5000 IU/L). These groups were compared in terms of time taken until extubation, ICU LOS, incidence of CRF and 90 d patient survival. Peak AST levels within 24 h post OLT were correlated with the presence of early AKI and organ status.

Clinical outcomes (time taken until extubation, ICU LOS, CRF and 90 d patient survival) were then compared

between those with neither AKI nor HIRI, either complication or both AKI and HIRI. In this context, HIRI was identified as those with moderate or severe HIRI.

### Statistical analysis

Continuous parametric variables were expressed as means with SDs, and compared using student's *t* test and ANOVA analysis of variance. Continuous non-parametric variables were expressed as medians with interquartile ranges, and compared using the Mann Whitney *U* test and Kruskal Wallis analysis of variance. Normality of data was confirmed using both Shapiro Wilk test and histogram analysis. Categorical variables were analysed using  $\chi^2$  test or Fisher's exact test and correlations between variables were analysed using Spearman's or Pearson's rank correlation for non-parametric and parametric data respectively. Kaplan Meier plots were used to analyse survival with log rank tests for differences and logistic regression analysis was performed to identify variables associated with AKI. A *P* value of < 0.05 was considered statistically significant unless otherwise stated. Statistical analysis was carried out using Microsoft Excel and IBM SPSS Statistics Version 24.

## RESULTS

One hundred and forty OLTs were performed in adult recipients over the study period, using either the caval replacement technique or the piggyback technique, with or without a temporary porto-caval shunt. Veno-venous bypass was not used in any of these cases. Twenty patients underwent urgent transplantation, 3 received a combined liver-kidney transplant and 1 patient died intra-operatively. These patients were excluded from further analysis. The remaining 116 patients were then grouped according to the absence or presence of post-operative AKI and compared with regards to demographics, aetiology, severity of liver disease and relevant co-morbidity (Table 1). These groups were further compared in context of the donor graft and intraoperative characteristics (Tables 2 and 3 respectively).

### Incidence of AKI

Out of the 116 patients included in the study, 58 (50%) developed early AKI post OLT using the AKIN criteria<sup>[22]</sup>. In those sustaining this post-operative complication, 19 were classified as stage 1, 7 as stage 2 and 32 as stage 3 acute kidney injuries. Twenty-eight/116 (24%) patients required CVVHF during the post-operative admission on ICU. The indication for commencement of renal replacement therapy was AKI in all these cases.

Ninety/116 (77.6%) patients had a baseline serum creatinine < 100 µmol/L and 26/116 (22.4%) had a baseline serum creatinine > 100 µmol/L. The incidence of AKI post OLT was 48/90 (53%) in the former group, compared to 10/26 (38%) in the latter group, this difference not being statistically significant ( $\chi^2 = 1.78$ , *P* = 0.182). However, a greater proportion of those with pre-existing renal impairment (baseline creatinine > 100 µmol/L)

**Table 1 Patient demographics**

Variables	No AKI ( <i>n</i> = 58)	Any grade of AKI ( <i>n</i> = 58)	<i>P</i> value
Patient characteristics			
Age (yr)	54 (18)	56 (6)	0.197
Female	17 (29%)	16 (28%)	0.837
Weight (kg)	75 (13)	79 (15)	0.163
Aetiology			
Alcohol	13	14	
Viral hepatitis	20	25	
NASH	2	7	
Autoimmune	16	7	
Hepatocellular carcinoma	10	13	
Other	7	6	
MELD score	16 (7)	16 (5.75)	0.421
Hypertension	8 (14%)	9 (16%)	0.733
Diabetes mellitus	12 (21%)	20 (34%)	0.074
Pre-operative Haemoglobin (g/L)	114 (20)	107 (26)	0.072
Baseline creatinine	76 (39)	75 (26)	0.932

Values expressed as mean (SD), median (interquartile range) and number (percentage) where appropriate. AKI: Acute kidney injury; NASH: Non-alcoholic steato-hepatitis; MELD: Model for End-Stage Liver Disease.

developed stage 3 AKI (35% vs 26%)  $P = 0.081$  and required CVVHF (35% vs 21%)  $P = 0.156$  during the post-operative admission to ICU, compared to the group with a normal baseline creatinine although again this was not statistically significant.

