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TOPIC HIGHLIGHT

2016 Advances in Hepatitis B Virus

Metabonomic window into hepatitis B virus-related hepatic diseases

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Abstract

Metabonomics has recently been widely used to discover the pathogenesis and find potential metabolic markers with high sensitivity and specificity. Furthermore, it develops new diagnosis and treatment methods, increases early phase diagnosis rates of certain diseases and provides a new basis for targeted therapy. This review mainly analyzes the research progress of the metabonomics of hepatitis B virus (HBV)-related hepatic diseases, hoping to discover some potential metabolic markers for identification of HBV-related hepatic diseases from other etiologies and for HBV-related hepatitis, liver cirrhosis and hepatocellular carcinoma. This can contribute to early discovery, diagnosis and treatment, eventually increasing the survival rate of HBV-related hepatic diseases.

Key words: Metabonomics; Hepatitis B virus-related hepatic diseases; Hepatitis B; Hepatitis B virus-related liver cirrhosis; Hepatitis B virus-related hepatocellular carcinoma

Core tip: This review mainly analyzes the research progress of the metabonomics of hepatitis B virus (HBV)-related hepatic diseases, hoping to discover some potential metabolic markers which can distinguish HBV-related hepatic diseases from other etiologies and discover potential metabolic markers of HBV-related hepatitis, liver cirrhosis and hepatocellular carcinoma, which can contribute to early discovery, diagnosis and treatment.

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METABONOMICS AND THE LIVER IN BRIEF

The main function of the liver is the synthesis and metabolism of various proteins, polysaccharides and fats and the detoxification of the body's normal metabolic wastes, such as uric acid, drugs and chemical products[1,2]. There are many hepatic diseases that threaten health. However, because of a lack of effective early diagnosis methods, a large number of the diseases are in the middle to late stages when detected, which seriously affects the prognosis. Therefore, it is important to find tumor markers with high sensitivity and specificity as well as to elucidate the pathogenesis.

Metabonomics, a branch of systematic biology, is a recent newly developing subject. It aims to explore biological systems, like the changes in metabolites of the cells, tissues and certain organisms in the environment of exogenous stimulations, especially studying metabolites weighing less than 1000. Metabonomics integrates gene regulation, post-transcriptional regulation and the interaction of the pathways together, which makes different metabolites manifest significant biological phenotypes through the stages of the cell directly. Compared to the vast information in genomics, transcriptomics and proteomics, there is more information about apparent learning[3]. Thus, metabonomics has recently been widely used to discover the pathogenesis, finding potential metabolic markers with high sensitivity and specificity and exploring new diagnosis and treatment methods in order to increase early phase diagnosis rates of certain diseases and provide a new basis for targeted therapy[4].

The morbidity of hepatocellular carcinoma (HCC) ranks 5th and its mortality ranks 3rd as a malignancy worldwide[5]. The incidence in southeast Asia and Africa is especially high, about 20 per 100000 population[6]. HCC has many risks with HBC as the primary one, causing 780000 death yearly[7]. The evolutionary progress of chronic hepatic disease is from chronic hepatitis B (CHB), hepatitis B virus (HBV)-related cirrhosis to HBV-related HCC. Nowadays, liver biopsy is the golden criteria in differentiating hepatic fibrosis, liver cirrhosis (LC) and HCC but cannot be used universally because of the invasiveness. Abdominal ultrasound is still the first screening method for hepatic diseases. It is widely used clinically because it is noninvasive and cheap. However, its sensitivity is affected by the machine, operators and different states of the disease. The sensitivity of diagnosing early cirrhosis is especially low, only 32% to 65% in HCC[8,9]. However, as a widely used clinical serum biomarker for HCC, alpha fetoprotein shows no increase in 80% of small HCC and its overall sensitivity is just 70%[8-11]. Some liver fibrosis indexes, such as hyaluronic acid, procollagen type Ⅲ, procollagen type Ⅳ and laminin, can indicate early hepatic cirrhosis by analyzing the proliferation and degeneration of hepatic fibrosis. However, its sensitivity and specificity remain unknown[12]. As an essential metabolic organ, any organic disease of the liver will lead to changes in the whole body's metabolism, causing widespread concern for medical staff. Research on the relationship between hepatic diseases and metabonomics has been increasing yearly. This review mainly analyzes the research progress of the metabonomics of HBV-related hepatic diseases, hoping to discover some potential metabolic markers for identification of HBV-related hepatic diseases from other etiologies and for HBV-related hepatitis, LC and HCC. It can contribute to early discovery, diagnosis and treatment, eventually increasing the survival rate of HBV-related hepatic diseases.

THE METABONOMIC WINDOW INTO HBV-RELATED HEPATIC DISEASES

CHB

Chronic HBV infection is a global problem, mainly in developing countries and especially in southeast Asia and Africa. About 600000 people die of acute or chronic HBV infection each year[13]. Chronic HBV infection can result in hepatitis, hepatic fibrosis and even LC and HCC. Presently, the main treatment methods for chronic HBV infection are interferon treatment[14-16], nucleotide analogue treatment[17-19], immune treatment[20-22], *etc.* Although they can reduce the transformation from CHB to LC and HCC, their cure rates still need to improve. In the meantime, the pathogenic pathway of chronic HBV infection is still unclear. In the metabonomic study of patients with chronic HBV infection, some metabolites with a significant difference were found, which may provide some basis for discovering a pathogenic pathway and ideas for new targeted therapy.

As shown in Table 1, there are 2 studies concerning CHB. Zhou *et al*[23] analyzed the metabolites in serum from CHB patients and a control group by liquid chromatography-mass spectrometry (LC-MS) and discovered 12 metabolites with a difference that were involved in fatty acids, amino acids, bile acids and energy metabolism and other pathways[24]. To date, there are still few metabonomic studies about CHB so it is a research domain that needs to be expanded. Autoimmune hepatitis (AIH) is an inflammatory reaction of the liver caused by autoantibodies. Early diagnosis can result in successful treatment. However, due to the unknown pathogenesis, the diagnostic rate is low and the prognosis cannot be estimated. Wang *et al*[25] studied metabonomic characteristics of AIH by nuclear magnetic resonance (NMR) for the first time, providing a basis for researching the pathogenesis of AIH and discovering potential metabolic markers further. About 11% of patients with nonalcoholic steatohepatitis (NASH) will develop LC after 15 years and 7% will develop HCC through LC or directly after 6.5 years[26]. The metabolic changes of NASH refer to the metabolism of fatty acids, carbohydrates and bile acids[27-29]. The metabonomic research for chronic hepatitis C has discovered that the

Table 1 Summary of metabolomic studies of chronic hepatitis B

Ref.	Year	Species	Tissue	Platform	Up-regulated	Down-regulated
Zhou *et al*[23]	2012	Human CHB 30 N 30 CHB/N	Serum	LC-MS	Cortisol, GCA, GCDCA, LysoPC (15:0), LysoPE (22:6), C16:1-CN	Tryptophan, C10-CN, C10:1-CN, C8-CN, C6-CN
Soga *et al*[24]	2011	Human CHB 7 N53 CHB/N	Serum	CE-TOM LC-MS	γ-Glu-Thr	

CHB: Chronic hepatitis B; LC-MS: Liquid chromatography-mass spectrometry; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; LysoPC: Lysophosphatidylcholine; LysoPE: Lysophosphatidylethanolamine; CN: C16:1-acylcarnitine.

Table 2 Summary of metabolomic studies of hepatitis B virus-related liver cirrhosis

Ref.	Year	Species	Tissue	Platform	Up-regulated	Down-regulated
Liu *et al*[42]	2013	Human LC 42 N 18 LC/N	Serum	NMR LC-MS	L-phenylalanine, C16 sphinganine, alpha-CEHC, LysoPC (18:1), linoelaidic acid, PC (18:4/20:1), bilirubin	L-carnitine, decanoyl-L-carnitine, phytosphingosine, 3α, 6α, 7α, 12α-tetrahydroxy-5β-cholan-24-oic acid PC (14:1/14:1), LysoPC (16:0)
Wang *et al*[39]	2012	Human LC 63 N 31 LC/N	Urine	GC-MS UPLC-TOFMS	Prolile, citrate, aconitate, 3,4-dihydroxyphenylacetate, taurohyocholate, glycocholate, glycoursodeoxycholate	Threonine, hippurate, 2-aminobutyrate, cis-aconitate, pyroglutamate, alpha-hydroxyisobutyrate, 3-hydroxyisovalerate, alpha-hydroxyhippurate, estrone
Zhou *et al*[23]	2012	Human CIR 30 N 30 CIR/N	Serum	LC-MS	GCA, GCDCA, CN	Tryptophan, LysoPC (20:5), LysoPC (0:0/14:0), LysoPC (22:6), LysoPC (14:0/0:0), LysoPE (20:4), C10-CN, C10:1-CN, C8-CN, C6-CN
Yin *et al*[41]	2009	Human LC25 N25 LC/N	Serum	RRLC	Taurocholic acid fragment, GCA, bilirubin, TCDCA fragment, GCDCA, oleic acid fragment, taurocholic acid fragment, carnitine fragment, L-acetylcarnitine	Hypoxanthine, lysoPC C18:2, LPC C18:3, LPC C16:1, LPC C18:0, Hypoxanthine fragment, inosine, taurine, 6-methylnicotinic acid
Xue *et al*[40]	2009	HBV infected human LC20 non-LC 20 LC/non-LC	Serum	GC-MS	Acetic acid, hexanoic acid, 1-naphthalenamine, butanoic acid	Sorbitol, D-Lactic acid, phosphoric acid, D-glucitol, glucose

HBV: Hepatitis B virus; LC-MS: Liquid chromatography-mass spectrometry; GCA: Glycocholic acid; TCDCA: Taurochenodeoxycholic acid; GCDCA: Glycochenodeoxycholic acid; LC: Liver cirrhosis; PC: Phosphatidylcholine; NMR: Nuclear magnetic resonance; Alpha-CEHC: 2,5,7,8-Tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman; LysoPC: Lysophosphatidylcholine; LysoPE: Lysophosphatidylethanolamine; UPLC-TOFMS: Ultra-high performance liquid chromatography-time of flight-mass spectrometer; CN: C16:1-acylcarnitine; GC-MS: Gas chromatography-mass spectrometer.

up-regulation of AKR1B10 expression in urine leads to abnormal glucose metabolism[30]. In studies about acute alcoholic hepatitis, Rachakonda *et al*[31] detected metabolites that were distinctly different from those in alcoholic LC that were involved in the metabolic process of fatty acids, bile acids, proteins and carbohydrates.

LC

LC is the terminal stage of chronic liver diseases (CLD), with a high morbidity worldwide. Chronic HBV infection is an important pathogenic factor of LC[32] and the evolution of HBV-related LC is a gradual progress[33]. Due to a lack of specific diagnostic methods, the incidence rate of LC is 3.7 per 100 person-years in HBV carriers[34] and the 5 years survival rate of decompensated LC patients is only 14% to 35%[35,36], while 70% to 90% of HBV-related HCC developed from decompensated LC[37,38]. To date, there are still few valuable markers for early diagnosis of HBV-related LC and it is especially important to detect potential biomarkers with a higher sensitivity and specificity.

Table 2 shows 5 studies regarding the metabonomics of HBV-related LC, 4 of them based on serum and 1 based on urine. According to the Child-Pugh scores, all the LC patients were classified into three groups, A, B and C. Wang *et al*[39] carried out a urinary metabonomic study on the different stages of HBV-related LC and healthy controls using a gas chromatography-mass spectrometer (GC-MS) and ultra performance liquid chromatography time-of-flight mass spectrometry. They found metabolites with a significant difference in the three groups of LC, which may be potential metabolic markers in different stages of LC, providing a basis for the estimate of progress. Differently from the other three studies, Xue *et al*[40] chose patients with CHB as a control group and found nine metabolites with an obvious difference in total. The study also further verified the distinguishing ability by SAS software, showing that five out of twenty LC patients in Child-Pugh A were misdiagnosed as patients with CHB due to the small

sample size. Zhou *et al*[23] and Yin *et al*[41] analyzed the metabolites in the serum of a HBV-related LC group and healthy control group by LC-MS and NMR, with both methods discovering metabolites with differences[42]. Among these five articles, only one used hepatitis B patients as a control group and the others chose healthy volunteers. In these present studies, we still lack research that uses CHB patients as a control group. The identification sensitivity of potential metabolic markers in patients with early HBV-related LC and patients with CHB found in present studies should be further discussed.

Primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are two diseases relevant to metabolic disorders of bile acid. Due to the insidious onset and lack of effective diagnostic methods with high specificity, patients are usually in an advanced stage when diagnosed[43]. Trottier *et al*[44] analyzed the metabolic changes of 17 bile acids in patients with these two diseases by LC-MS. Compared to healthy volunteers, the primary bile acid in serum in the two diseases increased significantly, which may be associated with impairment of the enterohepatic circulation. Compared with PBC, the levels of secondary bile acid in the PSC group decreased obviously. It suggests that PBC is only relevant to the impairment of the extrahepatic bile duct, while PSC involves both the intrahepatic and extrahepatic bile duct. Furthermore, Bell *et al*[45] also drew similar conclusions by LC-MS. Acute-on-chronic liver failure (ACLF) is acute liver failure resulting from the acute deterioration of liver function on the basis of CLD, which can be accompanied by multiple organ failure at the same time. Due to its yearly increasing incidence and high mortality rate, ACLF is receiving more and more attention from the medical profession[46]. Amathieu *et al*[47,48] studied the metabonomic characteristics of LC patients with and without ACLF and detected obvious differences in the metabolic features of the two groups. Nie *et al*[49] discovered 17 potential markers by comparing HBV-related ACLF with HBV-related LC in Child-Pugh A and 11 of them had improved survival after treatment, which has implications for the early diagnosis and prognosis assessment of ACLF. Lian *et al*[50] researched metabolic differences in alcoholic LC and HBV-related LC by LC-MS and found that oleamide and myristamide increased significantly in patients with alcoholic LC but decreased distinctly in patients with HBV-related LC, which indicated that they both could be used as specific metabolic markers to distinguish alcoholic LC from HBV-related LC. By GC-MS and LC-MS, Fitian AI, Soga *et al*[24] and Fitian *et al*[51] found that some bile acids and dicarboxylic acids increased in hepatitis C virus (HCV)-related LC. Also, γ-glutamyl dipeptides were mentioned in both studies and there was thought to be some expressed differences in different types of hepatic diseases. Therefore, it can be used as a potential metabolic marker to differentiate various hepatic diseases. So far, metabonomics of various hepatic diseases is still in the primary stages, lacking the metabolomic difference analysis comparing the diverse types of hepatic diseases. Therefore, the field of metabonomics of hepatic diseases needs further research.

HCC

In China, over 80% of HCC cases resulted from chronic HBV infection, an evolutionary progress from CHB to LC and eventually to HCC[32,33]. To improve the diagnostic rate for early HCC, potential biomarkers with high specificity which can be adopted to screen the HBV-related LC need to be explored. Some metabolites which are specifically expressed in HBV-related HCC may provide a new horizon for the targeted treatment of HCC in the future.

In Table 3, 4 studies from China exploring metabolomics of HBV-related HCC are shown, complying with the regional differences of HCC. The potential metabolic markers found in these studies involve the metabolism pathways of fatty, amino and bile acids, energy and so on. Liu *et al*[52] researched the metabolomic characteristics of liver tissue in 10 patients with liver carcinoma by LC-MS. Based on the comparison of the central area of the tumor and distant tissue, 14 metabolites were found with obvious differences and 9 of them[53-55] have also been mentioned in other studies. However, beta-sitosterol, quinaldic acid, arachidyl carnitine, tetradecanal and oleamide have rarely been mentioned, possibly because the levels of these 5 metabolites are too low in serum to be detected. It indicates that although metabolic profiling of tissue cannot reflect the changes of systemic metabolism in the human body, it could actually reflect the changes of metabolic characteristics of certain tissues or organs. Li *et al*[56] compared the metabolomic characteristics of HBV infected HCC host cells HepG2.2.15 with HCC host cell HepG2 by NMR and found that 11 metabolites were obviously different. N-acetyl glucosamine kinase had a significantly increased expression in HepG2.2.15 and was involved in the hexosamine biosynthesis pathway, which demonstrated that the hexosamine biosynthesis pathway was activated in HBV infected cells, providing a new thought for studying targeted therapy for HBV infection in the future. Zhou *et al*[23] and Yin *et al*[41] analyzed the metabolites of HBV-related HCC and normal bodies by LC-MS and found some potential biomarkers of metabolism involved in the metabolism of fatty acid, phosphoric acid, amino acid and glucose. Both studies found that the expression of glycochenodeoxycholic acid, lysophosphatidylcholine and glycocholic acid were significantly different in patients with HCC.

Besides the infection with HBV, infection with HCV, the addition of alcohol and steatohepatitis are also important pathogenic factors in HCC. We found 3 studies regarding HCV-related HCC[51,57,58] from the United States. Compared to the research of HBV-related HCC, other body fluid samples were added, as well as serum, containing metabolomic characteristics of HCV-related HCC and LC. Bowers *et al*[57] analyzed the metabolomic

Table 3 Summary of metabolomic studies of hepatitis B virus-related hepatocellular carcinoma

Ref.	Year	Species	Tissue	Platform	Up-regulated	Down-regulated
Li *et al*[56]	2015	Human	Liver	NMR	Fructose-bisphosphatealdolase, glucose-6-phosphate isomerase, alpha-enolase, citrate synthase	4-hydroxyphenylpyruvate dioxygenase
		Hepatoblastoma cell line HepG2.2.15/HepG2	Host cell		Phosphoglycerate kinase 1 Triosephosphate isomerase Sussinate dehydrogenase Malate dehydrogenase	Fumarylacetoacetase
Liu *et al*[52]	2013	Human	Liver	UPLC-MS	Sitosterol-beta, L-phenylalanine, LysoPC [18:2 (9Z, 12Z)], quinaldic acid glycerophosphocholine, LysoPC (18:0)	Arachidyl carnitine
		HCC 10			LysoPE (18:0/0:0), chenodeoxycholic acid glycine conjugate	Tetradecanal
		Central/distant			LysoPE [18:3 (9Z, 12Z, 15Z)/0:0] LysoPC [22:6 (4Z, 7Z, 10Z, 13Z, 16Z, 19Z)] M LysoPC [20:4 (5Z, 8Z, 11Z, 14Z)]	Oleamide
Zhou *et al*[23]	2012	Human HCC 30 N 30 HCC/N	Serum	LC-MS	GCA, GCDCA, C16:1-CN	Tryptophan, C10:1-CN, C8-CN, C10-CN, C6-CN, LysoPC (20:5) LysoPC (0:0/14:0), LysoPC (20:3), LysoPC (14:0/0:0)
Yin *et al*[41]	2009	Human HCC 24 N 25 HCC/N	Serum	LC-MS	Taurocholic acid, GCA, bilirubin, TCDCA, GCDCA, oleic acid, taurocholic acid, carnitine, L-acetylcarnitine	Hypoxanthine, phytosphingosine, dihydrosphingosine, LPC C18:2, LPC C18:3, LPC C16:1, LPC C18:0 phytosphingosine, inosine, hypoxanthine, taurine, 6-methylnicotinic acid

LC-MS: Liquid chromatography-mass spectrometry; LysoPC: Lysophosphatidylcholine; LysoPE: Lysophosphatidylethanolamine; LPC: Lysophosphatidylcholine; GCA: Glycocholic acid; TCDCA: Taurochenodeoxycholic acid; GCDCA: Glycochenodeoxycholic acid; UPLC-TOFMS: Ultra-high performance liquid chromatography-time of flight-mass spectrometer; CN: C16:1-acylcarnitine; HCC: Hepatocellular carcinoma.

characteristics in serum and urine from HCV-related HCC and chronic hepatitis C patients by LC-MS. Fitian *et al*[51] and Baniasadi *et al*[58] also studied the diversity of serum metabolomics in patients with HCV-related HCC and LC, resulting in some potential metabolic markers with significant differences being detected. There are increasing numbers of people addicted to alcohol with the speeding pace of modern society and about 1/3 of HCC cases result from alcohol worldwide[59]. Nahon *et al*[60] analyzed the metabolic changes of alcoholic LC and HCC by NMR and discovered that the metabolites in a group of alcoholic LC without HCC were apparently different from that of alcoholic LC with large HCC. Glutamine decreased greatly, while metabolites such as glutamate and glycoprotein increased sharply. It indicated that glutamine degradation and glycolysis might be the main metabolic pathway of energy in hepatoma cells. With the improvement of living standards and the changes in lifestyle, the incidence of non-alcoholic fatty liver disease is increasing yearly and is currently up to 30% in developed countries[61,62]. Excessive deposition of fat in the liver can cause NASH, liver fibrosis, LC and even HCC[63]. Beyoğlu *et al*[64] specifically analyzed the research about non-alcoholic HCC in their review. Most of the research used healthy people as the control group, while a small part used patients with LC. The potential metabolic markers detected were involved in the metabolic processes of fatty acids, bile acids and so on. There are some differences between the metabolic markers found in this research and in the research on HBV-related HCC. More research is needed to find the pathogenesis in order to provide the basis for targeted treatment of HCC of different etiologies in the future.