### AKI and clinical outcomes

The median time to extubation was 37 h in the group sustaining AKI compared to 16 h in those without AKI ( $P < 0.0001$ ) (Figure 1). Additionally, median intensive care length of stay was 5 d in the AKI group compared to 2.5 d in the no AKI group ( $P < 0.0001$ ) (Figure 1). At the 6-mo follow-up period 39% of those with early post OLT AKI had developed CRF compared to 25% of those without AKI, although this did not reach statistical significance ( $P = 0.142$ ).

### Risk factors for AKI

Variables associated with AKI on regression analysis are described in Table 4. On univariate analysis surgical technique, transfusion of red cell concentrate, fresh frozen plasma, platelets and cell salvage blood and peak AST within 24 h after OLT were all associated with an increased risk of the development of AKI. In a multivariate model that included all clinically relevant variables, only peak AST within the first 24 h post OLT remained statistically significant in predicting early AKI ( $P = 0.020$ ).

### Incidence and severity and of HIRI

To assess the severity of HIRI, patients were divided into groups based on peak AST levels within the first 24 h after OLT: AST < 1000 IU/L (minor HIRI); AST 1000-5000 IU/L (moderate HIRI); AST > 5000 IU/L (severe HIRI)<sup>[23]</sup>. Thirty-five/116 (30%) of patients developed mild HIRI, 68/116 (59%) developed moderate HIRI and 13/116 (11%) developed severe HIRI. The effect of organ status

**Table 2 Donor graft characteristics**

Variables	No AKI ( <i>n</i> = 58)	Any AKI ( <i>n</i> = 58)	<i>P</i> value
Graft characteristics			
Donor age (yr)	50 (14)	47 (15)	0.219
Organ status			
DCD (%)	7 (12%)	10 (17%)	0.454
Cold Ischaemic time (min)	493 (133)	493 (106)	0.502

Values expressed as mean (SD), median (interquartile range) and number (percent) where appropriate. DCD: Donation after cardiac death; AKI: Acute kidney injury.

**Table 3 Intraoperative variables**

Variable	No AKI ( <i>n</i> = 58)	Any AKI ( <i>n</i> = 58)	<i>P</i> value
Intraoperative variables			
Surgical technique			
Piggyback	33 (57%)	22 (38%)	0.041
Intraoperative blood products			
RCC transfusion (units)	1 (3)	4 (5)	0.001
FFP transfusion (units)	0 (2)	2 (6)	0.001
Platelet transfusion (units)	0 (1)	0 (2)	0.024
Cell salvage (mL)	281 (550)	764 (929)	0.001
Noradrenaline infusion rate on arrival to ICU (µg/kg per minute)	0.16 (0.10)	0.18 (0.13)	0.343

Values expressed as mean (SD), median (interquartile range) and number (percent) where appropriate. AKI: Acute kidney injury; RCC: Red cell concentrate; FFP: Fresh frozen plasma; ICU: Intensive care unit.

and surgical technique on HIRI severity and the renal implications of increasing ischaemia reperfusion injury are summarised in Table 5.

### Correlation of renal dysfunction and HIRI

Median peak AST levels within the first 24 h post OLT were 1248 IU/L and 2059 IU/L in the No AKI and AKI groups respectively ( $P = 0.0003$ ). Furthermore, increasing levels of peak AST correlated well with increasing severity of AKI (Spearman's  $r = 0.334$ ,  $P = 0.0003$ ). Finally, increasing severity of HIRI was associated with both a higher incidence of AKI ( $P = 0.002$ ) and more frequent use of CVVHF ( $P = 0.003$ ) (Figure 2).

### Correlation of organ status and HIRI

Median peak AST levels within the first post-operative day were 1307 IU/L and 2060 IU/L in those who underwent DBD and DCD transplantation respectively ( $P = 0.001$ ). A spearman's rank-order correlation was run to examine the relationship between organ status and HIRI, which revealed a positive correlation between the two (spearman's  $r = 0.322$ ,  $P = 0.0005$ ). Five thirteenths (38.5%) of those with severe HIRI had received a DCD graft, compared to only 1/35 (3%) of those with mild HIRI,  $P = 0.007$  (Table 5).

### HIRI and clinical outcomes

Increasing severity of HIRI was associated with a trend

**Table 4** Logistic regression analysis of variables associated with early acute kidney injury after liver transplantation

Variables	Univariate analysis		Multivariate model	
	Odds ratio (95%CI)	P value	Odds ratio (95%CI)	P value
Male gender	1.088 (0.49-2.44)	0.837	0.430 (0.07-2.55)	0.352
Weight	1.020 (0.99-1.05)	0.164	1.026 (0.96-1.10)	0.455
Age	1.034 (0.999-1.07)	0.058	1.121 (1.02-1.23)	0.019
Pre-transplant				
Diabetes mellitus	2.130 (0.92-4.92)	0.077	2.429 (0.48-12.41)	0.286
Hypertension	1.197 (0.43-3.36)	0.733	0.593 (0.04-8.04)	0.694
Serum creatinine	1.000 (0.99-1.01)	0.937	0.979 (0.96-1.002)	0.078
MELD	1.010 (0.97-1.05)	0.639	0.992 (0.91-1.08)	0.845
Haemoglobin	0.985 (0.97-1.002)	0.076	0.977 (0.93-1.02)	0.311
Graft characteristics				
DCD organ	1.488 (0.52-4.23)	0.455	2.638 (0.30-23.12)	0.381
Donor age	0.984 (0.96-1.01)	0.218	0.964 (0.92-1.01)	0.160
Cold ischaemic time	1.001 (0.99-1.004)	0.498	1.000 (0.99-1.01)	0.984
Peak serum AST < 24 h post OLT	1.001 (1.00-1.001)	0.001	1.001 (1.00-1.001)	0.020
Perioperative course				
Caval replacement surgical technique	2.160 (1.03-4.54)	0.042	3.289 (0.71-15.25)	0.128
Noradrenaline infusion rate on ICU arrival	4.830 (0.19-125.48)	0.343	47.468 (0.12-1836.27)	0.204
RCC transfusion	1.137 (1.03-1.26)	0.014	0.917 (0.64-1.32)	0.645
FFP transfusion	1.201 (1.07-1.35)	0.003	1.118 (0.84-1.48)	0.440
Platelet transfusion	1.499 (1.04-2.16)	0.030	1.948 (0.871-4.36)	0.104
Cell salvage volume	1.001 (1.00-1.001)	0.025	1.00 (0.999-1.002)	0.808

CI: Confidence interval; MELD: Model for End-Stage Liver Disease; DCD: Donation after Cardiac death; ICU: Intensive care unit; RCC: Red cell concentrate; FFP: Fresh frozen plasma; AST: Aspartate aminotransferase; OLT: Orthotopic liver transplantation.

**Table 5** The effect of organ status and surgical technique on hepatic ischaemia reperfusion injury severity and the renal implications of increasing ischaemia reperfusion injury *n* (%)

	Severity of HIRI			P value
	Mild ( <i>n</i> = 35)	Moderate ( <i>n</i> = 68)	Severe ( <i>n</i> = 13)	
DCD organs	1/35 (2.9)	11/68 (16.2)	5/13 (38.5)	0.007
Caval replacement	16/35 (45.7)	36/68 (52.9)	9/13 (69.2)	0.348
Incidence of AKI	12/35 (34.3)	34/68 (50.0)	12/13 (92.3)	0.002
Need for CVVHF	5/35 (14.3)	15/68 (22.1)	8/13 (61.5)	0.003
Development of CRF	6/30 (20.0)	19/53 (35.8)	5/11 (45.5)	0.195

HIRI: Hepatic ischaemia reperfusion injury; DCD: Donation after Cardiac death; AKI: Acute kidney injury; CVVHF: Continuous veno-venous haemofiltration; CRF: Chronic renal failure.

towards longer median time to extubation (Figure 3)  $P = 0.07$ , longer median ICU length of stay (Figure 3)  $P = 0.01$ , and a trend towards a higher incidence of CRF at 6 mo (Table 5)  $P = 0.195$ .