PROSPECTS

Metabonomics is still in the beginning and developing stage but it has drawn wide attention from the medical community. There are some short comings in its analysis technology and data analysis methods which require further completion and improvement. At present, metabonomics is just applied to common diseases. In our review, there are some obvious metabonomic differences between HBV-related hepatic diseases and other liver diseases, which have some research value and may provide evidence for detecting specific markers and elucidating the pathogenesis of HBV-related hepatic diseases. With the continuous development of medical technology, the prospect of metabonomics is immeasurable. It is expected to develop and enhance clinical diagnosis and treatment in the future, with genomics, transcriptomics and proteomics.

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TOPIC HIGHLIGHT

2016 Advances in Hepatitis C Virus

Chaperones in hepatitis C virus infection

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Abstract

The hepatitis C virus (HCV) infects approximately 3% of the world population or more than 185 million people worldwide. Each year, an estimated 350000-500000 deaths occur worldwide due to HCV-associated diseases including cirrhosis and hepatocellular carcinoma. HCV is the most common indication for liver transplantation in patients with cirrhosis worldwide. HCV is an enveloped RNA virus classified in the genus *Hepacivirus* in the *Flaviviridae* family. The HCV viral life cycle in a cell can be divided into six phases: (1) binding and internalization; (2) cytoplasmic release and uncoating; (3) viral polyprotein translation and processing; (4) RNA genome replication; (5) encapsidation (packaging) and assembly; and (6) virus morphogenesis (maturation) and secretion. Many host factors are involved in the HCV life cycle. Chaperones are an important group of host cytoprotective molecules that coordinate numerous cellular processes including protein folding, multimeric protein assembly, protein trafficking, and protein degradation. All phases of the viral life cycle require chaperone activity and the interaction of viral proteins with chaperones. This review will present our current knowledge and understanding of the role of chaperones in the HCV life cycle. Analysis of chaperones in HCV infection will provide further insights into viral/host interactions and potential therapeutic targets for both HCV and other viruses.

Key words: Hepatitis C; Hepatitis C virus; Chaperones; Heat shock proteins; Viral life cycle

Core tip: Interaction of viral proteins with host chaperones is critical for the hepatitis C viral (HCV) life cycle. Some of these chaperones, such as cyclophilins have been studied in detail recently and have led to the advent of new therapies for HCV infection with high success rates. Further investigation of the role of chaperones in the viral life cycle may allow for development of novel therapies both for HCV and related viruses.

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INTRODUCTION

The hepatitis C virus (HCV) infects approximately 3% of the world population or more than 185 million people worldwide[1,2]. While infection is less prevalent in developed countries including North America, other areas face prevalence rates as high as 3.5% or more[1]. Each year, an estimated 350000-500000 deaths occur worldwide due to HCV-associated diseases[1-3]. HCV is mainly responsible for liver transplantation in patients with cirrhosis worldwide[4-6]. Furthermore, HCV is the most common chronic bloodborne pathogen in the United States affecting 1.5% of the population and is the major etiologic factor responsible for the recent doubling of hepatocellular carcinoma[5,7-9].

HCV is an enveloped RNA virus classified in the genus *Hepacivirus* in the *Flaviviridae* family. It possesses an approximately 9.6 kb positive-sense RNA genome that is translated as a single polypeptide approximately 3000 amino acids in length[10,11]. It is subsequently proteolytically cleaved into 10 viral proteins including the structural proteins core, E1, and E2 as well as the non-structural (NS) proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B[12]. Core is the viral nucleocapsid protein that encapsidates the viral genome in the virion. E1 and E2 are glycoproteins on the viral envelope that are involved in receptor-mediated viral entry. p7 is an integral membrane ion channel also called viroporin that functions to protect virions from acidification during maturation by allowing protons to flow[13]. NS2, NS3, and NS4A are the viral proteases, while NS4B is a helicase. NS5A, a 56-59 kDa multifunctional phosphoprotein, lacks any known enzymatic activity, is a component of the viral replicase complex, and has been implicated in regulation of HCV genome replication, internal ribosomal entry site (IRES)-mediated viral protein translation, and infectious virion assembly[14-18]. NS5B is the viral RNA-dependent RNA polymerase. In addition to these originally identified 10 proteins, another viral protein called the HCV F protein was observed[19,20] and later identified[21-23] to be expressed as a result of a ribosomal frameshift near the beginning of the core protein coding sequence. The F protein has been implicated in the regulation of protein degradation, inhibition of apoptosis, and immunoregulation[24-31].

The 5′ non-coding region (NCR) of the viral genome possesses an IRES, a cis-acting element found in some host RNA transcripts as well as in viruses that allows ribosomal translation initiation to occur internally within a transcript in lieu of 5′ 7-methylguanylate cap-dependent translation[12,32]. The HCV viral life cycle in a cell can be divided into six phases: (1) binding and internalization; (2) cytoplasmic release and uncoating; (3) viral polyprotein translation and processing; (4) RNA genome replication; (5) encapsidation (packaging) and assembly; and (6) virus morphogenesis (maturation) and secretion[33] (Figure 1).

The viral life cycle begins with the attachment of the enveloped virion to the cell followed by entry, which is mediated by interaction of the E1 and E2 glycoproteins in the viral membrane with a number of hepatocyte cell surface receptors and proteins which include the low-density lipoprotein receptor (LDLR), glycosaminoglycans (GAGs), CD81, scavenger receptor B1 (SR-B1), claudin 1, occludin, and the cholesterol absorption receptor Niemann-Pick C1-like 1[34]. Subsequently, the viral particle is internalized through clathrin-mediated endocytosis or an alternative clathrin-independent pathway after which, the viral and cellular membranes fuse through acidification of the endosomal compartment, and the core-encapsidated viral genome is released into the cytosol, uncoated, and subsequently translated[35,36]. The resulting polyprotein is cleaved with the help of the cellular proteases signalase and signal peptide peptidase and the viral proteases NS2-NS3 and NS3-NS4A[37]. Viral genome replication is carried out by NS5B utilizing a negative-sense viral genome intermediate[38]. New virions are assembled at the sites of cytosolic lipid droplets in the vicinity of endoplasmic reticulum (ER) membrane where core protein encapsidates the viral genome followed by budding of the nascent virion into the lumen of ER[39]. The virions follow the Golgi-dependent secretory pathway during which they undergo maturation by addition of lipid components significantly decreasing their buoyant density[40,41]. Finally the mature virions are secreted through exocytosis[42].

In order to establish successful infection, HCV depends on numerous host factors during its entire life cycle. In addition to performing virus-specific functions such as viral genome replication and virion assembly, HCV proteins alter cellular metabolism, critical signaling pathways, and organellar morphology and function to establish persistent infection and to escape the immune responses. Accumulation of misfolded viral proteins in the ER leads to ER stress and the unfolded protein response (UPR) which is a cellular program to help restore ER protein homeostasis by shutting down cellular protein synthesis, properly folding the misfolded proteins, targeting them to ER-associated degradation (ERAD) if folding is unsuccessful, and inducing apoptosis if the

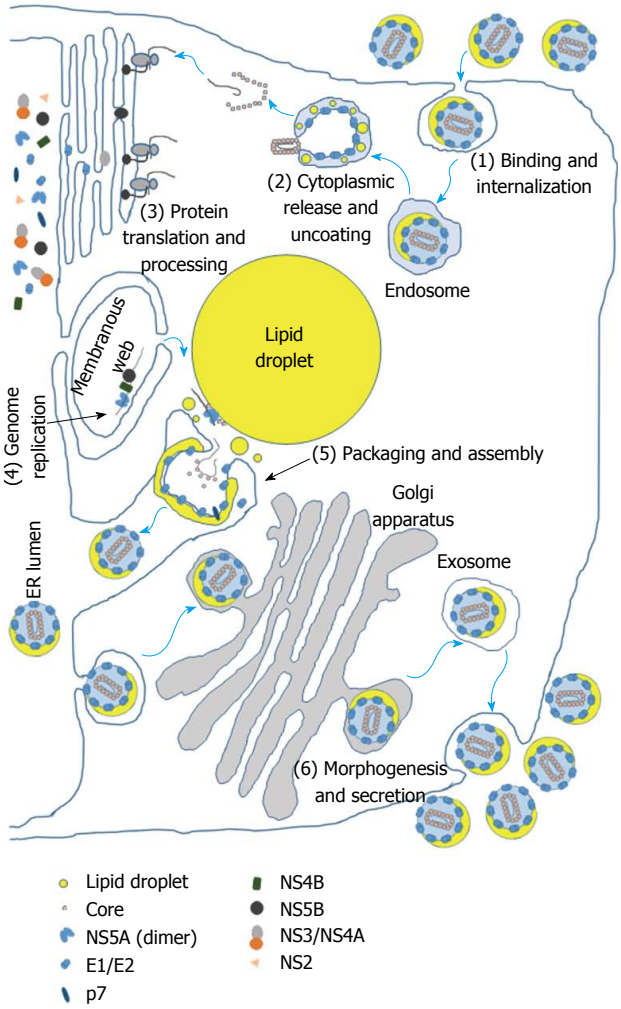

Figure 1 A schematic of the hepatitis C virus life cycle. The six steps of the viral life cycle are indicated in colored boxes with numbers. (1) Binding and internalization. HSC70 is part of the viral particle and may play a role in viral entry. Also HCV internalization occurs at least in part through clathrin-mediated endocytosis which involves HSC70; (2) Cytoplasmic release and uncoating. The chaperone activity of E1 and E2 may be involved in membrane fusion that releases the core-encapsidated viral genome into the cytosol; (3) Protein translation and processing. HSP70, together with the DNAJA2 member of HSP40 co-chaperones, is the main chaperone involved in IRES-mediated translation of the viral genome, while HSP90 may play some role as well. Calnexin, calreticulin, and CypA are also involved; (4) Genome replication. HSP90, some members of HSP40 co-chaperones, TRiC/CCT, FKBP38, SigR1, and some Cyps are involved in viral genome replication. Core and NS3 may play some roles in genome replication as well; (5) Packaging and assembly. HSC70, PDI, and MTTP are the principal chaperones involved in infectious virion assembly, and Cyps also play important roles; and (6) Morphogenesis and secretion. MTTP which is involved in the VLDL pathway also plays important roles in viral particle maturation and secretion. Cyps are also involved. Cyp: Cyclophilin; ER: Endoplasmic reticulum; FKBP: FK506-binding protein; HCV: Hepatitis C virus; HSC70: Heat shock cognate protein 70; HSP: Heat shock protein; MTTP: Microsomal triglyceride transfer protein; NS: Non-structural; PDI: Protein disulfide isomerase; SigR1: Sigma non-opioid intracellular receptor 1; TRiC/CCT: TCP-1 ring complex/chaperonin-containing TCP-1; VLDL: Very low-density lipoprotein.

cell cannot cope with the ER stress[43]. HCV suppresses ERAD and apoptosis thereby maintaining cells under ER stress in order to persistently produce its own proteins. However, HCV maintains a balance between ER stress and the UPR and virus production through different mechanisms some of which are presented in this review[44-46]. Additionally, HCV replication in cells disrupts mitochondrial homeostasis leading to formation of irregular mitochondrial morphology, overproduction of reactive oxygen species (ROS), and oxidative stress[47]. Oxidative stress leads to activation of antioxidant programs to cope with the stress, and if unsuccessful, apoptosis is triggered. As is the case with ER stress, HCV not only induces oxidative stress, but also activates antioxidant programs and suppresses mitochondria-induced apoptosis[44,47,48]. Again, this leads to persistent infection and benefits virus production[44]. Thus, while HCV infection and some viral proteins may be capable of inducing apoptosis[49-51], it is generally agreed that apoptosis is effectively suppressed during infection. A few mechanisms that HCV utilizes to suppress apoptosis are also discussed in this review.

Virus infection of hepatocytes leads to rearrangements of ER membranes to generate double-membrane vesicles (DMVs) and to a lesser extent multimembrane vesicles that are collectively referred to as the membranous web[52]. Viral genome replication occurs within the membranous web in replication complexes (RCs). Infection by all positive-strand RNA viruses results in the formation of membranous web. It is thought that the membranous web benefits viral replication by: (1) protecting viral RNA and proteins from degradation and intracellular antiviral defense; (2) increasing the local concentration of the factors involved in RNA replication; and (3) ensuring spatial proximity of viral RNA translation, viral genome replication, and virion assembly for efficient progression through the viral life cycle[39].

HCV also hijacks the hepatocyte very low-density lipoprotein (VLDL) pathway for the maturation and secretion of infectious viral particles[53]. Lipid secretion is reduced during infection, and maturing viral particles acquire VLDL characteristics, while secreted viral particles are bound to VLDL particles[40,54].

An important group of host factors intimately involved in essentially all steps of the HCV life cycle are molecular chaperones. The term chaperone reflects the significant role of these cytoprotective proteins in: (1) assisting client proteins to achieve native/functional conformation that is required for their function; (2) assembling/disassembling protein subunits; (3) preventing newly synthesized proteins or assembled protein subunits from forming nonfunctional aggregates and molecular crowding; (4) transporting proteins to particular subcellular compartments which is referred to as intracellular protein trafficking; and (5) targeting proteins to degradation if attempts to (re)fold or (re)assemble are not successful[55-57]. Newly synthesized proteins are assisted to fold properly by chaperones. Under stress conditions such as heat shock or viral infection, proteins can become misfolded, and chaperones attempt to refold such proteins. If folding is not successful, the protein gets targeted for proteasome-mediated degradation.

A large number of molecular chaperones belong

to the family of heat shock proteins (HSPs) originally identified as proteins that helped refolding proteins that were denatured as a result of heat stress[58]. HSPs are a highly evolutionarily conserved family of proteins that are typically classified into four different systems based on their molecular weight: HSP70, HSP90, HSP60, and small HSPs[57]. The HSP70, HSP90, and HSP60 systems consist of the adenosine triphosphate (ATP)-dependent main chaperones that utilize their enzymatic activity to induce conformational changes in the client polypeptide by hydrolyzing ATP to adenosine diphosphate (ADP). In addition, a number of co-chaperones may assist and regulate the activity of the main chaperones. Small HSPs, on the other hand, do not possess enzymatic activity, and instead, perform their chaperone function by functioning as holdases, *i.e.*, binding to client polyproteins, preventing their aggregation, and directing them to one of the ATP-dependent HSPs.

HCV has evolved a remarkable ability to interact with numerous chaperones to coordinate the diverse molecular systems and pathways that it requires for its propagation in hepatocytes (Table 1). This review presents our current knowledge and understanding of the chaperones that are involved in the HCV life cycle. First, HSPs are presented covering all four HSP systems HSP70, HSP90, HSP60, and small HSPs. Next, a diverse group of other molecular chaperones are discussed including BAG3, FK506-binding proteins (FKBPs), p23, prefoldin, apolipoprotein J [apoJ or clusterin (CLU)], protein disulfide isomerases (PDIs), microsomal triglyceride transfer protein (MTTP), calnexin (CANX), calreticulin (CALR), "endoplasmic reticulum degradation enhancer, mannosidase alpha-like 1" (EDEM1), EDEM3, sigma non-opioid intracellular receptor 1 (SigR1), prohibitin (PHB), and cyclophilins (Cyps). Finally the chaperone activity of the HCV proteins core, E1, E2, NS3, and NS4A are described. The gene names for the chaperones are also included in parentheses.

HSP70/HSP40 SYSTEM

The HSP70 family of chaperones consists of a large number of proteins that are ubiquitously expressed throughout the cell. They play important roles in proper protein folding, protection of proteins from stress-induced damage, recovery/renaturing of damaged/aggregated proteins, protein degradation, protein translocation, and disassembly of protein complexes such as the DNA replication machinery[59,60]. This family of HSPs typically functions as a group of three proteins where the main HSP70 chaperone interacts with the client polypeptide through its substrate-binding domain (SBD), while the nucleotide-binding domain (NBD) binds to an ATP hydrolyzing it to ADP to induce conformational changes in SBD for its chaperone function. The hydrolysis is stimulated by substrate binding the chaperone resulting in a closed state where it tightly binds the substrate and helps with (re)folding it. Cofactor HSPs also known as co-chaperones, such as HSP40, typically interact with the NBD to modulate chaperone activity and to determine the clients of HSP70s *via* their specificity in binding particular target proteins. A nucleotide exchange factor (NEF) assists with the removal of hydrolyzed ADP which causes the chaperone to revert to its open conformation releasing the substrate.

HSP70 (HSPA1A)

HSP70 is an inducible chaperone that is expressed in conditions of stress such as heat shock and viral infection. HSP70 has been identified as one of the numerous host factors important for HCV production[61-64]. Knockdown of HSP70 led to decreased virus production[61,63] or replication in subgenomic replicon (SGR) systems[62,63]. Both HSP70 overexpression and autoantibodies against HSP70 in the sera of HCV-infected patients have also been reported[65]. Huh7 cells harboring an HCV SGR demonstrated upregulation of HSP70[66]. It was also found that expression of NS5A alone in huh7 cells was sufficient for upregulation of HSP70[67]. This upregulation was the result of NS5A-induced increased levels of nuclear factor of activated T cells 5 (NFAT5), one of the transcription factors responsible for HSP70 expression. The increased NFAT5 levels itself is mediated by NS5A-driven ROS production.

Our laboratory has shown NS5A to colocalize with HSP70 and HSP40 as well[63]. We further showed that knockdown of HSP70 inhibited NS5A-augmented IRES-mediated translation. The HSP synthesis inhibitor quercetin, a bioflavonoid, also suppressed the NS5A-augmented IRES-mediated translation[63,68]. In addition, we demonstrated that the NS5A/HSP70 interaction is direct and identified the site of NS5A/HSP70 interaction on NS5A to be a hairpin moiety at the C terminus of NS5A domain I [17]. Treatment of cells with a synthetic peptide corresponding to this hairpin moiety, which we termed the HSP-binding domain[69], disrupted the NS5A/HSP70 interaction and suppressed NS5A-augmented IRES-mediated translation and virus production[17]. Others have shown that overexpression of HSP70 leads to increased viral RNA and protein levels, while knockdown of HSP70 has the opposite effect[64]. HSP70 was found to interact with NS3-NS4A protein and NS5B as well. HSP70 increases RC formation by interacting with viral proteins in RCs, increasing the stability of viral proteins, and enhancing NS5A-driven viral IRES-mediated translation. Further, HSP70 was found to interact with the 3′ NCR of the viral genome[70].

Heat shock cognate protein 70 (HSPA8)

Heat shock cognate protein 70 (HSC70) is a constitutively-expressed housekeeping gene with diverse cellular functions including protein folding, signal transduction, apoptosis, autophagy, and many others[71]. Viral entry occurs at least in part through the HSC70-dependent clathrin-mediated endocytosis[35]. HSC70 activity was found to be significantly increased in an HCV SGR system[72], and HSC70 levels were increased in a proteomic analysis of RCs[73]. HSC70 was also identified

Table 1 Chaperones and their roles in the hepatitis C virus viral life cycle

Chaperone	Subcellular localization	Function in HCV infection/stage of viral life cycle
HSP70 family		
GRP75 (HSPA9)	Mitochondrial	Varied expression/activity[66,72] Interacts with NS5A[105]
GRP78 (HSPA5)	ER	Regulation of viral protein homeostasis and maintaining a balance between viral and cellular translation to prevent viral protein overload (involves induction of ER stress and the UPR)[43,85-96] Increased expression and activity[72,85,88,93,95] Associated with the viral genome[70,76]
HSC70 (HSPA8)	Cytosolic	Infectious virion assembly[18,74] Potentially contributes to stability of virion structure and viral entry through clathrin-mediated endocytosis[35,74] Associated with the viral genome[70,76] Increased expression and activity[72,73] Knockdown decreases lipid droplet size and virion assembly[18,74]
HSP70 (HSPA1A)	Cytosolic	IRES-mediated translation of viral genome[17,63,64,68,69] Increased expression[65-67] Knockdown decreases IRES activity and virus production[61,63]
HSP70B′ (HSPA6)	Cytosolic	Associated with 3′ NCR of HCV genome[70]
HSP40 family		
DNAJA1	Cytosolic	Co-immunoprecipitates with NS3-NS4A[105]
DNAJA2	Cytosolic	IRES-mediated translation of viral genome[63]
DNAJA3	Mitochondrial	Potentially HCV-induced mitochondrial dysfunction[61,127]
DNAJB1	Cytosolic	Potentially regulates apoptosis[61,117] Knockdown decreases virus production[61]
DNAJB6	Cytosolic	Potentially viral RNA replication[105] Interacts with NS5B[105] Potentially overexpressed[108] knockdown decreases viral RNA replication[105]
DNAJB9	ER	Potentially regulates apoptosis[124] Varied expression[108]
DNAJC1	ER	Interacts with E1 and E2[107]
DNAJC7	Cytosolic	Potentially regulates apoptosis[118] Co-immunoprecipitates with NS3-NS4A[105]
DNAJC8	Cytosolic	Upregulated[119]
DNAJC10	ER	ER protein homeostasis likely benefiting virus production[126] Proper folding of LDLR (viral entry)[126] Likely overexpressed[125]
DNAJC14	ER	Viral RNA replication[62,121,122]
HSP110 family		
HSP105 (HSPH1)	Cytosolic	Overexpressed[129]
HSP70RY (HSPA4)	Cytosolic	Overexpressed[66,130] Knockdown decreases viral RNA replication[130]
Hip (HSPBP1)	Cytosolic	Knockdown decreases virus production[62,134]
HSP90 family		
GRP94 (HSP90B1)	ER	Regulation of viral protein homeostasis and maintaining a balance between viral and cellular translation to prevent viral protein overload (involves induction of ER stress and the UPR)[95,97,101] Suppression of HCV-induced apoptosis[50] Potentially HCV-induced liver fibrosis and autoimmune disease[155] Overexpressed[95,101,130]
HSP90 (HSP90AA1/HSP90AB1)	Cytosolic	Knockdown decreases viral RNA replication[130] HCV RNA replication[138,139,148,149] Maturation and stability of HCV proteins[140-143] IRES-mediated translation of viral genome[144] Circumventing IFNβ response in peripheral B cells[151] Potentially regulates miRNA levels in conjunction with GW182[145] Interacts with NS5A and NS5B[105,107,143] Overexpressed[130,152] Knockdown decreases RNA replication[138]
HSP60 family (chaperonins)		
HSP60 (HSPD1/HSPE1)	Mitochondrial	Regulates ROS production and apoptosis[159] Interacts with core, NS3-NS4A, and viral genome[76,105,107,159] Varied expression[66,130]
TRiC/CCT (TCP1/CCT2-8)	Cytosolic	Viral RNA replication by assisting in RC assembly[73] Increased activity[129,130] Increased TCP1, CCT2, and CCT5 expression[130] Decreased CCT4 expression[129] CCT4 co-immunoprecipitates with NS3-NS4A[105]

		Knockdown of CCT5 decreases viral RNA replication[73]
Small HSPs		
HSP22 (HSPB8)	Cytosolic	Potentially blocks apoptosis[166] Overexpressed[119]
HSP27 (HSPB1)	Cytosolic	Potentially decreases apoptosis[164] Binds NS5A[164] Overexpressed[66]
Other chaperones		
ApoJ (clusterin) (CLU)	Cytosolic	Binds to and stabilizes core and NS5A[190] Overexpressed[190]
BAG3 (BAG3)	Cytosolic	Co-chaperone of HSP90 family Likely blocks ER-stress-induced apoptosis[104]
Calnexin (CANX)	ER	E1/E2 folding and glycosylation[98,107,219,220,223-225] HCV-induced ER stress and viral protein homeostasis[98] Knockdown decreases virus production[62]
Calreticulin (CALR)	ER	E1/E2 glycosylation[98,107] HCV-induced ER stress and viral protein homeostasis[98,101] Overexpressed[101,130,226] Knockdown decreases virus production[62]
Cyp40 (PPID)	Cytosolic	Lipid trafficking and virion secretion[303]
CypA (PPIA)	Cytosolic	RC formation and viral RNA replication[263,270] NS5A and NS5B activation[276,280] Viral polyprotein cleavage[283,301] Regulates IFN response[304] Lipid trafficking and virion assembly and secretion[291,303]
CypB (PPIB)	Cytosolic	RC formation and viral RNA replication[271,272] NS5A and NS5B activation[271,272,274,276]
CypD (PPIF)	Mitochondrial	Inhibits mitochondrial function leading to ROS production[308]
EDEM1 (EDEM1)	ER	Downregulated[103,231] Binds E1 and E2[230] HCV-induced ER stress[230] Targets misfolded glycoproteins to ERAD (viral protein homeostasis)[227,228]
EDEM3 (EDEM3)	ER	Binds E1 and E2[230] HCV-induced ER stress[230] Targets misfolded glycoproteins to ERAD (viral protein homeostasis)[227,228]
Erp72 (PDIA4)	Cytosolic	Increased activity[72]
FKBP38 (FKBP8)	Cytosolic	Co-chaperone of HSP90 family[137] HCV RNA replication[137] Blocks apoptosis[177] Potentially regulates Ca^{2+} homeostasis by interacting with S100 proteins[175] Interacts with NS5A[105,169] Knockdown decreases HCV RNA replication[137]
FKBP54 (FKBP5)	Cytosolic	Interacts with NS5B[105]
GRP58 (PDIA3)	Cytosolic	Overexpressed[125,130] Knockdown decreases viral RNA replication[130]
MTTP (MTTP)	Cytosolic	Part of the PDI/MTTP heterodimer involved in VLDL biogenesis[193] Potentially causes HCV-induced liver steatosis[193,198] Viral maturation and secretion[210,211] Decreased expression and activity[193,198-200]
p23 (PTGES3)	Cytosolic	Co-chaperone of HSP90 family[179] Potentially regulates telomerase activity[180,181]
PDI (P4HB)	ER	Folding and transfer of MTTP to ER as a PDI/MTTP heterodimer involved in VLDL biogenesis[193] Increased activity[129]
PDIR (PDIA5)	Cytosolic	Increased activity[72]
Prefoldin (PFDN1-2/VBP1/PFDN4-6)	Cytosolic	Co-chaperone of TRiC/CCT[182] Binds F protein[183] Regulates cytoskeleton likely to balance virus production in hepatocytes[183]
Prohibitin (PHB/PHB2)	Mitochondrial	Inhibits mitochondrial respiratory function leading to ROS production[237-240] Binds core[238] Overexpressed[236,237]
SigR1 (SIGMAR1)	Cytosolic	Viral RNA replication immediately after entry[44,234] Interorganellar communication between ER and mitochondria[44]
HCV chaperones		
Core		Viral RNA stabilization, dimerization, and structural rearrangement[311-315] Folding of E1[316]

E1	Proper folding of E2[224,318-320]
E2	Proper folding of E1[317]
NS3	Interconversion of viral RNA species[322]
NS4A	Directs NS3 to ER[323]
	Increases NS3 stability[323]

Apo: Apolipoprotein; BAG: BCL2-associated athanogene; Cyp: Cyclophilin; EDEM: Endoplasmic reticulum degradation enhancer, mannosidase alpha-like; ER: Endoplasmic reticulum; ERAD: ER-associated degradation; FKBP: FK506-binding protein; GRP: Glucose-regulated protein; GW: Glycine-tryptophan; HCV: Hepatitis C virus; Hip: HSP70-interacting protein; HSC70: Heat shock cognate protein 70; HSP: Heat shock protein; IFNβ: Interferon beta; IRES: Internal ribosomal entry site; LDLR: Low-density lipoprotein receptor; MTTP: Microsomal triglyceride transfer protein; NCR: Non-coding region; NS: Non-structural; ROS: Reactive oxygen species; PDI: Protein disulfide isomerase; RC: Replication complex; SigR1: Sigma non-opioid intracellular receptor 1; TRiC/CCT: TCP-1 ring complex/chaperonin-containing TCP-1; UPR: Unfolded protein response; VLDL: Very low-density lipoprotein.

to be part of the HCV viral particles, and the viral E2 protein was found to contain the HSC70-interacting histidine-proline-aspartic acid (HPD) motif[74] which is required for the interaction of the HSP40 co-chaperones with HSP70 family of chaperones[75]. Pretreatment of the virus with HSC70 antibody significantly diminished infectivity suggesting that HSC70 is a part of the viral particle[74]. In addition, HSC70, core, and E2 were found to colocalize around lipid droplets, the site of virion assembly. RNAi-mediated knockdown of HSC70 significantly decreased the volume of lipid droplets and viral secretion, but not viral RNA replication levels. These results suggest that HSC70 plays an important role during virion assembly and may play a structural role for the virion as well. It has been observed that HSC70 associates with positive-strand subgenomic viral RNA (corresponding to domains Ⅲ and Ⅳ of the 5′ NCR and 36 nucleotides of core) and the 3′ NCR of the viral genome as well[70,76].

A number of compounds including IMB-DM122, N-substituted benzyl matrinic acid derivatives, and (+)-lycoricidine were shown to downregulate HSC70 mRNA expression leading to decreased virus production[77-79]. Our lab demonstrated that HSC70 directly binds to NS5A *in vitro* and colocalizes with NS5A in infected cells[18]. We further showed that knockdown of HSC70 significantly impacted intracellular infectious virion assembly thereby establishing distinct functions of HSC70 and HSP70 in the HCV life cycle. This is further supported by the fact that HSC70 and HSP70 do not interact with each other. Based on the available evidence, therefore, it seems that HSC70 is important for virion assembly.

HSP70B′ (HSPA6)

HSP70B′ is another member of the HSP70 family which is highly similar to HSPA1A in terms of sequence homology (82%) and function[80]. Both chaperones are stress inducible and work in conjunction to protect cells from stress. However, HSP70B′ is the secondary responder to stress after HSPA1A, and proteasome inhibition is a potent inducer of HSP70B′ expression[81]. HSP70B′ was found to be associated with the 3′ NCR of the HCV genome[70].

Glucose-regulated protein 78 (HSPA5)

Glucose-regulated protein 78 (GRP78), also known as the binding immunoglobulin protein (BiP), is another member of the HSP70 family and is the major molecular chaperone in the ER[82]. The ER is involved in vital cellular processes including protein folding, protein transport, the UPR, and calcium homeostasis. The UPR is an adaptive signaling program that is activated in response to accumulation of unfolded or misfolded proteins in the ER, referred to as ER stress. Proteins that are not successfully folded are either sent for refolding or tagged for degradation through the ERAD pathway[83]. If the UPR program is unable to successfully relieve cells from ER stress, it initiates mitochondria-mediated apoptosis[84]. Under certain conditions such as heat stress and pathogen infection, unfolded or misfolded proteins can accumulate in the ER leading to ER stress and activation of UPR. Stimulation of GRP78 transcription is an indication of ER stress and induction of UPR, which occurs in HCV infection likely to repress cellular protein translation in order to utilize cellular resources for the IRES-mediated translation of viral proteins and to suppress innate immunity in order to establish persistent infection[43,85-96]. GRP78 activity was also found to be significantly increased in an HCV SGR system[72].

UPR signaling can be initiated by three factors: Activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase R-like ER kinase (PERK)[43,92]. These three factors act as ER stress sensors and lead to induction of expression of GRP78, which is itself a negative regulator of the three ER stress sensors. ER stress may lead to the proteolytic cleavage of ATF6, an ER membrane-associated transmembrane protein. The 90 kDa ATF6 precursor, also known as pATF6α(P), is cleaved to form an approximately 50 kDa N-terminal fragment pATF6α(N) which translocates to the nucleus and activates transcription of ER chaperone genes such as GRP78 involved in the UPR. ER stress also leads to phosphorylation of IRE1 which results in the splicing of unspliced X-box-binding protein 1 to spliced XPB1 (sXBP1), a transcription factor that can induce expression of GRP78 and other genes involved in the UPR. Upon initiation of ER stress, PERK can also get activated and phosphorylate the eukaryotic initiation factor 2 alpha (eIF2α). Phosphorylated eIF2α (peIF2α) results in global inhibition of cellular protein synthesis and enhanced ATF4 expression which leads to induction of UPR genes. HCV can activate all three ER stress

sensors.

It was found that the viral glycoprotein E2, and not E1, can induce transcription of GRP78 and that only E2 bound to GRP78[97]. Another group reported that both E1 and E2 bind GRP78[98]. However, it seems that GRP78 tends to bind to E1/E2 aggregates rather than monomeric glycoproteins. Expression of both E1 and E2 was also shown to lead to the UPR[99,100]. The HCV core protein has also been reported to induce expression of GRP78[101]. Induction of core, E1, E2, and p7 in mice liver led to ER stress and overexpression of GRP78[95]. Expression of HCV NS genes led to upregulation of GRP78[102]. The NS2 alone also induces ER stress and leads to upregulation of GRP78 protein levels[46]. NS4B alone can also induce ER stress and the UPR and upregulate GRP78 expression[87,103]. NS5A weakly binds GRP78, enhances GRP78 expression, and protects hepatocytes from ER stress-induced apoptosis leading to persistent infection[104,105]. It was also shown that HCV bearing certain mutations in NS5A and NS5B proteins (C2441S, P2938S or R2985P) displayed higher levels of GRP78 expression[94]. However, it was not clear whether NS5A alone can induce ER stress in these studies. Another group reported that NS5A does not lead to ER stress and the UPR[89,106]. An SGR system expressing all the NS proteins led to the UPR as well[106]. Thus, it is not clear whether the NS5 proteins alone can cause ER stress and the UPR. GRP78 was also shown to benefit virus production in a genome-wide expression analysis of multiple huh7-derived cell lines where interactions with core, E1, E2, p7, NS3, NS4B and NS5A were implicated[107]. Furthermore, GRP78 is a target of miR-30a, miR-30c, and miR-30e that were found to be downregulated in acute HCV infection potentially leading to GRP78 overexpression[108].

In addition to the ER-targeted E1 and E2 proteins, cytosol-targeted E1 and E2 proteins have also been described with opposing functions in the context of ER stress[109-112]. In the cytosol, E1 binds to the cytoplasmic domain of PERK. Furthermore, cytosolic E1 leads to downregulation of GRP78. Similarly, E2 binds to PERK as well, inhibits its kinase activity, reverses PERK-mediated global translation repression, and confers resistance to ER stress. In addition, NS2 leads to phosphorylation of eIF2α and decreased protein synthesis as well as reduction of IRES-mediated translation suggesting that NS2 can also provide a negative feedback regulation of ER stress by decreasing viral protein translation that is responsible for inducing ER stress[46].

Thus, it seems that GRP78, as well as other ER-resident chaperones, play an important role in regulating and maintaining viral protein homeostasis to ensure the availability of sufficient viral proteins to establish a persistent infection while minimizing cellular protein expression and preventing viral protein overload. GRP78 was also found to be associated with positive-strand subgenomic viral RNA (corresponding to domains Ⅲ and Ⅳ of the 5′ NCR and 36 nucleotides of core) and the 3′ NCR of the viral genome[70,76].

A recent study reported that there was no significant difference in the mRNA levels of GRP74 and a number of other genes involved in ER stress and UPR between infected patients and healthy controls[113]. No difference in GRP78 protein levels were observed either. This may be attributed to the fact that typically HCV infects a small percentage of hepatocytes, and therefore, changes may not be detected.

GRP75 (HSPA9)

GRP75 also known as mtHSP70 or mortalin is the mitochondria-resident HSP70 family member. It plays a number of critical roles in the cells including anti-apoptosis, protein transport into mitochondria which may involve HSP60 as well, protection of cells from ROS, and mitochondrial biogenesis[114]. It has also been implicated in membrane trafficking and human immunodeficiency virus (HIV) virion release[115]. In the context of HCV, it has been reported that GRP75 activity was significantly increased in one HCV SGR system[72], while GRP75 protein was significantly downregulated in another SGR system[66]. These different results may reflect the HCV-mediated modulation of GRP75 activity/expression to accommodate its needs during the viral life cycle. Furthermore, NS5A was shown to co-immunoprecipitate with GRP75[105].

HSP40 family

The HSP40 family are co-chaperones of HSP70 proteins that regulate the activity of HSP70s and determine their client range by binding specific target proteins[60,116]. This large family of proteins are homologous with the bacterial DnaJ chaperone, and the term DNAJ is utilized in the gene nomenclature of the isoforms of this family. DNAJA1 and DNAJA2 are the most abundant cytosolic HSP40 co-chaperones[116]. DNAJA1 was reported to co-immunoprecipitate with the NS3-NS4A protein[105]. We have shown that DNAJA2 participates together with HSP70 in regulating the NS5A-augmented IRES-mediated translation of the viral genome[63]. The interaction of viral proteins with these co-chaperones may, therefore, modulate chaperone activity to benefit the viral life cycle. A genome-wide siRNA screening identified DNAJB1 to be important for HCV production[61]. DNAJB1 plays important roles in regulating apoptosis and cell proliferation[117]. DNAJC7 co-immunoprecipitates with NS3-NS4A protein[105]. DNAJC7 also regulates apoptosis by binding to the pro-apoptotic p53 protein and increasing its activity and stability[118]. Thus, it can be speculated that binding of NS3-NS4A may prevent the pro-apoptotic function of DNAJC7/p53 thereby suppressing apoptosis and contributing to persistent HCV infection. DNAJC8 was reported to be upregulated in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) as well as microarray analyses of host gene expression in infected huh7 cells[119]. DNAJC8 has been shown to play an important role in regulating pre-mRNA splicing by the spliceosome[120]. This is achieved by the binding of DNAJC8 with "serine/arginine-rich splicing

factor protein kinase 1". DNAJB6 interacts with NS5B, and shRNA-mediated knockdown of DNAJB6 led to a significant decrease in viral RNA replication[105]. DNAJB6 may, therefore, be required for the stability or activity of NS5B for viral RNA replication. In addition, miR-17, miR-106a, and miR-106b with DNAJB6 as their target were found to be downregulated in acute HCV infection[108].

There are seven ER-resident HSP40 co-chaperones: DNAJB9, DNAJB11, DNAJC1, DNAJC3, DNAJC10, DNAJC23 and DNAJC25. DNAJC14 was found as a host factor involved in HCV replication in an siRNA screen where knockdown of DNAJC14 led to increased viral replication[62]. Further, DNAJC14 has been reported to be involved in RNA replication of yellow fever virus (YFV) and other flaviviruses including HCV[121] and has been shown to be important for RC assembly in YFV[122]. Overexpression of DNAJC14 blocked viral RNA replication in all flaviviruses tested including HCV, while NS2/3 cleavage was not inhibited. siRNA-mediated knockdown of DNAJC14 also demonstrated similar results indicating that both elevated and reduced levels of DNAJC14 interferes with viral RNA replication. Also DNAJC14 is recruited to YFV RCs consistent with the normal cellular function of DNAJC14 as an ER-localized co-chaperone involved in protein transport[121,123]. DNAJB9 was identified in a microarray analysis as one of the host genes with most consistently modified expression as a result of acute HCV infection[108]. Further, miR-17, miR-106a, and miR-106b that target DNAJB9 were found to be downregulated. DNAJB9 has been shown to be involved in regulation of apoptosis[124]. DNAJC10 expression was found to be increased in HeLa cells expressing HCV polyprotein[125]. DNAJC10 is also a member of the PDI family of chaperones (discussed below) which is responsible for removing non-native disulfide bonds in conjunction with BiP and targeting misfolded proteins for degradation[126]. Interaction of DNAJC10 with EDEM1, an ER chaperone (discussed below), is required for disulfide bond reduction. Interestingly, DNAJC10 is also required for the correct folding of LDLR, one of the cell surface receptors utilized by HCV for entry. DNAJC1 was identified as an antiviral protein in a genome-wide expression analysis of multiple huh7-derived cell lines where interactions with E1 and E2 were implicated[107].

Five members of the HSP40 family have been identified in mitochondria: DNAJA3, DNAJC11, DNAJC15, DNAJC19, and DANJC20. DNAJA3 was identified as an HCV-interacting protein[61]. DNAJA3 is normally involved in maintaining mitochondrial morphology, and altering DNAJA3 levels leads to mitochondrial fragmentation and reduced cell viability[127]. HCV infection leads to mitochondrial dysfunction, and DNAJA3 may play a role in this process.

NEFs

NEFs play an important role in normal chaperone functioning by facilitating replacement of the hydrolyzed ADP with an ATP[128]. Three families of NEFs have been identified for the HSP70 chaperones: (1) HSP110/GRP170; (2) HSP70-interacting protein (Hip) (HSPBP1)/BiP-associated protein (SIL1); and (3) the BCL2-associated athanogene (BAG) family of proteins. The HSP110/GRP170 family consists of three cytosolic members HSP105 (HSPH1), HSP70RY (Apg-2) (HSPA4), and OSP94 (Apg-1) (HSPA4L), and one mitochondrial member GRP170 (HYOU1).