### The combined impact on clinical outcomes of AKI and HIRI

To examine the clinical impact of having both the complications of AKI and HIRI, the cohort was divided into 4 groups: Those with neither AKI nor HIRI (group 1); HIRI but no AKI (group 2); AKI but no HIRI (group 3) and those with both complications (group 4). The presence of HIRI included any patient that sustained either moderate or severe HIRI (peak AST within 24 h post OLT > 1000 IU/L). These groups were then compared for median time to extubation, median ICU length of stay and the incidence of CRF.

The median time to extubation (hours) differed between the groups ( $P < 0.001$ ) with the lowest time observed in group 1 and the highest observed in group 4 (Figure

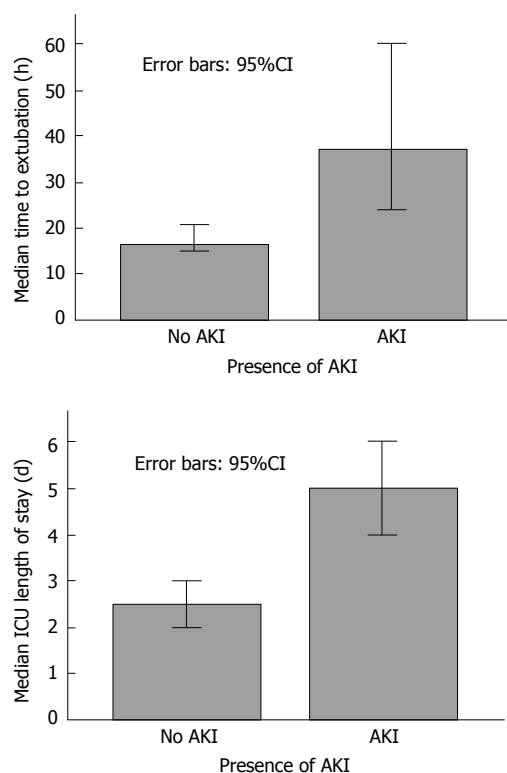
4). Pairwise comparisons revealed statistically significant differences between groups 1 and 4 ( $P = 0.001$ ) and groups 2 and 4 ( $P = 0.003$ ).

Similarly, the median ICU length of stay (days) increased between the groups ( $P = 0.001$ ), with the highest value observed in the patients sustaining both AKI and HIRI (Figure 4). Pairwise comparisons revealed statistically significant differences between groups 1 and 3 ( $P = 0.04$ ), groups 1 and 4 ( $P < 0.0001$ ) and groups 2 and 4 ( $P = 0.005$ ). Finally, there was a trend towards a higher incidence of CRF in those with any one of, or both AKI and HIRI compared to those with neither, with an incidence of only 15% in group 1 and 45% in group 4 ( $P = 0.238$ ).

### Survival

Kaplan Meier analysis revealed a reduction in 90-d patient survival associated with the presence of early AKI compared to no AKI (91.4% vs 100% respectively,  $P = 0.024$ ) and increasing severity of HIRI (severe 84.6%;





**Figure 1** Bar graphs demonstrating median time to extubation and intensive care unit length of stay in the absence or presence of acute kidney injury. ICU: Intensive care unit; AKI: Acute kidney injury.

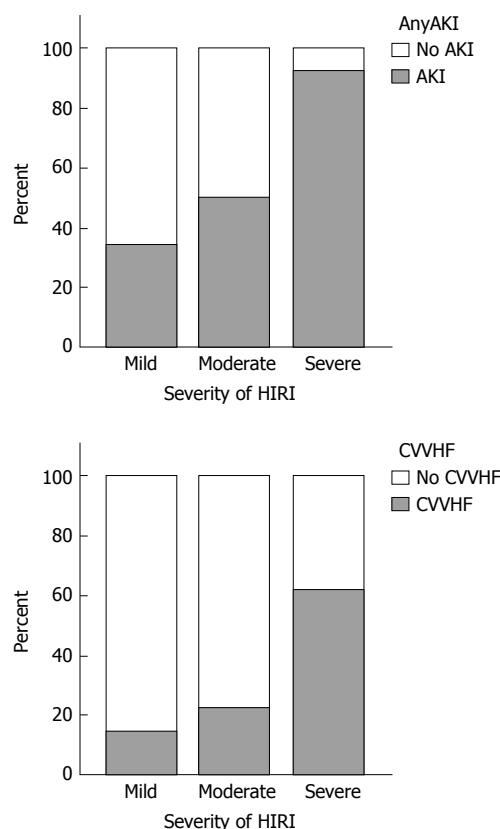
moderate 95.5%; mild 100.0%,  $P = 0.053$ ) (Figure 5). Furthermore, early AKI and moderate/severe HIRI occurring in combination had a greater impact on 90-d patient survival than either complication when occurring in isolation (89% vs 100% respectively,  $P = 0.049$ ) (Figure 6).

## DISCUSSION

This single centre study has allowed detailed analysis of factors influencing post-operative AKI in United Kingdom patients undergoing LT, allowing strategies for intervention to be designed. In particular it has investigated the impact of HIRI, which is becoming more prevalent in an era that has seen a steady rise in the use of marginal grafts. The aetiology of AKI following OLT is complex and multifactorial, so our study has benefitted from the analysis of details that are not collected in national databases.

The importance of AKI and HIRI to the outcome of patients undergoing OLT is emphasised by major differences being demonstrated in this small single centre study in important patient centred outcomes. Patients who sustained both AKI and HIRI had a longer time to extubation, longer ICU length of stay and a lower 90-d patient survival. Furthermore, in a multivariate model of all clinically relevant variables, HIRI was shown to be the single most important factor predicting post-operative AKI, suggesting that it plays a critical role in the pathogenesis of renal dysfunction after LT.

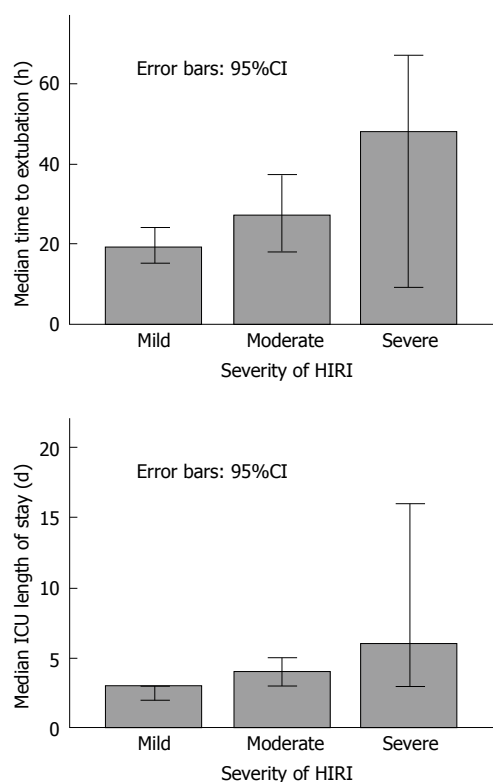
The association between HIRI and AKI has previously been reported, with Leithead *et al*<sup>[16]</sup> demonstrating