It was found that HSP105 and HSP70RY expression levels increase in HCV SGR systems[66,129,130]. Also knockdown of HSP70RY in an SGR system decreased viral RNA replication levels[130]. This is expected as the levels and activity of HSP70 family members increase during HCV infection which may require more NEFs for their function. Furthermore, HSP110 levels increase in stressed cells likely to assist in coping with stress, and in the context of HCV infection, increased HSP110 levels may help cells with HCV-induced ER stress. Similar effects of overexpression of HSP110 has been reported in cancer and gastric ulcer where targeting HSP110 had beneficial effects[131-133]. In siRNA screens, it was found that knockdown of Hip led to a significant decrease in virus production levels[62,134]. The role of BAG3 in HCV infection is discussed below.

HSP90 SYSTEM

The HSP90 proteins are highly conserved evolutionarily and are involved in the folding of proteins especially those involved in signal transduction[135]. Thus, HSP90 possesses a more discrete range of clients compared with the HSP70 system. Like HSP70, HSP90 also undergoes conformational changes to assist with the folding of client proteins, a process which is driven by ATP hydrolysis, and co-chaperones also assist in regulating HSP90 function. HSP90 has been shown to be important for a large group of viruses including HCV[136]. The HSP90 family consists of the inducible cytosolic isoform HSP90α (HSP90AA1), the constitutively expressed cytosolic isoform HSP90β (HSP90AB1), the inducible ER isoform GRP94 (HSP90B1), and the mitochondrial isoform "tumor necrosis factor (TNF) receptor-associated protein 1" (TRAP1) (HSP90L).

HSP90 (HSP90AA1 and/or HSP90AB1)

HSP90 has been shown to be important for virus production[137]. siRNA-mediated knockdown of HSP90 as well as HSP90 inhibitors geldanamycin, "17-dimethylaminoethylamino-17-demethoxygeldanamycin" (17-DMAG), herbimycin A, and radicicol resulted in dose-dependent suppression of HCV in a replicon system[138]. Further, viral levels in chimeric mice with a humanized liver treated with 17-DMAG were significantly reduced. Other derivatives of geldanamycin as HSP90 inhibitors have also been reported to block HCV RNA replication[139].

HSP90 is required for the maturation of the viral polyprotein complex specially to generate functional NS2/3 protease[140]. HSP90 inhibitors were shown to block NS2/3 cleavage. Expression of HCV core in *Saccharomyces cerevisiae* impaired the growth of yeast cells, and it was found that HSC82, the yeast homolog

of HSP90, is required for the stability of core protein[141]. Treatment of yeast cells with the HSP90 inhibitors geldanamycin, radicicol, herbimycin A, and herbimycin C suppressed core-induced growth impairment. HSP90 directly binds to NS3 through the NS3 helicase region and is required for NS3 stabilization[142,143]. In an SGR system, the HSP90 inhibitor "17-N-allylamino-17-demethoxygeldanamycin" (17-AAG) resulted in NS3 degradation specifically[142]. In the same SGR system, 17-AAG also suppressed HCV RNA replication in a dose-dependent manner. However, it was not clear if replication was affected directly or through decreased IRES translation. A subsequent study demonstrated the indirect interaction of HSP90 with the subunit C of eIF3c which involves and is dependent on the viral IRES RNA[144]. This interaction prevents the ubiquitination and the subsequent proteasome-dependent degradation of eIF3c which is required for IRES-mediated translation of the viral genome. Therefore, treatment with HSP90 inhibitors may prevent the chaperoning of eIF3c by HSP90 which leads to its degradation. Knockdown of eIF3c inhibited IRES-mediated translation, but not cellular 5' 7-methylguanylate cap-dependent translation.

HSP90 was found to colocalize and co-immunoprecipitate with glycine-tryptophan (GW) 182, an important component of GW bodies which are involved in mRNA degradation and translational repression *via* miRNAs[145]. Both HSP90 and GW182 also colocalized with NS3, core, and NS5A. Knockdown of GW182 significantly decreased HCV RNA levels in infected cells, while overexpression of GW182 resulted in a significant increase in viral RNA levels. The HSP90 inhibitor 17-DMAG and knockdown of HSP90 significantly decreased GW182 and miR-122 levels leading to decreased HCV RNA levels. Ethanol was shown to upregulate both GW182 and HSP90 thereby facilitating HCV RNA replication. Interestingly, the same group discovered infectious exosomes from sera of HCV-infected patients or supernatants of infected huh7.5 cells that contained negative-strand viral RNA in association with Argonaute 2 [a component of the RNA-induced silencing complex (RISC)], HSP90, and miR-122[146]. These exosomes are capable of transmitting HCV infection in a CD81, SR-B1, and apolipoprotein E (apoE) receptor-independent manner, which was blocked by miR-122 and HSP90 inhibitors. An interaction between NS5A and HSP90 was also implicated in a genome-wide expression analysis of multiple huh7-derived cell lines[107]. Thus, viral proteins may modulate GW182 activity in an HSP90-dependent manner in order to regulate viral RNA replication and miRNA levels. A number of miRNAs have been shown to be modulated by HCV infection[108].

Treatment with the HSP90 inhibitor 17-DMAG was shown to destabilize phosphoinositide-dependent kinase 1 (PDK1), an upstream kinase of protein kinase C-related kinase 2 (PRK2)[147]. The PDK1-PRK2 signaling pathway leads to phosphorylation of NS5B, which is required for HCV RNA replication[148,149]. 17-DMAG-driven destabilization and degradation of PDK1 diminished NS5B phosphorylation levels leading to suppression of viral RNA replication[147]. An interaction between NS5B and HSP90 has also been reported in a yeast two-hybrid system[143]. NS5B co-immunoprecipitates with both isoforms of HSP90 as well[105].

Peripheral B cells have been proposed to serve as reservoirs for persistent HCV infection[150,151]. It was found that peripheral B cells in patients with chronic HCV infection circumvent the interferon beta (IFNβ)-mediated antiviral response in part by downregulating HSP90 which acts as a stabilizer of TANK-binding kinase 1 involved in phosphorylation of the interferon-regulatory factor 3 (IRF3) transcription factor that induces IFN expression[151]. Thus, by using this HSP90-mediated strategy, HCV in B cells evades detection by the immune system contributing to recurring infection even after liver transplant.

The constitutively expressed isoform of HSP90, HSP90AB1, was found to be significantly overexpressed in the mononuclear cells of HCV-infected patients[152]. Co-infection with HIV decreased the overexpression of HSP90AB1 in the same study. HSP90AB1 was also reproducibly enriched in the detergent-resistant membrane fraction of an SGR system[130].

HSP90 also plays an important role in HCV RNA replication in conjunction with FKBP38, a co-chaperone of HSP90 family, which is a member of the immunophilin family of proteins. The role of FKBP38 and its interaction with HSP90 is discussed in detail in the FKBP38 section below. Another HSP90 co-chaperone p23 is also involved in the HCV life cycle and is discussed below as well.

GRP94 (HSP90B1)

GRP94 is the ER-resident HSP90 isoform which is involved in folding of secreted proteins, ER stress, and the UPR[153]. It was found that the viral glycoprotein E2, and not E1, can lead to the ER stress response and induce transcription of GRP94[97]. This leads to activation of nuclear factor kappa B and induction of anti-apoptotic proteins[50]. In addition, knockdown of GRP94 abolished the anti-apoptotic activity of E2 suggesting that E2 inhibits apoptosis induced by HCV infection and leads to persistent viral infection in hepatocytes. The HCV core protein also contributes to ER stress by inducing the expression of GRP94[101]. Increased expression of GRP94 was also observed in the liver of mice conditionally expressing HCV structural proteins core, E1, E2 and p7[95]. No binding of GRP94 to either E1 or E2 glycoproteins was observed[98]. GRP94 was reproducibly enriched in the detergent-resistant membrane fraction of SGR cells[130]. HCV utilizes GRP94 as well as other ER-resident chaperones especially GRP78 to maintain viral protein homeostasis in the ER in order to establish persistent infection and suppress cellular protein translation. GRP94 was also shown to be beneficial for virus production in a genome-wide expression analysis of multiple huh7-derived cell lines where interactions with core, E2, NS3, and NS4B were implicated[107]. Knockdown of GRP94 in an SGR system led to a significant decrease

in viral RNA replication levels as well[130].

GRP94 is prevented from translocating to the cell surface by "aminoacyl tRNA synthetase complex-interacting multifunctional protein 1" (AIMp1)/p43[154], which is a cofactor of aminoacyl tRNA synthetase complex and is involved in regulating transforming growth factor beta (TGF-β) signaling. Translocation of GRP94 to the cell surface leads to activation of dendritic cells and leads to autoimmune diseases. The HCV E2 protein has been reported to directly bind AIMp1/p43 and lead to its degradation through ubiquitination and the proteasome pathway[155]. In addition, E2 interferes with the AIMp1/p43-GRP78 interaction leading to lower cellular AIMp1/p43 levels. Decreased AIMp1/p43 levels in cells leads to elevated TGF-β signaling and cell surface expression of GRP94. Therefore, these mechanisms may be responsible for HCV-induced liver fibrosis and autoimmune diseases.

HSP60 SYSTEM

HSP60 chaperones also known as chaperonins are an important family of HSPs involved in protein folding and macromolecular assembly[156]. The HSP60 family consists of mitochondrial and cytosolic proteins. The mitochondrial HSP60 (encoded by *HSPD1* and *HSPE1* genes), also known as mtHSP60, is thought to have originated in the bacterial ancestors that were engulfed by early eukaryotic cells giving rise to the mitochondrial organelle. HSPD1 (the homolog of bacterial GroEL) forms tetradecamers, composed of two stacked heptameric rings with a central cavity that accommodates the target protein. HSPE1 (the homolog of bacterial GroES) forms one heptameric ring that serves as a cap for the HSPD1 structure. The HSPD1/HSPE1 complex functions in protein folding in an ATP-dependent manner. The eukaryotic/cytosolic chaperonin, also known as "TCP-1 ring complex/chaperonin-containing TCP-1" (TRiC/CCT), is homologous to the Archean thermosome complexes forming hexadecamers consisting of two octameric rings to assist in oligomeric protein assembly[157] and folding of approximately 10% of the proteome[158]. TRiC/CCT is composed of eight paralogous subunits encoded by *TCP1* and *CCT2-8* genes. The TRiC/CCT complex lacks a GroES-like homolog and instead uses a built-in cap system. Typically, the term HSP60 is used to refer to the mitochondrial chaperonin, whereas the eukaryotic cytosolic homolog is referred to as TRiC/CCT.

HSP60 (HSPD1/HSPE1)

Proteomic analyses of huh7 cells harboring an HCV SGR demonstrated downregulation of HSP60[66], while it was shown to be reproducibly enriched in the detergent-resistant membrane fraction of another SGR system[130]. However, these studies did not validate HSP60 levels by Western analysis or in the context of viral infection. HSP60 has been shown to interact with core[107,159]. This interaction led to production of ROS and sensitization of cells to TNFα-induced apoptosis[159]. Further, overexpression of HSP60 decreased ROS production and prevented apoptosis in core-expressing cells. Thus, binding of core to HSP60 seems to impair the function of HSP60 in regulating ROS production and apoptosis as a possible pro-oncogenic process. However, significant research is still required to elucidate the function of the HSP60 system in the context of HCV infection. Nevertheless, HSP60 has been shown to be important for Dengue virus production (also a positive-stranded RNA virus) although the exact function has not been elucidated[160]. Further, HSP60 is overexpressed in HBV and HIV infection[156,161]. Autoantibodies against HSP60 have been detected in sera of chronic HCV infected patients[162]. HSP60 has also been shown to co-immunoprecipitate with the NS3-NS4A protein[105] and associate with positive-strand subgenomic viral RNA (corresponding to domains Ⅲ and Ⅳ of the 5′ NCR and 36 nucleotides of core)[76].

TRiC/CCT (TCP1/CCT2-8)

The activity of TRiC/CCT, the cytosolic chaperonin, was reported to be increased in an SGR system[129]. Also TCP1, CCT2, and CCT5 were reproducibly enriched in the detergent-resistant membrane fraction of an SGR system[130]. TRiC/CCT also plays an important role in the assembly of RCs which mediate HCV RNA replication[73]. This may be facilitated by an interaction between the subunit CCT5 of TRiC/CCT and NS5B. siRNA-mediated knockdown of CCT5 suppressed viral RNA replication. Treatment with an antibody against CCT5 also suppressed HCV RNA synthesis in an *in vitro* cell-free assay. These observations suggest that NS5B may recruit TRiC/CCT to the RCs to assemble components of RCs in order to facilitate HCV RNA replication. It was also reported that that CCT4 can co-immunoprecipitate with the NS3-NS4A protein[105]. CCT4 activity was decreased in an SGR system[129].

TRiC/CCT is regulated by a number of co-chaperones including prefoldin. The role of prefoldin in the HCV life cycle is discussed below.

SMALL HSPS

Small HSPs constitute a family of ten proteins with molecular mass in the range of 12-43 kDa with diverse functions including protein folding, development, and eye lens tissue formation to name a few[163]. They lack enzymatic activity and work as holdases in conjunction with the ATP-dependent chaperones to carry out their functions[57].

HSP27 (HSPB1)

Proteomic analyses of huh7 cells harboring an HCV SGR have demonstrated upregulation of HSP27[66]. HSP27 was found to bind NS5A (and not NS5B) in co-immunoprecipitation studies and colocalize by immunofluorescence under heat shock conditions[164]. The N-terminal regions of both proteins were found to be involved in the interaction (amino acids 1-122 of

HSP27 and 1-181 of NS5A). While the function of this interaction is not known, it has been speculated that it may decrease infection-induced apoptosis. This is likely as HCV is known to modulate apoptosis in order to establish persistent infection. In fact, HSP27 is overexpressed and has anti-apoptotic roles in several cancers as well[165].

HSP22 (HSPB8)

HSP22 is a multifunctional chaperone involved in regulation of protein folding, macroautophagy, carcinogenesis, and apoptosis[166]. HSP22 was reported to be significantly overexpressed in infected huh7 cells as determined by qRT-PCR as well as microarray analyses of host gene expression[119]. HSP22 is an anti-apoptotic protein, and its upregulation by HCV may be one of the mechanisms that HCV utilizes to block apoptosis in hepatocytes.

OTHER CHAPERONES

In addition to HSPs, cells possess a number of other molecular chaperones and co-chaperones that play critical roles in numerous cellular functions by assisting with protein folding and stability in their respective pathways.

BAG3 (BAG3)

BAG3 is one of the BAG family of proteins and serves as a NEF for the HSP70 family of chaperones. BAG3 is the only heat stress-inducible BAG isoform and plays important roles in cell proliferation, apoptosis, adhesion, and migration[167]. It acts as an anti-apoptotic protein in different cancers. In the context of HCV infection, it was found that overexpression of NS5A in HepG2 cells upregulated a number of anti-apoptotic genes including BAG3 when the cells were treated with thapsigargin, an inducer of ER stress[104]. GRP78 was also overexpressed.

FKBP38 (FKBP8) and FKBP54 (FKBP5)

FKBP38 is a co-chaperone of the HSP90 family and a member of the immunophilin family of chaperone proteins which possess peptidylprolyl isomerase (PPIase) activity and also serve as receptors for the immunosuppressive drug FK506[168]. FKBP38 was identified as an NS5A interacting protein in a fetal liver cDNA library screen, and both NS5A and FKBP38 colocalize to mitochondria and the ER[169]. NS5A and FKBP38 were also shown to co-immunoprecipitate[105]. FKBP38 interacts with HSP90 and plays an important role in HCV RNA replication. FKBP38 forms a complex with HSP90 and NS5A where FKBP38 binds to both HSP90 and NS5A through different sites in its tetratricopeptide repeat domain[137]. Both knockdown of FKBP38 and treatment with geldanamycin suppresses HCV RNA replication in a replicon system indicating that the HSP90/NS5A/FKBP38 complex is important for the regulation of HCV RNA replication. In fact, the FKBP38/NS5A interaction is so critical for the virus that a single amino acid mutation in NS5A that disrupts its binding with FKBP38 impairs virus production[170]. The same group found that HSP90 binds to human butyrate-induced transcript 1 (hB-ind1)[171], which is a member of the Rho family of GTPases and a component of the Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling pathway[172,173]. hB-ind1 was found to bind to NS5A and is involved in viral RNA replication through its interaction with HSP90. Thus, by interacting with NS5A, hB-ind1 recruits HSP90 and FKBP38 to the RCs. In addition, through immunofluorescence analyses, it was found that hB-ind1 colocalizes with NS5A, FKBP38, and double-stranded viral RNA at the site of the membranous web[174]. These results further support the role of HSP90 in viral RNA replication. Moreover, treatment with an HSP90 inhibitor decreased the HCV-induced UPR which points to a potential involvement of HSP90 in an hB-ind1-mediated protein folding mechanism in the membranous web in order to circumvent the virus-induced UPR.

It was also found that a few members of the S100 family of proteins, S100A1, S100A2, S100A6, S100B and S100P directly bind FKBP38 in cell-free *in vitro* assays in a Ca^{2+}-dependent manner[175]. The S100 proteins are a family of 24 Ca^{2+} binding proteins which are involved in regulating inflammation, cell proliferation and differentiation, apoptosis, cell migration and invasion, and Ca^{2+} homeostasis[176]. The S100/FKBP38 interactions blocked both NS5A/FKBP38 and HSP90/FKBP38 interactions[175]. Furthermore, overexpression of S100A1, S100A2 and S100A6 suppressed HCV RNA replication. S100P was identified as one of the proteins with most consistently modified expression in acute HCV infection[108].

FKBP38 has also been reported to be involved in HCV suppression of apoptosis[177]. NS5A plays an important role in HCV pathogenesis by activating the mammalian target of rapamycin (mTOR) pathway. This leads to suppression of apoptosis and hepatocyte cell survival which is required for persistent infection. NS5A exerts its anti-apoptotic activity by blocking the interaction between FKBP38 and mTOR.

FKBP54 (p54), another FKBP family member, was reported to co-immunoprecipitate with NS5B[105]. FKBP54 is an important co-chaperone involved in regulating a number of signaling pathways, steroid hormone receptors, and autophagy[178].

p23 (PTGES3)

p23 (prostaglandin E synthase 3) is another HSP90 co-chaperone and an inhibitor of HSP90 ATP turnover[179]. In addition, p23 together with HSP90 are essential telomerase components, and telomerase activity as well as expression of multiple telomerase components were reported to be significantly induced in HCV infection of huh7.5 cell[180]. The same group also showed that expression of the La protein (Sjogren syndrome antigen B), a regulator of HCV IRES-mediated translation[181], significantly correlated with the expression of telomerase components including telomerase RNA, p23 and HSP90 in HCV-infected patient tissues. Thus, HCV may regulate

telomerase activity in an HSP90-dependent manner which may potentially be linked to HCV-induced hepatocarcinogenesis.

Prefoldin (PFDN1-2/VBP1/PFDN4-6)

Prefoldin is the co-chaperone of the cytosolic chaperonin TRiC/CCT. It is a hexameric protein complex consisting of the six subunits encoded by the PFDN1-2, VBP1 (PFDN3), and *PFDN4-6* genes[182]. Newly synthesized proteins at ribosomes bind to prefoldin which in cooperation with HSP70/HSP40 transports them to TRiC/CCT for proper folding and preventing protein aggregation. Prefoldin also plays an important role in clearing aggregated proteins as a result of ER stress or proteasome inhibitor treatment.

The HCV F protein, a 17 kDa product of ribosomal frameshift at the beginning of the core protein coding sequence, was found to bind prefoldin 2[183]. Prefoldin is involved in the proper folding of actin and tubulin subunits and plays an important role in the formation of the cytoskeleton. It was found that overexpression of the HCV F protein interfered with the prefoldin 1 and 2 interaction and resulted in an aberrant tubulin cytoskeleton. It was speculated that since an intact cytoskeleton is needed for HCV production in infected cells[184-187], the HCV F protein may modulate and decrease virus production in order to establish a persistent chronic infection[183].

ApoJ/clusterin (CLU)

ApoJ, also known as clusterin, is another chaperone with both intracellular and extracellular functions including protein folding and extracellular protein degradation and is involved in a number of age-related diseases including cardiovascular and neurodegenerative diseases and cancer likely by interacting with HSP60[188,189]. HCV infection led to increased clusterin expression both in cell culture and serum of infected patients[190]. siRNA-mediated silencing of clusterin led to decreased virus production without affecting viral RNA replication levels suggesting a subsequent step such as translation, assembly, or secretion is affected. It was found that clusterin binds to and stabilizes core and NS5A.