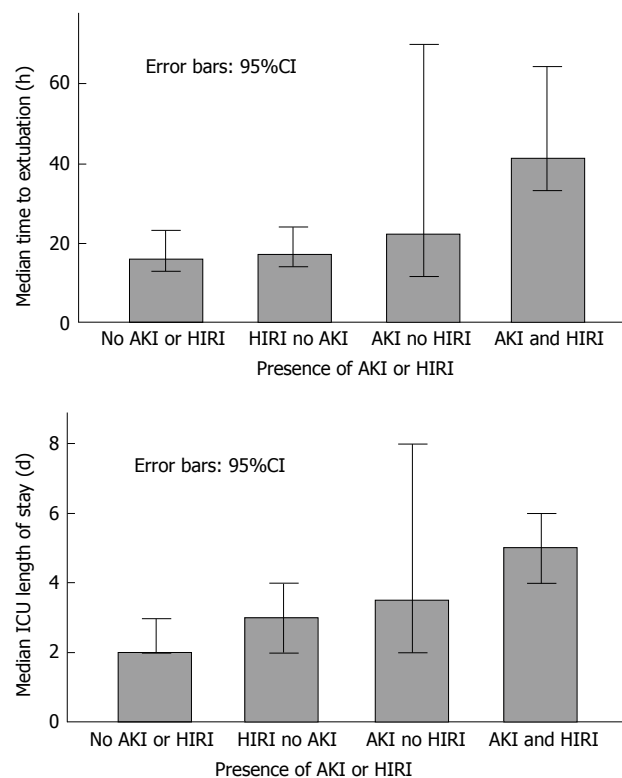
**Figure 2** Bar graphs demonstrating the presence of acute kidney injury and use of continuous veno-venous haemofiltration in context of the severity of hepatic ischaemia reperfusion injury. Mild HIRI  $n = 35$ ; moderate HIRI  $n = 68$ ; severe HIRI  $n = 13$ . AKI: Acute kidney injury; CVVHF: Continuous veno-venous haemofiltration; HIRI: Hepatic ischaemia reperfusion injury.

peak postoperative AST as the main predictor of renal dysfunction after DCD transplantation. Renal outcomes were examined for those undergoing DCD transplantation, but not specifically correlated with the degree of HIRI. Glanemann *et al*<sup>[23]</sup> examined the clinical implications of increasing severity of hepatic preservation injury, and found it to be associated with initial graft non-function and, as in the current study, to be correlated with an increased duration of post-operative ventilation and haemodialysis. However Glanemann *et al*<sup>[23]</sup> did not define the cohort that developed AKI. For the first time we have shown that the combination of early AKI and moderate to severe HIRI leads to worse post OLT outcomes than either complication alone.

The aetiology of AKI following LT is thought to be multifactorial, and contributory causes include exposure to high levels of toxic free radicals, renal ischaemia, use of nephrotoxic medications and the effects of end stage liver disease on the kidneys. Perioperative risk factors for the development of AKI post OLT have included pre-existing renal dysfunction, diabetes mellitus, hypertension, previous ascites, MELD score, surgical technique, intraoperative transfusion of blood products, ischaemia time, post-reperfusion syndrome and post OLT immunosuppression<sup>[24-26]</sup>. In our study, peak serum AST within 24 h of OLT, surgical technique and transfusion of



**Figure 3** Increasing severity of hepatic ischaemia reperfusion injury compared with median time to extubation and median intensive care unit length of stay. ICU: Intensive care unit; HIRI: Hepatic ischaemia reperfusion injury.



**Figure 4** Median time to extubation and median intensive care unit length of stay in groups with the combined absence or presence of acute kidney injury and hepatic ischaemia reperfusion injury. ICU: Intensive care unit; AKI: Acute kidney injury; HIRI: Hepatic ischaemia reperfusion injury.

blood products were all statistically significant in univariate analysis in being predictors of early AKI. However only peak serum AST within 24 h of OLT remained so in the multivariate model.

Previously it has been shown that DCD transplantation is associated with post-operative renal dysfunction<sup>[16]</sup>. This would be expected as the use of DCD grafts is associated with increased warm ischaemia, incidence of poor and non function of the graft and patient and graft mortality<sup>[27]</sup>. In our study we were not able to reproduce these results. This perhaps was secondary to the fact that only 15% of our cohort received DCD grafts, or that to compensate for the use of DCD organs the donors may have been younger, had lower cold ischaemia times or were transplanted into younger, fitter patients.