PDI (PDI family) and MTTP (MTTP)

The PDI family of proteins are ER chaperones that are responsible for disulfide bond formation[191]. The term PDI typically refers to the beta subunit of the prolyl 4-hydroxylase (P4H) enzyme, PDIA1 (P4HB), which is the first characterized member of the PDI family[192]. P4HB is involved in the folding and transfer of MTTP, a chaperone itself, from the cytosol into the lumen of ER[193,194]. P4HB and MTTP subsequently form a heterodimer, and MTTP then lipidates and stabilizes apolipoprotein B (apoB), a component of the VLDL produced by hepatocytes. ApoB associates with triglyceride containing particles generating VLDLs, and MTTP is involved in VLDL secretion as well[194,195].

It has been shown that core expression leads to decreased MTTP activity, in an HCV genotype 3-dependent manner[196] thereby reducing VLDL formation and secretion, which leads to accumulation of lipids in HCV-infected hepatocytes and subsequently liver steatosis[193,197,198]. Viral NS proteins have also been shown to decrease MTTP expression and activity and implicated in inhibition of VLDL secretion likely due to interaction of NS5A and apoB[199]. NS5A overexpression was also shown to decrease the expression of MTTP and increase lipid droplet size[200]. Furthermore, MTTP gene polymorphisms contribute to the accumulation of lipids in hepatocytes and may predict sustained virological response (SVR) to antiviral therapy in patients infected with genotype 4[201-204]. Thus, HCV infection is highly dependent on modulation of lipid metabolism, possibly in a genotype-specific manner[205-207], through interactions with MTTP[208]. During maturation, the newly assembled virions acquire low-density configuration prior to being secreted, a process that requires MTTP, and the secreted viral particles are bound to VLDL[54,209,210]. Secretion of viral particles depends on the apoB-positive lipoprotein particles in an MTTP-dependent manner, while virion assembly (and infectivity through LDLR and GAGs) requires apoE and is not MTTP and VLDL dependent[34,211-216].

P4HB activity was found to be increased in an HCV SGR system[129]. GRP58 (PDIA3), an important ER chaperone[191,217], was found to be overexpressed in HeLa cells expressing HCV polyprotein[125]. Further, GRP58 was reproducibly enriched in the detergent-resistant membrane fraction of an SGR system, and knockdown of GRP58 led to a significant decrease in viral RNA replication[130]. The activity of two other PDI family members ERp72 (PDIA4) and PDIR (PDIA5) were also significantly increased in an HCV SGR system[72,191]. ERp5 (PDIA6) activity was reduced in an SGR system. It should be noted that SGR systems do not produce infectious virus, and the activity/expression of PDIs may, therefore, not correspond with the context of viral infection.

The PDI family also includes DNAJC10, an HSP40 family member, which is discussed in the HSP40 section above.

Calnexin (CANX) and calreticulin (CALR)

Protein glycosylation among other post-translational modifications is carried out in the ER/Golgi apparatus. Calnexin and calreticulin are ER-resident chaperones that play a crucial role in the proper folding and glycosylation of glycoproteins. Both chaperones are part of a quality control mechanism in the ER that occurs in a cyclical manner[218]. Both HCV E1 and E2 being glycoproteins undergo the same cycles of quality control until they achieve the proper folding conformations required for the assembly of virions[98]. siRNA-mediated knockdown of calnexin and calreticulin decreased virus production[62].

Both E1 and E2 rapidly associate with calnexin immediately after synthesis in the ER, but dissociate slowly[61,98,107,219]. While E2 folding occurs rapidly and is complete upon cleavage of the E2-NS2 precursor

polyprotein, folding of E1 is slow. Their association with calnexin parallels this timing suggesting that calnexin plays a role in proper folding of the E1/E2 glycoprotein complexes[220]. Calreticulin binds to E1 and E2 glycoproteins as well[98,107]. Whereas calnexin preferentially binds to monomeric glycoproteins, calreticulin seems to bind to E1/E2 aggregates. The N-linked oligosaccharides on these glycoproteins are important for the formation of E1/E2 complexes and for their interactions with some chaperones as treatment with tunicamycin, a glycosylation inhibitor, blocked the interaction of E1/E2 complexes with calnexin and calreticulin preventing their maturation and suppressing virus production[98,221,222]. Virus infectivity may also be impaired due to incorporation of immature glycoproteins in some virions[222]. Rather than being secreted, the E1/E2 complexes seem to remain in the ER and do not migrate past the cis-Golgi apparatus and are subsequently utilized in assembly of virions after undergoing proper folding and complex formation. Properly folded E1/E2 heterodimers no longer interact with calnexin[223-225].

NS2 was reported to co-immunoprecipitate with CANX in infected cells[105]. All viral NS proteins were found to colocalize with the newly synthesized HCV RNA and calnexin at RCs which are ER-derived perinuclear structures[52]. In agreement with this observation, calnexin was reported to be associated with positive-strand subgenomic viral RNA (corresponding to domains Ⅲ and Ⅳ of the 5′ NCR and 36 nucleotides of core)[76]. Calnexin is also a target of miR-130a, miR-130b and miR-310 that were shown to be downregulated in acute HCV infection[108]. HCV core protein causes ER stress thereby inducing the expression of calreticulin[101]. Calreticulin was reproducibly enriched in the detergent-resistant membrane fraction of an SGR system[130]. HCV infection was also found to increase calreticulin expression[226].

EDEM1 (EDEM1) and EDEM3 (EDEM3)

EDEMs that consist of three proteins EDEM1, EDEM2, and EDEM3 are lectin chaperones and regulators of ERAD that are involved in targeting misfolded glycoproteins to the ERAD pathway[227,228]. EDEMs binds to the target glycoproteins that are destined for degradation[229]. EDEMs also bind GRP78 and appear to provide the signal for degradation of the target glycoprotein[227]. EDEM1 and EDEM3, but not EDEM2, directly bind HCV glycoproteins and increase their ubiquitination[230]. Knockdown of EDEM1 and EDEM3 as well as treatment with kifunensine, an ERAD inhibitor, increased the half-life of E1 and E2 and virus production, and overexpression of the two EDEMs decreased virus production.

As mentioned above, misfolded proteins in the ER are targeted to the ERAD pathway if attempts to properly fold these proteins are unsuccessful[83]. While HCV production in cells leads to ER stress and the UPR, the virus has evolved strategies to prevent its proteins from being degraded through the ERAD pathway[44]. The ERAD pathway is activated downstream of the IRE1 pathway, and the IRE1 pathway is activated in response to HCV-induced ER stress and activation of the UPR[92]. However, despite activation of the IRE1 pathway, activation of the ERAD pathway is inhibited in HCV infection[231]. Thus, although sXBP1 is produced indicating activation of the IRE1 pathway, expression of EDEM1 is suppressed. This seems to be unique for HCV as other flaviviruses do not suppress EDEM expression in presence of sXBP1 production[83,231]. HCV NS4B similarly leads to production of sXBP1, but suppresses EDEM expression[103]. The lack of EDEM induction may also lead to increased IRES-mediated translation of viral proteins[231]. These results suggest that EDEMs may play a crucial role in regulating viral protein homeostasis and maintaining a balance in viral protein production to establish persistent infection.

SigR1 (SIGMAR1)

SigR1 is a cholesterol-binding chaperone in lipid-rich areas of ER and mitochondrion-associated ER membranes (MAMs)[232]. MAMs play an important role in pathogenesis of HCV by serving as interorganellar communication sites between ER and mitochondria both of which are crucial for HCV production[44]. SigR1 is normally involved in crucial processes including cellular response to stress, lipid and protein trafficking, cell survival, and neuroprotection[232,233]. SigR1 has been reported to play an important role for viral RNA replication immediately after virion entry, but not afterwards during persistent infection[44,234]. siRNA-mediated knockdown of SigR1 reduced viral RNA replication only in early stages of infection.

Prohibitin (PHB) and prohibitin 2 (PHB2)

The mitochondrial chaperone prohibitin is involved in a variety of processes including mitochondrial protein folding and membrane potential, cell cycle, and apoptosis[235]. It forms a ring structure composed of two subunits encoded by the *PHB* and *PHB2* genes. The HCV core protein as well as viral infection lead to overexpression of prohibitin[236,237], which is a target of the HCV core protein[238]. Core binds to prohibitin and impairs its chaperone function thereby preventing the proper function of mitochondrial respiratory chain leading to overproduction of ROS which may result in hepatocarcinogenesis[237,238]. This is likely caused by the core-mediated suppression of the interaction between prohibitin and subunit Ⅰ and Ⅳ of cytochrome C oxidase[239,240].

Cyps (PPI family)

Cyps are an important family of molecular chaperones most of which possess PPIase activity and are involved in diverse cellular processes including protein folding, scaffolding, protein trafficking, and apoptosis[241]. The genes that encode Cyps are referred to as PPIs. Cyps have been reported to be important for replication of HCV as well as other flaviviruses[242], and Cyp inhibitors such as cyclosporine A (CsA) have been shown to effectively block virus production when used alone

or in combination with other antiviral agents such as IFN[243-262]. Cyps have been suggested to play important roles in the HCV life cycle including viral RNA replication, membranous web formation, viral polyprotein cleavage, lipid trafficking, virion assembly, suppression of IFN-based antiviral response, and induction of mitochondrial dysfunction.

It has been suggested that NS5B is recruited to the RCs in the membranous web by cyclophilin A (CypA) (PPIA) likely to ensure NS5B retains its proper conformation for viral RNA replication[263]. In fact, both NS5B and CypA share a common binding site on NS5A[264] suggesting that CypA delivers NS5B to the RCs at which point NS5B binds NS5A. This function of CypA is supported by the finding that treatment of cells with CsA reduces the levels of NS5B in RCs, but not NS5A or NS3[263]. In addition, mutant NS5B from CsA-resistant replicons retained their RC incorporation in presence of CsA. Other published Cyp inhibitor-selected mutations in NS5B have been reported to increase its RNA binding capacity[265-267]. Also the observed CsA resistance of the JFH1 strain (genotype 2a) is NS5B dependent[268]. PPIase mutant CypA maintained its NS5B binding[263]. However, the mutant CypA was unable to rescue HCV replication in CypA knockdown cells implicating its PPIase activity is important for HCV replication. Another study reported that CypA does not recruit NS5B or NS5A to RCs as CsA treatment did not affect the RC association of NS5B and NS5A, concluding the possibility of a CypA-independent recruitment of NS5B and NS5A to RCs[269]. A recent report seems to resolve this discrepancy[270]. It was found that Cyp inhibitor treatment did not affect the replicase activity of RCs after active RCs are established. This suggests that Cyp inhibitors exert their antiviral activity prior to formation of active RCs supporting the originally proposed CypA-mediated NS5B recruitment model.

In addition, NS5B binds to CypB (PPIB) which is required to stimulate the RNA-binding activity of NS5B and RNA synthesis[271-273]. Both CypA and CypB activate NS5B replicase function, particularly RNA binding, *in vitro* where CypB demonstrates viral genotype 1b specificity[274]. It was shown that the lack of PPIase activity in mutant CypA and CypB had some effect on NS5B activation, but the PPIase mutant CypA and CypB were still capable of activating NS5B to a significant extent suggesting that the PPIase activity is dispensable for NS5B activation. However, these experiments were performed in a cell free system, whereas the previous experiments showing the importance of PPIase activity in HCV replication were performed in a replicon system. Others have shown NS5B/CypB interaction to be mediated by CsA-associated helicase-like protein in GST pulldown assays[275].

Significant evidence also points to a role of Cyps in viral RNA replication through their PPIase activity likely inducing conformational changes in viral and/or host proteins for optimal functioning. NS5A is a substrate for the PPIase activity of CypA and CypB through many proline residues in NS5A domain Ⅱ and the linker region between NS5A domains Ⅱ and Ⅲ (known as the low-complexity sequence Ⅱ or LCS-Ⅱ)[276-278]. A three amino acid structural motif, a proline-tryptophan turn, is essential for HCV RNA replication and proper interaction with CypA and influences the PPIase activity of CypA on NS5A domain Ⅱ[279]. CypA also binds NS5A domain Ⅲ and has PPIase activity towards some peptidylprolyl bonds in NS5A domain Ⅲ[280]. The NS5A/CypA interaction and the PPIase activity of CypA, which are both disrupted by Cyp inhibitors, have been shown to be critical for HCV production[280-289], and the PPIase activity of CypA is required for the NS5A/CypA interaction[281]. Further, wild-type CypA rescued viral RNA replication under CypA knockdown, but a PPIase mutant did not[284]. Indeed, it was found that CypA interacts with NS5A and stimulates RNA binding of NS5A domain Ⅱ in a PPIase-dependent manner[290,291]. Furthermore, some SNP mutations in the PPIase domain of CypA render hepatocytes resistant to HCV replication likely by decreasing the intracellular stability of CypA[292]. Mutant NS5A from Cyp inhibitor resistant virus still binds to CypA as wild-type NS5A *in vitro*[281,282,286], whereas in cell culture the interaction appears much stronger than with wild-type NS5A implying other cellular proteins are important for this interaction[170]. NS5B was found to further strengthen this interaction as well. Others have provided an alternative mechanism for resistance through NMR analyses showing that the resistant NS5A exhibited a trans to cis conformational shift possibly rendering NS5A less dependent on the PPIase activity of CypA for isomerization[285]. Importantly, the Cyp inhibitor-induced NS5A mutation can rescue viral replication under CypA knockdown conditions[282] although it still requires CypA at lower levels[293]. Thus, most of the evidence to date suggests that CypA is the most important Cyp in the context of HCV replication and that CypA and NS5A are the main targets of Cyp inhibitor-mediated antiviral activity as knockdown of CypB, CypC (PPIC), and CypD (PPIF) failed to suppress viral replication, and NS5A mutations have the major role in Cyp inhibitor resistance compared with NS5B and other viral proteins[263,265,283,284,293-298].

Cyp inhibitor treatment also prevents formation of DMVs that are required for RNA replication at RCs suggesting that Cyps are involved in formation of RCs as well[270]. While the NS3-NS5B polyprotein and even NS5A alone suffices for formation of DMVs, knockdown of CypA prevents DMV formation suggesting that Cyps and, in particular, CypA is required for DMV formation. In addition, the PPIase activity of CypA was found to be required for DMV formation indicating that both NS5A and CypA are crucial for formation of DMVs.

The JFH1 SGR (lacking NS2) is not very sensitive to CsA or NIM811 (another Cyp inhibitor)[299], and it was shown that full-length JFH1 was inhibited much more efficiently by CsA implicating NS2 to be important for CsA-mediated viral inhibition in a CypA-dependent manner[283,300,301]. Subsequently, it was found that NS2

itself is not a target of CsA, but that the rate-limiting NS2-NS3 cleavage determines sensitivity to CsA[301]. It has been suggested that NS3 also binds Cyps and that mutations in NS3 may also lead to CsA resistance[297,302]. Also it was found that the CypA dependence of HCV replication correlates with the NS5A-NS5B cleavage kinetics as demonstrated by substitution mutants at this cleavage site[283]. These findings indicate that viral polyprotein cleavage may at least in part be dependent on Cyps especially CypA.

CsA has also been shown to affect hepatocyte lipids pointing to an additional role of Cyps in lipid trafficking and in HCV pathogenesis[303]. Cyp inhibitor treatment disrupts the VLDL pathway of virus maturation described above resulting in increased lipid droplet size, accumulation of apoB on lipid droplets, removal of NS5A from lipid droplets, and inhibition of infectious virion assembly[291,303]. The Cyps involved were found to be CypA and Cyp40 (PPID).

Yet another role of CypA in viral infection has been suggested in the context of the IFN pathway[304]. It was found that CypA and IRF9, a component of the JAK/STAT pathway, directly bind each other *via* the PPIase domain of CypA and the newly-identified CypA binding site in the IRF-association domain of IRF9. Cyp inhibitors prevent this complex formation. Interestingly, NS5A and IRF9 compete for binding to CypA, and CypA inhibition led to increased IFN-induced transcriptional activity through interferon-sensitive response elements (ISREs). Thus, it seems that HCV utilizes NS5A to dampen the IFN response by replacing IRF9 in the CypA/IRF9 complex, in order to establish persistent infection in hepatocytes. Furthermore, it was observed that Cyp inhibitor treatment blocks phosphorylation of protein kinase R (PKR) and its target eIF2α which inhibits translation of interferon-stimulated genes[305,306]. Cyp inhibitors also blocked stress granule formation. CypA binds PKR, and this interaction was disrupted by Cyp inhibitor treatment as well[305]. However, it was reported that Cyp inhibitor-mediated inhibition of PKR phosphorylation is due to suppression/clearing of viral infection rather than being a direct effect[306]. Thus, the significance of the CypA/PKR interaction and its disruption by Cyp inhibitors is not clear.

It is also reported that CsA treatment of uninfected huh7 cells induces the UPR and upregulation of GRP78[307]. Further, treatment of cells with UPR-inducing agents suppressed HCV replication. This may suggest that CsA may also exert its antiviral activity by inducing UPR which likely leads to improper viral glycoprotein/protein folding, their aggregation, and subsequent degradation.

The Cyp inhibitor alisporivir has also been found to prevent and to some extent reverse the negative impacts of HCV infection on mitochondrial function revealing another potential role for Cyps in the context of viral infection[308]. In particular, alisporivir prevents HCV-mediated collapse of the mitochondrial membrane potential, overproduction of ROS, and mitochondrial calcium overload through inhibition of CypD-mediated opening of the mitochondrial permeability transition pore[308-310].

HCV PROTEINS AS CHAPERONES

Remarkably, some HCV proteins possess chaperone functions that are critical for virus production. For example, core, in particular the N-terminal domain I, has been shown to play important chaperone roles for viral RNA stabilization, dimerization, and structural rearrangements[311-315]. Also core appears to be involved in folding of the E1 glycoprotein[316]. Both viral glycoproteins E1 and E2 have been reported to possess chaperone functions. E2 has been reported to be required for proper E1 folding[317]. The disulfide bonds in E1 have been shown to be required for the proper function of E2 during viral assembly and entry[318], and E2 does not seem to be able to reach a native structure in the absence of E1[319]. Further, a monoclonal antibody was reported to recognize properly folded E2 only when complexed with E1[224]. Also the ectodomain of E2 was shown to fold only in presence of E1[320]. CANX may be important for the chaperone activities of HCV glycoproteins[220]. This is in agreement with the observation that E2, unlike E1, did not associate with cellular chaperones such as CANX in an infection-free system[319]. In many class Ⅱ enveloped viruses, of which HCV is a member, one viral glycoprotein acts as a chaperone for the folding of the other one which carries out the membrane fusion after viral entry in order to release viral genome in the cytosol[321]. However, for HCV, the mechanism of membrane fusion and the role of glycoproteins is not fully understood. The NS3 protein which possesses a helicase domain has been reported to mediate functions beyond the known helicase activity as it is involved in "intermolecular annealing, resolves three-stranded RNA duplexes, and assists dsRNA and ssRNA inter-conversions to establish a steady state among RNA structures"[322]. NS4A directs NS3 to ER and increases the intracellular stability of NS3[323].

CONCLUSION

Chaperones play crucial roles in HCV infection, and essentially all phases of the viral life cycle depend on chaperone functions and the interaction of viral proteins with chaperones (Table 1). The critical roles of Cyps and HSP90 in HCV RNA replication among others, HSP70 in viral protein translation, HSC70 in virion assembly, and the ER chaperones GRP78 and GRP94 in viral protein stability and persistent infection are important examples. Better understanding of the role of chaperones in the viral life cycle will provide further insights into the mechanism of virus production and suppression of immune response. Recently, significant advancements have been achieved in HCV therapy, and IFN-free therapies utilizing combinations of direct-acting antivirals (DAAs) with or without ribavirin (RBV) are being used successfully to achieve SVR in the majority of cases.

Besides very high costs associated with some therapies, other issues include variability in activity across different genotypes, such as genotype 3 that can result in failure to achieve SVR[324]. If RBV is required, significant side effects can occur such as hemolytic anemia[325]. Treatment with DAAs can also result in resistant virus as targeting viral proteins puts direct selective pressure for resistant mutants. Furthermore, a small percentage of patients are infected with intergenotypic recombinant strains of HCV which may not respond optimally to the current standard treatments[326,327]. Analysis of the role of chaperones in the viral life cycle may allow for development of novel strategies to target HCV infection. Targeting host factors may reduce selective pressure on the virus to generate resistant mutants. Furthermore, insights obtained by studying chaperones in HCV infection may allow for development of therapies for other viruses especially flaviviruses.

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TOPIC HIGHLIGHT

2016 Advances in Liver Transplantation

Vascular complications following liver transplantation: A literature review of advances in 2015

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Abstract

Although vascular complications (VCs) following orthotopic liver transplantation (OLT) seldom occur, they are the most feared complications with a high incidence of both graft loss and mortality, as they compromise the blood flow of the transplant (either inflow or outflow). Diagnosis and therapeutic management of VCs constitute a major challenge in terms of increasing the success rate of liver transplantation. While surgical treatment used to be considered the first choice for management, advances in endovascular intervention have increased to make this a viable therapeutic option. Considering VC as a rare but a major and dreadful issue in OLT history, and in view of the continuing and rapid progress in recent years, an update on these uncommon conditions seemed necessary. In this sense, this review comprehensively discusses the important features (epidemiological, clinical, paraclinical, prognostic and therapeutic) of VCs following OLT.

Key words: Vascular complications; Orthotopic liver transplantation; Liver transplantation; Endovascular intervention

Core tip: Although vascular complications (VCs) following orthotopic liver transplantation (OLT) seldom occur, they are the most feared complications with a high incidence of both graft loss and mortality, as

they compromise the blood flow of the transplant (either inflow or outflow). Diagnosis and therapeutic management of VCs constitute a major challenge in terms of increasing the success rate of liver transplantation. This review comprehensively discusses the important features (epidemiological, clinical, paraclinical, prognostic and therapeutic) of VCs following OLT.

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INTRODUCTION

Although vascular complications (VCs) following orthotopic liver transplantation (OLT) are seldom, they are one of the most dreaded complications with a high incidence of both graft loss and mortality, as they compromise the blood flow of the transplant (either inflow or outflow). Khalaf[1], in 2010, reported that patient who presented VCs had significantly inferior graft and patient survival rates. The overall incidence of VCs in adults varies widely among transplant centers worldwide, but remains around 7% in various series of deceased donor liver transplantation (DDLT), and around 13% involving living donor liver transplantation (LDLT)[1-5]. Bleeding, stenosis and thrombosis can arise at any of the vascular anastomoses, as well as aneurysms at the arterial anastomosis and exceptionally on the portal vein[6,7], with an overall reported incidence of 7.2%-15% in adults (mainly arterial 5%-10%, following by portal 1%-3% and caval < 2%) (Table 1)[5,8-10]. In this sense, diagnosis and therapeutic management of VCs constitute a major challenge in terms of increasing the success rate of liver transplantation. This explains why, currently, many transplant teams perform close surveillance of all vascular anastomoses using Doppler ultrasonography, which allows prompt detection and treatment before ineluctable graft failure. All vascular problems must be treated aggressively, particularly in- or out-flows and sudden vascular occlusions (*i.e.*, thrombosis or kinking), such as hepatic artery thrombosis (HAT) and portal vein thrombosis (PVT), which are the most common, and more rarely hepatic veins or cavo-caval thrombosis. Indeed, they can suddenly interrupt hepatic blood supply with both high graft loss and retransplantation rates[1,5,10]. Usually, therapeutic options include surgical revascularization, percutaneous thrombolysis, percutaneous angioplasty, retransplantation and a conservative approach. Although surgical treatment used to be considered the first choice for management, advances in endovascular intervention have increased to make this a viable therapeutic option following OLT. In recent decades, huge advances in the field of interventional radiology have radically changed the diagnostic and therapeutic approaches to VCs in liver transplant patients. For example, technical improvements made in the catheterization of hepatic vessels and computed imaging allow a specific and localized intervention on these pathological vessels, in a less invasive way[1,5,11-18]. As a matter of fact, percutaneous endovascular therapies (*i.e.*, catheter-based thrombolytic intervention, balloon angioplasty and stenting) provided by an experienced interventional radiologist are commonly employed and have supplanted surgery as the therapy of choice in almost all cases[18-20].

Considering VCs as rare but as major and dreadful issues of OLT history, and in view of the continuing and rapid progresses in recent years, an update on these uncommon conditions seems necessary. In this sense, this review comprehensively presents the important features (either epidemiological, clinical, paraclinical, prognostic and therapeutic) of VCs following OLT. In this review, only VCs following adult OLT (DDLT or LDLT) are presented, excluding pediatric liver transplantation. Taking into account that biliary complications following OLT also constitute a major therapeutic challenge, and that they are intrinsically linked with hepatic arterial pathology, they are beyond the subject of this article and therefore will not be discussed herein.

ARTERIAL COMPLICATIONS

Arterial complications are still a major source of morbidity and mortality after OLT. Normally, the liver allograft maintains a dual inflow blood supply: Portal and arterial. Hepatic artery (HA) plays a major physiological role, because it provids the blood supply for both the liver parenchyma and the biliary tree. Arterial reconstruction is a frequent therapeutic option after the ligation of different collaterals until, finally, the celiac trunk remains the only arterial vascular supply to the transplanted liver[21]. In patients with traumatic liver rupture with currative ligation of the hepatic artery, it has been reported that bile duct necrosis is not always associated[22]. On the contrary, the interruption or the reduction of arterial flow during liver transplant is frequently associated with biliary tree complications due to ischemic processes (*i.e.*, bile duct necrosis, liver abscesses and graft dysfunction)[23]. This discrepancy can be explained by the absence of collaterals in an OLT recipient[2,24]. In the native liver, HAT or even acute ligation, is usually well-tolerated due to the abundant arterial collateral sources which avoid ischemia of the liver parenchyma. In contrast, disruption of these collaterals inevitably occurs when performing total hepatectomy for OLT. Thus, the allograft may survive by portal and arterial inflows *via* portal and hepatic artery anastomoses. In cases of HA complications (HAC) perturbing the arterial inflow, the allograft may survive by portal inflow, but only if arterial collaterals exist[2,24,25]. These facts explain why recognition and prompt management of HAC is of great importance

Table 1 Vascular complications following orthotopic liver transplantation

Type	Delay (incidence)	Clinical presentation	Diagnosis	Treatment
Arterial complications				
HAT incidence: 3.5%	Early HAT (2.9%)	Abnormal transaminase Fever Biliary complications Graft failure Coagulopathy	DUS ce-MDCT Angiography	Emergent revascularization by endovascular intervention or surgical revascularization or rLT
	Late HAT (2.2%)	Asymptomatic Fever Abnormal transaminase Bile leak Hepatic abscess Cholangitis		
HAS incidence: 2%-13%	Early HAS	Graft failure Biliary complications	DUS ce-MDCT Angiography	Endovascular intervention or surgical revascularization
	Late HAS	Asymptomatic Fever Abnormal liver function	DUS ce-MDCT Angiography	Endovascular intervention or surgical revascularization
HAP incidence: 2.5%		Asymptomatic Abdominal pain Fever	DUS ce-MDCT Angiography	Endovascular intervention or surgical resection and revascularization
HAR incidence: 0.64%		Gastrointestinal bleeding Massive bleeding through abdominal drains Hemorrhagic shock	None in emergency	Emergent surgical hemostasis and surgical repair
Portal vein complications				
PVT incidence: < 3%	Early	Abnormal transaminase Graf dysfunction Multi-organe failure Variceal bleeding	DUS ce-MDCT (portal phase) Portography	rLT or surgical repair or endovascular interventions
	Late	Ascite Portal vein hypertension Splenomegaly Variceal bleeding	DUS ce-MDCT (portal phase) Portography	Curative anticoagulant therapy
PVS incidence: 2%-3%	Early	Asymptomatic Portal vein hypertension Abnormal transaminase	DUS ce-MDCT (portal phase) Portography	Endovascular interventions
	Late	Asymptomatic Ascite Abnormal liver test function	DUS ce-MDCT (portal phase) Portography	Anticoagulant therapy and/or Endovascular interventions
Caval anastomosis complications				
Caval resection and end-to-end cavo-caval anastomosis	Early	Acute Budd-Chiari syndrome Graf failure Intestinal congestion Renal dysfunction Lower limb edema	DUS ce-MDCT Cavography	Endovascular intervention or surgical repair or rLT
	Late	Moderate Budd-Chiari syndrome Ascite	DUS ce-MDCT Cavography	Endovascular intervention
Piggy-back	Early	Acute Budd Chiari Graf failure Intestinal congestion Renal dysfunction Lower extremity edema	DUS ce-MDCT Cavography	Surgical repair or rLT
	Late	Moderate Budd-Chiari Ascite Lower extremity edema Renal dysfunction Abdormal liver test function	DUS ce-MDCT Cavography	Endovascular intervention

Clinical characteristics of arterial and caval complications. rLT: Re-liver transplantation; DUS: Doppler ultrasound; HAT: Hepatic artery thrombosis; HAS: Hepatic artery stenosis; HAP: Hepatic artery pseudoaneurysm; HAR: Hepatic artery rupture; PVT: Portal vein thombosis; PVS: Portal vein stenosis; MDCT: Multi-detector computed tomography.

Table 2 Hepatic artery thrombosis highlights

Summary of the clinical characteristics about HAT
HA supplies exclusively the bile duct, so HAT is associated with a high frequency of biliary complications
HAT represents more than 50% of all arterial complications following OL
The incidence of HAT following OLT is 3.5% with early and late HAT incidences of 2.9% and 2.2%, respectively
HAT carries an incidence of graft failure and mortality of more than 50% without prompt treatment
The median time to detection of early and late HAT was 6.9 d (range: 1-17.5 POD) and 6 mo (range: 1.8-79 mo), respectively
No differences in HAT incidences were observed between DDLT and LDLT
Clinical presentation spectrum: Mild elevation of serum transaminase and bilirubin levels (75%), biliary complications (15%), fever and sepsis (6%), graft dysfunction or failure (4%)
Risk factors of early HAT are mainly represented by technical problems, LDLT, cigarette smoking and hypercoagulability state, while late HAT is usually related to ischemic or immunologic injury: CMV positive donor, female donor and male recipient and hepatitis C seropositive recipient
Early diagnosis is achieved by assessing the serum transaminase level and performing Doppler ultrasound monitoring in the postoperative period and confirmed by contrast-enhanced abdominal CT scan and/or visceral angiography
Currently, the literature on the curative management of early HAT suggests the following procedures: First endovascular radiological intervention (IAT, PTA and stent placement), secondly open surgical revascularization, and finally retransplantation, which is associated with the best survival rate compared with revision or thrombolysis, but is a limited therapeutic option due to organ shortage

HA: Hepatic artery; HAT: Hepatic artery thrombosis; OLT: Orthotopic liver transplantation; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation; CMV: Cytomegalovirus; IAT: Intra-arterial thrombolysis; PTA: Percutaneous transluminal angioplasty; CT: Computed tomography.

for graft and patient survival. The etiology underlying most HAC involves the anastomosis, including: (1) HAT: 1.9%-16.6% (the most frequent and pejorative); (2) anastomotic stricture [*i.e.*, hepatic artery stenosis (HAS)]: 0.8%-9.3%; (3) pseudoaneurysm formation [*i.e.*, hepatic artery pseudoaneurysm (HAP)]: 0%-3%; and (4) hepatic artery rupture (HAR): 0.64%[8,9,18,26]. These complications can be classified into two categories (Table 1): Early (< 1 mo) or late (delayed, *i.e.*, > 1 mo). Very particular attention should be focused on early complications, because they are associated with graft loss and a high mortality rate. In different studies, the definition of early and late complications continues to be discussed. Most of the authors have defined late complications as those occurring after 4 wk, and others after 6 mo[13,25,27,28]. In this review, we consider the recent consensus which defines early complication when it appears within the first month[10,13,18,27,28].

HAT

HAT represent more than 50% of all arterial complications. It is the most frequent and severe vascular complication following OLT. Table 2 usually more frequent after pediatric liver transplantation[5,10,16,17,28-31]. It is the first cause of primary non-function of the liver transplant, which can lead to allotransplant loss and patient death in the early postoperative period. HAT is associated with a high incidence of liver transplant failure (more than 50%) and carries a mortality of more than 50% in the absence of revascularization or retransplantation. In recent years, early revascularization by means of endovascular catheter-based intervention has been a viable option for graft salvage before considering retransplantation. Indeed, the retransplantation rate is very high in untreated HAT (25%-83%) compared to graft revascularization treated patients (28%-35%)[3,10,13,16,17,30,32-40].

Definition: HAT is defined as a thrombotic occlusion of the hepatic artery. It has been classified, as described above, into two types depending on the time of presentation following OLT: Early HAT [within the first 30 d of liver transplantation (LT)] and late HAT (after 30 d of LT)[13,17,28]. The hepatic artery supplies the biliary tree of the transplant, explaining the high frequency of biliary complications in HAT (*i.e.*, biliary ischemia, necrosis, stricture, sepsis) and eventually hepatic insufficiency and graft loss[31].

Incidence: The true incidence of early HAT following OTL is unknown, but it varies widely from 0% to 12% in adults[5,9,24,25,27,30,38,41]. Bekker *et al*[28] (2009) reported in a systematic review comprising 21822 OLT cases an incidence of 843 cases (adults and children) of early HAT with an overall incidence of 4.4%. In adults, the incidence of HAT was 2.9%. They also showed that the incidence of early HAT had decreased over time since the first report in 1982 by Starzl (6.9% in 1996 *vs* 3.2% in 2006) with improvements in perioperative care. They reported that there were no differences in incidence among transplantation centers worldwide[2,28]. Median times to the occurrence detection of early and late HAT were respectively 6.9 postoperative days (range: 1-17.5) and 6 mo (range: 1.8-79 mo)[17].

In literature, it does not confirm that HAT incidence in LDLT is significantly lower or higher compared to HAT incidence in DDLT. Many studies show contradictory results[1,9,17,28,41] but, a meta-analysis on HAT found no significant difference with an incidence of 3.1% and 4.6% in LDLT and DDLT, respectively[28]. Furthermore, it was reported that arterial anastomosis with operation microscope or loupe mangnification did not show any difference in incidence HAT[9,17,28,41].

Late HAT shows a lower incidence, ranging from 1% to 25%[38,42]. Torras *et al*[34] (1999) reported an

incidence of 7.5% (35/413) following OLT. Sixteen cases occurred during the first month (early HAT): Diagnosis made from 1 to 13 d after OLT (median: 2.5). Nineteen cases were late HAT (> 30 d, from 2 to 79 mo after OLT (median: 5 mo)[34].

Clinical presentation: The clinical presentation of HAT range from a mild elevation in serum amino transferase (most frequently in patients with HAT) and bilirubin levels to fulminant hepatic necrosis. HAT is associated with elevated transaminases in 75%, biliary complications in 15%, fever and sepsis in 6% and graft dysfunction or failure in 4% of cases[5]. The clinical expression depends on the timing of the onset of HAT as well as on the existence of collaterals[5,25,27]. Usually, initial non-function or severe allograft dysfunction predominately occurs in patients with early HAT. This explains the importance of symptomatic expression, whereas biliary tract complications (*i.e.*, bile duct strictures or bile leaks sometimes leading to biliary hepatic abscesses) are more frequently, but not exclusively, associated with late HAT. Indeed, clinical expression depends on the existence of collaterals, which can develop as early as within two weeks[17,24,27]. Therefore, two main forms of HAT are recognized: (1) acute presentation (early HAT) characterized by a severe clinical course; and (2) delayed presentation (late HAT) generally associated with a milder clinical course[25].

In every cases, early HAT clinically manifests with fever, increase leukocytosis and a important elevation in liver enzyme levels. The natural history of early HAT could be summarized as biliary tract necrosis followed by uncontrolled septic shock in the immunosuppressed population, and even by the patient's death[17,27,28,31,38]. The pathophysiological process of early HAT results in injury to the bile duct epithelium and to hepatocytes. This leads to massive necrosis in the allograft, partly due to the disruption of arterial inflows (*i.e.*, main flow by HA and accessory physiological collaterals), explaining the high incidence of biliary sepsis in early HAT[25,27,28].

It is usually assumed that late HAT is due to ischemic or immunological damages with a more insidious onset. Up to 50% of patients with late HAT can be asymptomatic with elevated liver function tests only[10,19,27,36]. Symptomatic patients often present with biliary complications including recurrent cholangitis, bile duct stricture/stenosis, biliary leakage, biliary tract necrosis and abscess formation revealed by relapsing fever and bacteremia. The presentation may be insidious. Liver graft ischemia and liver failure are other classical insidious clinical outcomes revealing late HAT[17,27,28,36,38,42,43].

Risk factors: Several reports studied the risk factors associated with HAT[5,10,17,19,25,27,28,34,44,45]. They can be divided into several categories. It is usually considered that technical problems are mainly associated with early HAT. Conversely, risk factors for late HAT are less well-defined. However, a donor positive CMV status and a recipient negative cytomegalovirus (CMV) status have repeatedly been shown to be a possible risk factor for late HAT[27,45]. Moreover, specific factors of late HAT reported include the association of female donor and male recipient, hepatitis C virus positive recipients, episodes of rejection, tobacco consumption and retransplantation[10,17,27,45,46]. Besides, while some authors believe that HAS and hepatic artery kinking are the initiating factors, others suggest a perioperative hypercoagulable state as a possible underlying cause[5,10,17,28,29].

Truly, the cause of early HAT is still under debate and remains unknown in most cases. Up to 20% of HAT cases are probably due to surgical causes (technical problems) in the arterial anastomosis, such as difficult anastomosis, technical imperfections with the anastomosis, kinking, stenotic anastomosis, small vessel size, reduction in a disparate diameters of the arteries, dissection of the hepatic arterial wall, celiac stenosis or compression by the median arcuate ligament, the presence of multiple arteries, aberrant or complex donor/recipient arterial anatomy or arterial abnormalities requiring complex arterial reconstructions, complex backtable arterial reconstruction of the allograft, poor quality donor and recipient vessels and high-resistance microvascular arterial outflow caused by rejection or severe ischemia-reperfusion injury. Those problems are more common among centers performing fewer than 30 OLT a year; the incidence of HAT diminishes with the surgical team's experience. Therefore, surgical causes probably do not represent the main risk factor for HAT[17,28,29,31,38].

It has been reported that HAT can occurs within a few hours after LDLT, which indicates a population at higher risk of HAT. Indeed it has been shown that these patients displayed a higher rate of VCs explained by the complexity vascular reconstructions linked to smaller and shorter caliber of donor and recipient vessels[1,10,47].

Regarding the non-surgical risk factors involved in the occurrence of HAT, donor age > 60 years, extended cold ischemia time, lack of ABO compatibility, cigarette smoking, hypercoagulability state, donor positive for CMV in a CMV-negative recipient, rejection, regrafts and transplant for primary sclerosing cholangitis have been shown to be statistically linked with the occurrence of HAT[17,28,38,46]. However, the literature review dealing with this issue displayed conflicting results. Indeed, some authors reported that some parameters like cold ischemic time, donor age and the presence of rejection were not found to be factors related to the development of HAT[34]. This emphasizes the difficulty in accurately determining the risk factors associated with early HAT. In a recent study, Panaro *et al*[48] (2014) have shown a statistical association between TACE and the radiological and histological arterial wall injury, as in the past 25 years TACE has been widely used in the treatment of HCC. This procedure may potentially cause vascular lesions in the arterial wall (catheterization and drug infusion), suggesting that previous transarterial chemoembolization (TACE) could constitute a risk factor

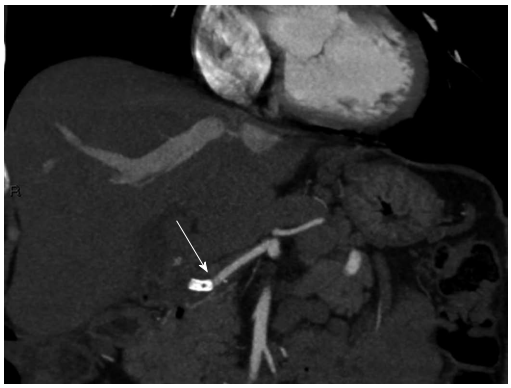

Figure 1 Contrast-enhanced-multidetector-row computed tomography-scan showing hepatic artery thrombosis after an endovascular intervention with stent placement. Thrombus (arrow).

of HAT when future OLT is performed[5,48].

Some practices could prevent the occurrence of HAT, and the data reported by Duffy *et al*[5] (2009) demonstrates that arterial reconstructions which restore the normal anatomy and gentle handling of vessels are of great importance in the accomplishment of hepatic arterial anastomosis. Some studies reported that recipients with multiple anastomoses for arterial reconstruction should receive aspirin and Doppler ultrasound (DUS) assessment to screen the patency of the reconstructed hepatic artery. Moreover, the use of aortic conduits for arterial reconstruction is a risk factor that warrants the initiation of prophylaxis in the post-transplant period[5,10,17,19,25,31,44]. For patients with inheritable thrombophilic diseases; given the devastating effects of HAT on graft outcomes, it should be necessary to identify these to prevent thrombotic complications. It is likely that patients who present both hematological and operative factors are most at risk, and routine anticoagulation in the post-OLT setting should be instituted. In sum, many studies recommand peritransplantation anticoagulation with heparin or an antiplatelet agent in patients with extraanatomic conduits, complex backtable reconstruction, or pre-OLT TACE. However, the best prophylactic approach is controversial, and this should be clarified by randomized, controlled trials[5,10,17,19,31,44,25]. An interesting report by Marín-Gómez *et al*[40] (2012) demonstrates that intraoperative blood flow allows for a prediction of the occurrence of HAT when it is less than 100 mL/min with 84.5% sensitivity and a predictive positive value of 97.8%.