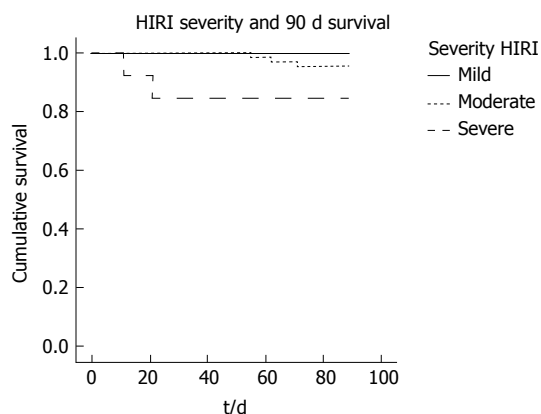
It has been reported that pre-operative renal dysfunction is an independent predictor of post-OLT AKI and the need for CVVHF<sup>[28,29]</sup>. However, in our study, pre-operative serum creatinine was not a predictor for early post-OLT AKI in logistic regression analysis. In fact, a greater proportion of those with a pre-operative creatinine < 100  $\mu\text{mol/L}$  developed early AKI (53%) than those with a pre-operative creatinine > 100  $\mu\text{mol/L}$  (38%). One possibility to explain this may have been that better quality grafts with a lower donor risk index were matched to the higher risk recipients.

Interestingly though, in those with pre transplant CRF who did develop AKI it was likely to be severe (stage 3 AKI) and require CVVHF suggesting a predisposition

to an increased severity of the complication in the setting of pre-operative dysfunction. The difference in outcome of our logistic regression analysis may reflect the discrepancies in the definitions used to categorise AKI. For example, Cabezuolo *et al.*<sup>[30]</sup> categorised post-op AKI as an increase in pre-operative serum creatinine > 50% (compared to our definition of > 150%). In addition, their team defined pre-operative acute renal impairment as an increase in creatinine > 50% from baseline, compared to our pre-operative renal function being defined by the serum creatinine on the day of transplantation alone.

The main limitation of this study lies in its retrospective nature and the inability to control for factors with inter-individual variability, such as the indications and timing in use of CVVHF, which remained reliant on the judgment of the clinician. Also, the variable definitions of AKI mean that interpretation of results needs to be considered in context of the methodologies used.

The frequency of AKI has increased in recent years, and this increase has occurred in parallel with a marked increase in the use of high-risk grafts. In the United Kingdom 29% of donors are over 60 years of age, over 20% are DCD, and clinically obese donors have doubled in the last 10 years. It is known that grafts from these extended criteria donors are more prone to HIRI and poor outcome<sup>[31]</sup>. HIRI is often more severe when implanting steatotic organs, and it has been reported that the incidence of AKI is significantly higher in patients



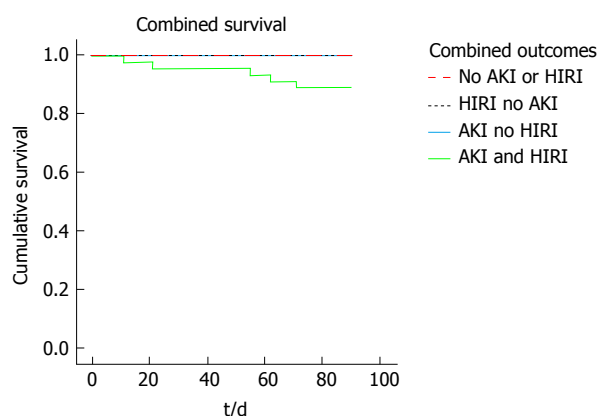
**Figure 5** Kaplan Meier plot of 90-d patient survival in those with mild, moderate and severe hepatic ischaemia reperfusion injury. HIRI: Hepatic ischaemia reperfusion injury.

receiving grafts from donors with a high BMI, although long term survival was not significantly different when corrected for other variables, such as diabetes<sup>[32]</sup>. The accelerated search in recent years for methods to expand the organ donor pool has lead to the increasing use of higher risk grafts. This trend in activity has important implications on the recipient population in terms of increased morbidity and mortality post OLT, however, as extended criteria donor grafts are usually allocated to patients with lower MELD scores, this may impact on increased hospital stay, complications and costs but not necessarily poorer graft or patient survival figures.