Diagnosis: Early diagnosis is mandatory to allow immediate treatment and to prevent graft loss. The detection of these patients includes biological (serum transaminase levels) and morphological (DUS) exams, while visceral angiography allows to confirm the diagnosis. DUS is a proven non-invasive technique and the gold standard investigation to assess hepatic artery patency. It detects the absence of hepatic artery flow, even in its intrahepatic branches. The DUS diagnosis comprize the lack of HA signal (Se = 92%) or an increased resistive index (RI)[25,17,38]. Even though the screening protocol varies between liver transplant centers, a DUS surveillance protocol of the hepatic artery can detect reduced hepatic arterial flow and to allow for prompt revascularization management, which may result in transplant salvage[17]. In sum, in case of an abnormal elevation in liver enzymes and suggestive findings on DUS, abdominal computed tomography (CT) angiogram or angiography confirmed diagnosis and it can precisely shows an underlying anatomical defects (stenosis or kinking) with a high sensitivity and specificity specificity (Figure 1)[17]. Pareja *et al*[38] (2010) established a screening protocol for early HAT, consisting of a first Doppler ultrasound within 48 h of OLT and in another Doppler ultrasound 7 d later. If the first examination is conclusive, they perform contrast ultrasound (microbubbles) or computed tomography. When HAT is confirmed, arteriography should be performed[38]. Intimal hyperplasia causing progressive HAS may precede late HAT and may be screen by regular (yearly) post-OLT DUS assessment. In some cases, HAS is likely to stimulate the development of arterial collaterals that protect the liver from ischemia at the time of HAT[25,48].

Therapeutic management: Classically, we consider several treatment modalities for HAT: (1) revascularization (surgical or endovascular); (2) retransplantation; and (3) observation. Currently, the most effective treatment approach remains controversial and the choice of any of these treatments depends on the time of diagnosis. Early diagnosis, prompt revascularization and retransplantation have been considered the only solution to rescue patients with HAT. Historically, retransplantation is the treatment of choice for most groups, offering the best survival results[5,16]. However, this possibility is strongly conditioned by the shortage of donors and by the patient's condition[16,17,27,38,39]. Percutaneous endovascular treatments including intra-arterial thrombolysis (IAT), percutaneous transluminal angioplasty (PTA) and stent placement have shown hopeful outcomes in the literature. Finally, some patients survive without revascularization or retransplantation by developing collateral circulation distal to the thrombosis, but this occurs in rare cases[17,20,24,38,39]. Despite these encouraging results of endovascular interventions, the efficacy and risk of complications (mainly represented by hemorrhage risk) make this therapeutic option still controversial. Moreover, in some cases these are ineffective and surgical intervention (including anastomotic revision and retransplantation) must be applied. The complications of PTA include thrombosis, vascular dissection and rupture. Thus, urgent revascularization by means of endovascular interventions as a primary option offers could give a chance to avoid rLT, but only in asymptomatic patients[8,10,17,20]. Despite the proof of efficacy and safety of thrombolytic treatment with different products and regimens (urokinase, streptokinase, alteplase), the best protocol is not still known and there are currently no specific guidelines for

thrombolytic therapy application. Furthermore, several studies recommend low dose of heparin in association with thrombolytic despite incresing the risk of adverse bleeding. Indeed, hemorrhage is the most frequent adverse effect and concern about 20% of patients: Ranging from blood in the drainage to intra-abdominal hemorrhage, which could be fatal in some cases. This is mainly true in early postoperative period, but selective thrombolysis *via* the hepatic artery presents several advantages, such as a smaller thrombolytic dose, a highly localized concentration and little influence on systemic coagulation[17,20]. Endoluminal IAT with restoration of flow should be associated with underlying anatomic defects treatment if present, including reduction of kinking, treatment of an anastomotic stenosis and often requires balloon angioplasty and/or stent placement[16,20]. Association of IAT with PTA and/or stenting showed better efficacy and survival rates when compared to IAT alone. In summary, PTA and stent placement are currently tried first to resolve the problem in many centers[10,20]. Open surgical revascularization of thrombosed liver transplant is considered a viable option to save the transplant and to avoid retransplantation. Open surgical revascularization can be performed in various ways depending on the length and on the integrity of the recipient and on the graft arterial stumps. The procedure in its simplest form can be a Fogarty thrombectomy and a primary resuture of the end-to-end hepatic artery anastomosis[16]. Duffy *et al*[10] evaluated 4234 LT from 1984 to 2007: 203 (5%) developed HAT including 133 early and 70 late HAT; the occurrence of HAT was 3.9% in adults. Overall 90 patients were treated with surgical exploration, thrombectomy, or anastomotic revision. Nine patients were treated with catheter-based thrombolysis and 13 patients received anticoagulation. Of the patients with early HAT who underwent thrombectomy and anastomotic revision, only 9 (10.5%) had graft salvage, and the remaining patients needed re-transplantation. Overall, re-transplantation was necessary in 153 (75%) patients with HAT. Therefore, retransplantation after HAT has a better survival rate compared with revision or thrombolysis[5,10].

In contrast, some patients with late HAT survive without revascularization or retransplantation by developing a collateral circulation distal to the thrombosis. The mean time between the diagnosis of HAT and the neovascularized liver is 4.1 mo (range: 3-5.5 mo). Four factors are associated with the development of a neovascularized liver: Late HAT, early HAS, site of thrombosis, and Roux-en-Y anastomosis[24,39]. These results confirm that a slow arterial obstruction process allows for the formation of arterial substitute pathways, but this striking neoangiogenesis capacity, only significant in cases of chronic ischemia, is insufficiently rapid in the case of early HAT. Given the improved outcome of the conservative treatment of liver transplant recipients, in whom late HAT develops without revascularization or retransplantation, revascularization in this condition is controversial. Based on two limitations (the relative lack of utility of revascularization of late HAT and the contraindication to early postoperative thrombolysis), Saad *et al*[16] (2007) proposed that the clinical window of the applicability of transcatheter thrombolysis should be most likely from 1 to 3 wk to 1 to 3 mo post-transplantation, respecting contraindications to avoid fatal bleeding complications. However, successful and safe pharmaceutical thrombolysis was described by Figueras *et al*[11] (1995) 3 d after OLT. In the literature, the time interval between the transplant and thrombolysis procedures ranges from 2 to 120 d (mean, 53 d)[11,16,27,49-51].

Prognosis: At the time of revascularization, survival rates is 40% in symptomatic *vs* 82% in asymptomatic patients[17]. The incidence of HAT has a significant impact on transplant and recipient survivals. Indeed, Silva *et al*[27] (2006) reported an overall mortality rate of 23% in those developing HAT post-OLT. In the meta-analysis reported by Bekker *et al*[28] (2009) HAT was a major cause of graft loss (53.1%) and mortality (33.3%) in the early postoperative period.

Conclusion: HAT is rare but it represent the most common vascular complication following LT. A definitive diagnosis is achieve by angiography, which may detect predisposing anatomical anomalies. Moreover, it allows prompt therapeutic management in the same time. IAT can be performed alone and an eventual anatomical anomaly may then be corrected by endovascular procedures such as balloon angioplasty and/or stent placement, or a surgical intervention. Currently, it seems reasonable to propose endovascular treatment first, mainly due to organ shortage and the high mortality related to retransplantation, considering the highly individualized outcome and depending of the competence of the transplant center. However, in the early post-transplant period, it is widely accepted that symptomatic patients with severe allotransplant dysfunction and symptoms related to arterial thrombosis need retransplantation.

Hepatic artery stricture/HAS

Definition: HAS following OLT is defined as a narrowing of the transverse diameter of the HA, more or less extended, resulting in graft ischemia mainly revealed by elevated liver function tests[2,16,52-56]. Significant HAS is usually defined as a narrowing of the transverse diameter > 50% on angiogram associated with clinical suspicion and a RI < 0.5 (defined by peak systolic flow-end diastolic flow/peak systolic flow) and a peak systolic velocity > 400 cm/s detected by DUS[16,57,58]. HAS and HAT are the most common hepatic arterial complications, with high rates of morbidity and mortality[56,58] (Table 3).

Incidence: HAS occurs in 2% to 13% of transplants and has been suggested to progress to HAT implicating, at least in part, that HAS and HAT are two contiguous components of the broader allotransplant ischemic spectrum[2,16,30,52,53,55,56,58-60]. Wozney *et al*[2] (1986) reported three cases in which untreated anastomotic

Table 3 Hepatic artery stenosis highlights

Summary of the clinical characteristics about HAS
Significant HAS is defined as a narrowing of the transverse diameter > 50% on the angiogram associated with clinical suspicion, with a resistive index < 0.5 and a peak systolic velocity > 400 cm/s detected by DUS
HAS occurs in 2% to 13% of transplants, at the level of the anastomosis (59% of cases), graft HA (41%) or recipient HA (2.6%)
HAS has been speculated to progress to HAT in 65% of cases at 6 mo for untreated HAS
The median time to diagnosis is 100 (range: 1-1220) d following OLT
Most of patients with HAS are asymptomatic and most commonly present only with abnormal liver function tests and in rare cases with graft failure
Routine screening by DUS during the postoperative period is mandatory because of the insidious clinical presentation
The risk factors are not really known, but among these, technical and surgical factors (vascular injury such as clamp injury, intimal dissection, faulty placement of anastomotic sutures, excessive length with kinking and angulation, differences in the vessel caliber that require and oblique anastomosis, vasa vasorum disruption) or acute cellular rejection
DUS is a non-invasive method for the assessment of HA patency, but a contrast-enhanced CT scan and angiography are required to confirm the diagnosis
Radiological endovascular intervention by PTA with or without stent placement is often used to treat post-transplant HAS and are both efficacious, with 7% to 12% of complications including dissection and arterial rupture, restenosis or thrombosis (25%) and 12% failed attempts
Surgical revision and retransplant showed a high rate of success, but the overall mortality rate was as high as 20%. In some case, HAS may be an early sign of chronic rejection

DUS: Doppler ultrasound; HA: Hepatic artery; HAT: Hepatic artery thrombosis; HAS: Hepatic artery stenosis; OLT: Orthotopic liver transplantation; PTA: Percutaneous transluminal angioplasty; CT: Computed tomography.

strictures of the hepatic artery progressed to HAT. Saad *et al*[52] (2005) emphasized the correlative progression of untreated significant HAS to HAT with an incidence rate of 65% at six months for untreated HAS[2,16,52]. Abbasoglu *et al*[57] (1997) reported an incidence of 4.8% in a cohort of 857 consecutive OLT from 1988 to 1995. The median time to diagnosis was 100 d (range: 1-1220 d) following OLT, which was also reported by Denys *et al*[60] (2002) with a mean time to diagnosis at 94 d post-OLT[57,60]. Similar to HAT, HAS may be divided in two groups: HAS occurring within 30 d after OLT (early HAS), and HAS occurring more than 30 d after OLT (late HAS). Chen *et al*[61] (2009) reported an overall HAS incidence of 2.8%, with an early HAS incidence of 40% *vs* a late HAS incidence of 60% (mean time elapsed between transplantation to diagnosis: 91 d; range: 1-430 d). Abbasoglu *et al*[57] (1997) reported that stenosis occurred in 59% of cases at the level of the anastomosis with a median time of diagnosis at 75 d post-OLT, in 41% of cases at the level of the graft HA with a median time of diagnosis at 160 d post-OLT, and in 2.6% at the level of the recipient HA[57]. Saad *et al*[52] (2005) did not confirm these results. Indeed, the literature has established that the anastomotic stenosis is the most common place for the development of HAS within three months after LT[10,62].

Clinical presentation: The clinical presentation of HAT range from normal liver function to transplant failure secondary to ischemia or necrosis. Moreover, HAS can lead to an insidious form of graft disorder, both in the early and later postoperative stages. Many patients with HAS are asymptomatic and most commonly present only with abnormal liver function tests (LFT)[16,52,57,58,60,63,64]. Indeed, Abbasoglu *et al*[57] (1997) reported that an elevation in LFT was the main clinical presentation. Most asymptomatic patients are detected during routine DUS screening. In fact, the non-specific and insidious clinical presentation of HAS mandates to perform routine screening DUS at regular time intervals. In contrast, it is obvious that DUS screening should be highly required for OLT asymptomatic patients presenting elevated LFT.

Compared with HAT, the risks of developing biliary complications, including biliary strictures and bile leaks, are less frequent with HAS. Ideally, HAS should be diagnosed before the occurrence of biliary complications, because of the significant impact on both graft and patient survival[10,19,57]. Indeed, incidence of biliary complications is reported to be as high as 67% in liver transplant recipients with HAS[52,63,64].

Risk factors: The risk factors of HAS are not really known and seem to have a multifactorial origin[60]. Many authors suggest perioperative factors (technical) of vascular injury (clamp injury, intimal dissection, faulty placement of anastomotic sutures), donor and recipient factors (excessive length with kinking and angulation, differences in vessel caliber that require oblique anastomosis), and others, such as extrinsic compression and microvascular injury, *i.e.*, vasa vasorum disruption or acute cellular rejection[52]. Abbasoglu *et al*[57] (1997) demonstrated that a low mean initial HA flow (less than 400 mL/min) after OLT is a risk factor for developing anastomotic HAS, but they did not identify a risk factor. Moreover, they showed that the presumed immunological bases, such as autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis for their OLT, were not risk factors for HAS[57].

Diagnosis: DUS is a well-established non-invasive method for the assessment of HA patency, and its efficiency in the early diagnosis of HAS has been reported in several studies[52,57]. Abbasoglu *et al*[57] (1997) showed a DUS sensitivity of 85% in detecting HA stenosis. DUS showed a sensitivity of 100%, a specificity of 99.5%, a positive predictive value of 95% and a negative predictive value of 100%, and an overall accuracy of 99.5% in early HAS diagnosis[10,57,62,65].

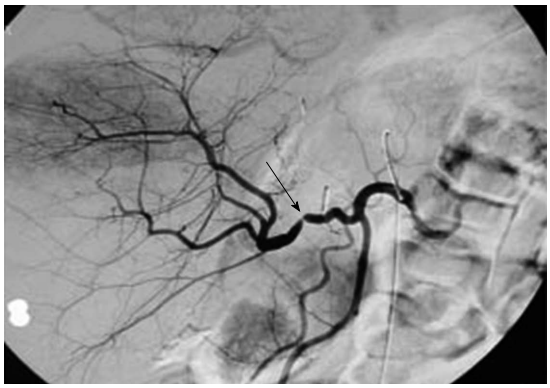

Figure 2 Arteriography showing an anastomotic hepatic artery stenosis after orthotopic liver transplantation. Stenosis (arrow).

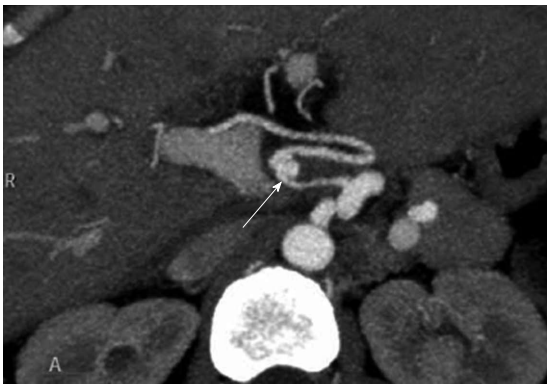

Figure 3 Contrast-enhanced-multidetector-row computed tomography-scan showing a hepatic artery pseudoaneurysm following orthotopic liver transplantation. Pseudoaneurysm (arrow).

Many teams also use MDCTA and standard angiography to confirm the diagnosis, which is the gold standard for HAS diagnosis[10,62,65].

Therapeutic management: The therapeutic management of HAS includes either surgical revision, retransplant or percutaneous endovascular interventions, such as PTA with or without stent placement[52,57,60,63,64,66] (Figures 2 and 3). Abbasoglu *et al*[57] (1997) reported 35 cases of surgical revision, including aortohepatic iliac artery graft (from banked donor vessels), autologous saphenous vein patch angioplasty and resection of the stenotic segment either with primary reanastomosis or with interposition of a banked iliac artery or saphenous vein graft. In this group, HA flow was reestablished successfully in all patients. At a mean follow-up of 25 mo, 67% of patients were asymptomatic with normal liver function. Six patients were treated with PTA. Five of them were found to be asymptomatic at a mean follow-up of 25 mo[57]. Indeed, balloon angioplasty can be an effective treatment option in these cases[10,19]. Similar to Abbasoglu *et al*[57] (1997), Saad *et al*[52] (2005) also reported 81% successful treatment of cases in a series of 42 cases of significant HAS treated by PTA, with an incidence of immediate complication of 7% including dissection and arterial rupture[52,57]. Delayed complications (*i.e.*, HAT) within 30 d of PTA occurred in 5% of cases, yielding a total complication rate of 12% and 12% total failed attempts without consequences. In this treatment modality, very different rates of restenosis have been reported from no restenosis to rates as high as 75%[60,63,64,67,68]. Denys *et al*[60] (2002) reported a low rate of HAT among 13 HAS patients treated by HA stent placement, which may be attributed to anticoagulation and/or antiplatelet regimens that were routinely given to their patients[52,60]. In their study, they also reported a post-HA stent placement HAT in one patient, and four patients with intra-stent restenosis in whom restenosis was dilated successfully. Other teams showed that primary stenting of the HA is feasible and offers a low complication rate with an acceptable one-year patency rate[60,69]. Ueno *et al*[69] (2006) reported an incidence of restenosis of 25% after stent placement, which is significant, but Sommacale *et al*[56] (2013) demonstrated that repeated endovascular treatment of recurring HA stenosis carries a high rate of success[56,69]. However the best time for the earliest endovascular intervention after liver transplant is currently still discussed. Boyvat *et al*[66] (2008) reported endovascular intervention performed within seven days after transplant in nine patients, with a mean intervention time of 34.6 d (range: 6 h-210 d). They experienced extravasation or HAR in five patients and used graft-covered stents to solve this issue in all patients. They suggested that this technique should allow for safer endovascular intervention with no restriction time after surgery and with an acceptable benefit/risk ratio[66]. Finally, a recent published meta-analysis of case series has reported that interventional radiological procedures are often used to treat post-transplant HAS, and that PTA with balloon dilation alone or associated to stent placement are both efficacious and show similar complication rates and decrease the retransplantation rate[55].

Prognosis: In the study by Abbasoglu *et al*[57] (1997) the overall mortality was 20%, mainly in the surgical revision group. Nineteen percent of patients with HAS had retransplantation with a median time of four months (range: 11 d-21 mo). It is interesting to note that among these, five had chronic rejection not diagnosed prior to HA revision, suggesting that HA stenosis should be an early sign of chronic rejection[57]. Therefore, Abbasoglu *et al*[57] (1997) recommended that every HAS patients should be screened for chronic rejection. The patient and graft survival rates at four years in the revised HA group were 65% and 56%, respectively; these rates were not significantly different from those of the control group[57].

Conclusion: To conclude, HAS requiring revision is an uncommon condition after OLT. Early diagnosis by means of systematic DUS in the postoperative period and prompt revascularization procedures, with percutaneous endovascular methods with or without stent placement first, are usually successful with long-term graft and patient survival[56]. Individualized therapeutic regimens

Table 4 Hepatic artery pseudoaneurysm highlights

Summary of the clinical characteristics about HAP
The reported incidence of HAP is ranging from 0.27% to 3% following OLT
In most cases, HAP is localized extra-hepatic and occurred in the early postoperative period around 1 mo post-OLT (69% within 20 d and 81% within 35 POD)
Clinical presentation varies from the asymptomatic state and incidental diagnosis to abdominal pain with fever and gastrointestinal bleeding (25% of cases, massive bleeding through the abdominal drain or acutely with hemorrhagic shock)
Risk factors include peritoneal infection, biliary leak, bilbo-digestive anastomosis and digestive leak
Diagnosis of HAP is confirmed by DUS (with lower performance), contrast-enhanced CT scan, magnetic resonance angiography or angiography
Treatment of HAP includes reoperation (urgent laparotomy for HA ligation: Mortality rate 60%; HAP excision and immediate revascularization with a cryopreserved arterial allograft: Mortality rate 28%) or interventional radiology (HA embolization with a coil or HAP exclusion with a covered stent)
HAP has a worse prognosis with an overall mortality of more than 50% (ranging from 53% to 100%)
Early recognition of HAP in the population at high risk is mandatory and allows for a successful therapeutic outcome in 100% of cases

DUS: Doppler ultrasound; HA: Hepatic artery; HAP: Hepatic artery pseudoaneurysm; OLT: Orthotopic liver transplantation; CT: Computed tomography.

should be applied to treat HAS according to the technical platform available within transplant centers. When endovascular intervention fails to rescue arterial blood inflow, surgical revascularization should be attempted, especially if HAS is associated with biliary complications before considering retransplantation, which carries a higher mortality rate[70]. Finally, a meticulous arterial anastomosis suture with careful attention of a sufficient arterial flow into the liver transplant seems prevent this complication.