The role of graft injury and HIRI injury in the pathogenesis of AKI is being increasingly recognised<sup>[16,30]</sup>. It is one of the most important causes of organ dysfunction, and is a major determinant of successful LT. The deleterious effects are not limited to the liver, but are seen in other organs, including the lungs and kidney<sup>[33]</sup>. IRI can trigger a systemic inflammatory response and subsequent multi-organ failure, the injury being characterised by intra-vascular oxidative stress and functional impairment of the mitochondria<sup>[34,35]</sup>. Peak serum AST is a surrogate marker of the severity of HIRI, and is closely correlated with the development of AKI, as confirmed in this study. Low values of AST following transplantation are associated with superior outcomes<sup>[36]</sup>, and a reduction in AST levels have been used as a primary end point for liver IRI studies in animal models. Preliminary results from a proof of concept study of normothermic machine perfusion compared to a standard cold preservation demonstrated a marked reduction in peak AST levels (417 IU/L vs 902 IU/L respectively), indicating that this method of preservation, by “reconditioning” the graft, may reduce HIRI and its attendant consequences, including AKI<sup>[37]</sup>.

### Conclusion

In summary, our study has shown that renal dysfunction and use of CVVHF after OLT is common, and rises in proportion to the level of hepatic-ischaemia-reperfusion-injury (as determined by AST levels) and its coexisting



**Figure 6** Kaplan Meier plot of 90-d patient survival in those with the combined absence or presence of acute kidney injury and hepatic ischaemia reperfusion injury. AKI: Acute kidney injury; HIRI: Hepatic ischaemia reperfusion injury.

systemic inflammatory response. Further work should focus on novel therapies that prevent and treat this graft-related injury to improve recipient outcomes and broaden the donor pool with more extended criteria grafts.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Acute kidney injury (AKI) is a common complication following liver transplantation (LT) and has significant clinical implications on patient outcomes. In recent years, the growing demand for organs in transplantation has prompted a search for methods to expand the donor pool, which has included the consideration of use of higher risk grafts, including those from donation after cardiac death donors. This has conferred an increased risk of hepatic ischaemia reperfusion injury (HIRI) to the recipients, of which one of the consequences is AKI.

### Research frontiers

It is not fully clear what additional extra-hepatic clinical impact the use of these higher risk grafts have on the recipient in LT. A few reports have addressed the association between HIRI and renal dysfunction post LT but none have explored the clinical impact of having both HIRI and AKI as a combined outcome.

### Innovations and breakthroughs

In this study, the authors have shown for the first time that not only do early AKI and moderate to severe HIRI as individual complications, lead to poorer patient outcomes, but combined have a worse impact on time to extubation, intensive care unit length of stay and 90-d survival when compared to each complication alone.

### Applications

This study has highlighted the adverse extra-hepatic consequences of HIRI and the subsequent need to develop novel therapies that prevent and treat this graft-related injury to improve recipient outcomes and broaden the donor pool with more extended criteria grafts.

### Terminology

AKI: Acute kidney injury, as defined by the AKIN criteria (multiple rise in creatinine from baseline or the need for renal replacement therapy); HIRI: Hepatic ischaemia

reperfusion injury; A graft related injury causing a systemic inflammatory response that has, in this study been categorised according to peak serum aspartate aminotransferase levels on day one post LT.

# Peer-review

The study was conducted well in terms of identifying the predictors for and impact of HIRI on early AKIs.

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