HAP

Definition: HAP is defined as a dilated hepatic artery, which occurs after iatrogenic injury in most cases, causing blood to leak and pool outside the artery wall into surrounding tissue, with a persistent communication between the HA and the resultant adjacent cavity (Table 4). This is a very unusual event, with a reported incidence of 0.27%-3%[26,30,71-80].

Incidence: In the retrospective cohort studied by Volpin *et al*[81] (2014) on 787 LT performed between January 1990 and 31 December 2005, a HAP incidence of 2.5% was reported, uniformly distributed over the 16-year period. The authors showed that this complication did not significantly affect any specific indication for liver transplantation. In the 16 patients that were concerned, the anatomical localization of HAP was extra-hepatic and occurred after the first liver transplant. In fact, most HAP occurred in the early postoperative period around one month post-OLT: 69% presented within 20 d and 81% within 35 d following LT. The median time of presentation of HAP was 13 d. This corresponds to the median time reported by many authors, varying from 13.4 to 29 d post-LT[26,30,78,80,81].

Clinical presentation: The clinical presentation of HAP varies from the asymptomatic state and incidental diagnosis upon imaging to abdominal pain associated with fever, gastrointestinal bleeding (25% of cases), massive bleeding through the abdominal drain in the very early post-LT period (31% of cases) and acutely with hemorrhagic shock (81% of cases, the most frequent in the series of Volpin *et al*[81], 2014). These imply additional investigations, such as emergent abdominal imaging.

Risk factors: Several predisposing factors have been suggested, including peritoneal infections, technical difficulties during the completion of arterial anastomosis and biliary leak[26,30,71-83]. The rate of patients with extra-hepatic HAP and with bacterial or fungal organisms isolated from the peritoneal fluid or from the arterial wall is very high. In the series of Volpin *et al*[81] (2014), these patients accounted for 81% of the total (microorganisms cultured from the HAP wall: 50% of cases; cultured from the abdominal fluid: 31% of cases), and other authors report a rate varying from 66% to 100%[26,30,71,73-81,84,85]. Four patients of the Volpin series had a biliary leak discovered before or at the same time as HAP. Indeed, bile leak and bilio-digestive anastomosis were found to be risk factors for HAP, suggesting that enterotomy, bile and digestive leaks could be a source of peritoneal contamination, be considered very seriously and treated promptly because of the risk of HAP formation. In contrast, LDLT, reduced size, split, auxiliary LT and re-transplantation were not risk factors for HAP.

Diagnosis: In the study by Volpin *et al*[81] (2014), the diagnosis of HAP was made by DUS, contrast-enhanced CT scan or angiography (Volpin *et al*[81], 2014) (Figure 4).

Therapeutic management: Treatment of HAP can be achieved by reoperation or interventional radiology[26,75,78,81,86]. In the series of Volpin *et al*[81] (2014), five patients underwent urgent laparotomy for HA ligation; three of them died in the immediate postoperative course with a mortality rate of 60%. The two survivors had biliary complications[81]. Among patients treated by HA ligation, other authors confirmed this unfavorable outcome: 28% mortality in the series of Madariaga *et al*[73] (1992), 75% in the series of Marshall *et al*[78] (2001) and 85% in the series of Bonham *et al*[74] (1999). Moreover, this treatment exposes survivors to impaired liver function, graft loss and finally retransplantation[81,85]. Despite these poor outcomes, Boleslawski *et al*[26] (2013) reported that HA ligation without revascularization is

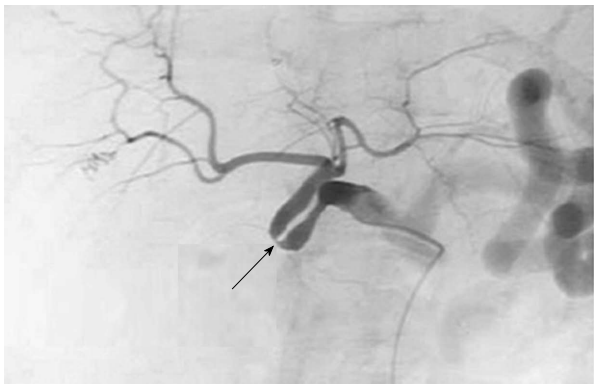

Figure 4 Arteriography showing a hepatic artery stenosis due to a kinking following orthotopic liver transplantation. Kinking stenosis (arrow).

regarded as a reasonable option, with no early mortality in 10 patients with HAP rupture treated by ligature without revascularization. Six of them were still alive without retransplantation after a median follow-up of 70 mo[26]; seven patients underwent HAP excision and immediate revascularization. The arterial continuity was directly restored in five cases and cryopreserved arterial allograft conduits were interposed in two cases. In three cases, concomitant biliary complication was treated simultaneously by bilio-enteric anastomoses. Two patients died postoperatively (mortality rate of 28%). In this subgroup of treated patients, 66% of cases had an uneventful outcome, which seems to offer the best outcome in an emergency setting. Finally, two patients were treated by interventional radiology. One patient underwent embolization with a coil for deliberate HA occlusion; at 10.5 years of follow-up, this patient has good liver function without biliary complications. Another patient had HAP exclusion with a covered stent inserted into the HA; this patient has good liver function at 10 years of follow-up[81].

Prognosis: Volpin *et al*[81] (2014) reported an overall mortality of 50%. Among patients who presented with HAP rupture, the mortality rate was 53%. The three patients treated before HAP rupture occurred are still alive after 10 years of follow-up[81]. In the literature, HAP is associated with a high mortality rate, ranging from 69% to 100%[26,30,71-81].

Conclusion: To conclude, the early recognition of HAP in a high risk population (patient presenting with a documented peritoneal infection, bacteremia, bile and/or digestive leak, or bilio-digestive anastomosis) is crucial to expressly carry out diagnostic assessment and therapeutic management by percutaneous endovascular techniques first. Surgical intervention for HAP excision should be followed by immediate revascularization, even in an infected field, if endovascular management has failed. Recognition before rupture should allow a successful outcome in 100% of cases. Keeping in mind that HAP is usually asymptomatic before rupture, that it occurs most often within the first five weeks post-LT and the poor performance of DUS[87], Volpin *et al*[81] (2014) suggested that a contrast-enhanced CT scan or magnetic resonance angiography should be performed.

HAR

Definition, incidence and risk factors: HAT is defined as a severe hemorrhage from the trunk or from a main branch of the HA. It is a very serious complication that results in the disruption of the arterial blood supply of the transplant. This is a very exceptional but a dramatic complication after OLT which carries very high incidence of liver transplant loss and high mortality rate. In most cases, this condition complicates a pseudoaneurysm of the HA, leading to major bleeding that requires emergency operation. Many reports reported the role of infectious pathogens as the cause in the development of pseudoaneurysms. Diagnosis of pseudoaneurysms is accessible with various radiological techniques, but in half of cases, HAP is not recognized before rupture, requiring immediate surgery[26] (Table 5).

In cases of acute bleeding, many therapeutic possibilities are available: endovascular intervention with embolization with or without stenting, surgical intervention for anastomotic revision, aorto-hepatic grafting, HA ligation or emergency/elective retransplantation. In case of HAR, mortality remains very high and currently there is no consensus on the indications for these procedures[26,73,78,80,88]. Boleslawski *et al*[26] (2013) published the largest series of ruptured post-transplant HAP; they highlighted the efficacy of primary HA ligation on both early and late survival. They reported an HAR incidence of 0.64% (17 patients out of 2649 OLTs from 1997 to 2007). The mean age was 47.9 years (range: 27-65 years; 13 men and 4 women). The median time between transplant and HAR occurrence was 29 d (range: 2-92 d), but the distribution of events was bimodal with only four late HA ruptures occurring after two months[26].

Clinical presentation and diagnosis: In the study by Boleslawski *et al*[26] (2013), clinical presentation was always sudden hemorrhage: Hemoperitoneum in ten patients, gastrointestinal bleeding in five patients, hematoma in one patient and hemobilia in one patient. The presence of a fungal infection in the arterial wall was confirmed in six patients. Biliary leak was observed in seven patients[26].

Therapeutic management: In the study by Boleslawski *et al*[26] (2013), immediate treatment included urgent laparotomy (15 patients) with definitive ligation of the HA (10 patients), anastomotic revision (3 patients) and aortohepatic grafting (2 patients). One patient had a percutaneous embolization and one patient died before treatment. Treatment of the associated biliary leak was performed either synchronously or after the first surgery in seven patients. In this series, the early mortality rate was 35% (0-80 d from HAR and 16-172 d from

Table 5 Hepatic artery rupture highlights

Summary of the clinical characteristics about HAR
HAT is defined as a severe hemorrhage from the trunk or from a main branch of the HA, resulting in disruption of graft arterial blood supply
This is a very rare (incidence of 0.64%) but a dramatic complication following OLT with a high mortality rate
In most cases, HAR complicates a pseudoaneurysm of the HA
The median time of HAR is 29 d (range: 2-92 d) following OLT
The clinical presentation is always a sudden hemorrhage: Hemoperitoneum, gastrointestinal bleeding, hematoma and hemobilia
Treatment comprises urgent laparotomy with definitive ligation of the HA, anastomotic revision and aortohepatic grafting or interventional radiology with percutaneous embolization

HA: Hepatic artery; HAT: Hepatic artery thrombosis; OLT: Orthotopic liver transplantation; HAR: Hepatic artery rupture.

transplantation) because of hemorrhagic relapse or sepsis[26].

Prognosis: Boleslawski *et al*[26] (2013) also studied the effect of HA ligation on survival. They compared patients with (n = 10) and without (n = 6) HA ligation treatment. Of the 6 patients that received percutaneous embolization or revascularization, only 1 survived beyond 90 d (mortality rate: 83%). The 10 patients with HA ligation survived after postoperative day 90. Additionally, the one- and three-year graft survival rates for patients without HA ligation were 14% and 14%, respectively, *vs* 80% and 70%, respectively, in patients with HA ligation. The one- and three-year overall survival probabilities were 14% and 14%, respectively, in patients without HA ligation *vs* 100% and 80%, respectively, in patients with HA ligation[26].

Conclusion: Finally, in this retrospective study, Boleslawski *et al*[26] (2013) recommended that HA revascularization should be avoided, especially when mycotic pseudoaneurysm is suspected (*i.e.,* if there was a gastrointestinal wound during liver procurement, documented systemic candidiasis prior to HAR, or if HAR occurred several weeks after transplant with associated lesions, such as biliary leak or gastroduodenal perforation). In contrast, HA ligation seems to be a reasonable life-saving option because it prevents hemorrhagic recurrence and should achieve a successful long-term outcome, with or even without retransplantation. Expected biliary complications, such as ischemic cholangitis, following HA ligation could be managed afterward by percutaneous and/or endoscopic interventions[26].

VENOUS COMPLICATIONS

Compared to arterial complications, venous complications are less frequent with an estimated overall incidence of less than 3%[4,5,8,9,62,89-91]. They can be potentially devastating and lead to graft failure, and therefore represent an important source of morbidity and mortality after OLT, especially if they occur in the early post-operative period[9,90,91]. Numerous literature reports have demonstrated that the incidence of venous complications in pediatric transplants is higher than in adult transplants[9,62,92,93]. Venous complications following OLT include: Portal (1%-3%) and caval (< 2%) problems[5,8,9,91]. The etiology underlying most of these involves the anastomosis, including: (1) PVT: < 3% (the most pejorative), portal vein stenosis (PVS): 2%-3%; and (2) caval and hepatic veins with specific complications depending to the type of anastomosis either end to end caval anastomosis: Thrombosis, stenosis (< 2%); or piggyback: Thrombosis, stenosis, kinking < 2%[4,5,8,9,91,94,95]. In the same fashion as HACs, they can be classified into two categories (Table 1): Early (< 1 mo) or late (delayed, *i.e.*, > 1 mo). In the recent years, the literature has been in favor of endovascular intervention management of venous complications, with very good outcomes[8,9,10,62,91].

Portal vein complications

The incidence of portal vein complications (PVCs) following liver transplantation is relatively uncommon, occurring in 1% to 3% of patients[4,5,8,9,89-91,96]. These complications are associated with high morbidity and graft loss[8,9]. An another important fact to mention is that PVCs are more common with split liver and LDLT and also in pediatric transplantation[91,97]. Regarding PVCs, DUS, contrast enhanced ultrasound (CEUS) and contrast-enhanced CT are the usual tools for diagnosis; more recently, magnetic resonance venography using the gadofosveset trisodium agent has been proposed[8,9,98]. Therapeutic PVCs management ranges from thrombectomy and anastomosis revision to retransplantation depending to the delay of occurrence after OLT. Nowadays, except early PVT, endovascular procedures are now considered to be the first line treatment for post-transplant PVCs, and many studies have shown highly successful results[62,93,99,100].

PVT: The incidence of PVT in OLT ranges from 0.3%-2.6%[1,90] (Table 6). From the UCLA experience, Duffy *et al*[5] (2009) reported a PVT incidence of 2% in more than 4200 patients. However, the incidence of PVT is close to 4% in adult LDLT due to technical difficulties in PV reconstructions, mainly related to a shorter vessel pedicle and limited vessel graft[101]. In LDLT, PVT occurs more frequently in the early period, defined as within 3 mo by Kyoden *et al*[101] (2008) (73% of cases from Kyoden's series; median, 58 d; range, 1-68 d).

The clinical presentation depends on the time the

Table 6 Portal vein thombosis highlights

Summary of the clinical characteristics about PVT
The incidence of PVT is uncommon and ranges from < 3% following OLT
PVT incidence is higher in pediatric transplantation, LDLT and split liver transplantation
Early PVT is more frequent than late PVT with a median time to diagnosis of 5 d following OLT (range: 1 to 15 d)
The clinical presentation of early PVT ranges from portal hypertension manifestations (abdominal pain, ascites, gastrointestinal bleeding, splenomegaly) to severe allograft dysfunction and multiorgan failure
The most common causes leading to PVT are technical errors and anatomic complications such as venous redundancy, kinking and/or stenosis of the anastomosis
Risk factors are the presence of portal thrombosis prior OLT, small diameter of the portal vein, previous splenectomy, large portosystemic collaterals and the use of cryopreserved venous conduits for PV reconstruction
DUS, CEUS, contrast-enhanced CT, MRI and portography are imaging tools used for a positive diagnosis
PVT treatment includes systemic anticoagulation therapy, catheter-based thrombolytic therapy by percutaneous radiological intervention (transhepatic or transjugular access depending of the coagulation state) with or without stent placement to portosystemic shunting (TIPS) to retransplantation in highly unresolvable cases
PVT is associated with poor survival without treatment, but with prompt management, outcomes in terms of morbidity and mortality are satisfying

DUS: Doppler ultrasound; PVT: Portal vein thombosis; OLT: Orthotopic liver transplantation; LDLT: Living donor liver transplantation; CEUS: Contrast enhanced ultrasound; MRI: Magnetic resonance imaging; CT: Computed tomography; TIPS: Transjugular intrahepatic portosystemic shunt.

thrombosis occurs. When it occurs early, severe acute liver insufficiency or graft failure predominates. If it occurs late, clinical symptoms depend of the portocaval collateral circulation existence. Portal hypertension manifestations including upper gastrointestinal bleeding due to esophagogastric varices and ascites are the most frequent clinical presentations. In contrast, liver failure is rare[30,90,96]. Langnas *et al*[30] (1991) reported a mean diagnosis time of 5 d following OLT (range: 1 to 15 d), which was confirmed by Kyoden *et al*[101] (2008), who reported that PVT occurred more frequently in the early period, *i.e*., 8/11 cases (72%).

The most common causes of PVT are technical errors related to venous redundancy and kinking and/or stenosis of the anastomosis[90]. Other reported risk factors include prior surgery on the portal or splanchnic venous system or a pre-transplant portal thrombosis requiring thrombectomy during the operation, a small diameter of the portal vein (< 5 mm), previous splenectomy, hypoplastic portal vein, large portosystemic collaterals and the use of venous conduits for portal vein reconstruction[90,96]. Specific risk factors found in adult LDLT are: Small PV size, liver graft position and the type of venous conduits used to connect the PV of the donor to the recipient such as a cryo-preserved vein, the use of which is discouraged by Kyoden *et al*[101] (2008)[30,90,96,102-105].

DUS should be the first imaging tool used and is easily employed to evaluate vascular patency. It allows, in most cases, for an immediate non-invasive diagnosis and provides a rapid evaluation of vascular flow patency. DUS protocols vary widely worldwide among liver transplant centers, but most teams recommend performing DUS daily (some authors recommend twice daily) in the immediate post-operative period until POD 5 or in the presence of abnormalities of liver function tests or a clinical suspicion of the diagnosis[106-109]. Recently, other authors have proposed the use of CEUS to avoid frequent false-positive results after DUS[108,110]. CEUS may help in assessing the severity of portal insufficiency, based on evidence of parenchymal perfusion status. It allows to show small thrombus in a peripheral portal branch[108,110]. In a retrospective evaluation of 23 patients, CEUS was used as an additional diagnostic method to DUS, CT and magnetic resonance imaging[110]. The authors reported new clinically relevant findings in 52% of cases, such as PVT confirmed during surgery or other radiological results.

Therapeutic options for PVT range from systemic anticoagulation to catheter-based thrombolytic therapy, to surgical revision until retransplantation. The three percutaneous options presented in the literature include transhepatic portal vein angioplasty (with or without stent placement), percutaneous thrombolytic therapy *via* transjugular intrahepatic portosystemic shunt (TIPS) creation and the transplenic approach[111-114]. In practice, three different therapeutic situations that require specific care may be distinguished: (1) complete PVT within the first 48 h post-OLT; (2) PVT (complete or partial) at 48 h and not more than at 30 d (early PVT); and (3) after more than at 30 d (late PVT).

Early complete PVT within the first 72 h post-LT: In a patient who shows signs of multiorgan failure, surgical revision of the anastomosis is mandatory. In the presence of kinking or twisting that caused the thrombosis, anastomotic revision and systemic anticoagulation are sufficient to resolve this condition. If this procedure is unsuccessful in obtaining satisfactory portal transplant revascularization, emergent retransplantation should be indicated.

Early PVT (PVT > 72 h and < 30 d): Independently of PVT presentation (partial or complete), non-surgical treatment should be reasonably attempted. The most frequent procedure is percutaneous thrombolysis associated with stent placement[111,113,115-117]. Cherukuri *et al*[113] (1998) reported the necessity that thrombolytic doses should be relatively low and maintained for only a few hours for efficacy and safety Concerning the modality for stent placement, two different possibilities are described in the literature: The classical percutaneous

Table 7 Portal vein stenosis highlights

Summary of the clinical characteristics about PVS
The true incidence of PVS is not really known, but is thought to be < 3%
The major complication of PVS is the evolution to PVT if not treated
The majority of patients with PVS are asymptomatic and the diagnosis of stenosis is an incidental finding detected on routine DUS screening
Risk factors of PVS are almost exclusively represented by technical errors, particularly if a tapered anastomosis is required in the case of a vessel size mismatch between donor and recipient
Pre-OLT radiotherapy is another major predisposing factor of PVS
DUS with the finding of a stenosis ratio > 50% or a portal velocity ratio > 3:1 defines PVS. Contrast-enhanced CT and portography are used to confirm the diagnosis
If PVS is asymptomatic, no therapeutic intervention with close surveillance is possible, but anticoagulation therapy is recommended
In patients with clinical manifestations, percutaneous radiological intervention is the method of choice by transhepatic or transjugular access to perform angioplasty with our without stent placement; this prevents recurrence with a high rate of success and low rate of complications

PVT: Portal vein thombosis; PVS: Portal vein stenosis; DUS: Doppler ultrasound; OLT: Orthotopic liver transplantation; CT: Computed tomography.

transhepatic approach and the transjugular approach. It is obvious that the latter should be preferred in patients with a coagulopathy or ascites, to minimize the risk of bleeding from transhepatic puncture[118-120]. This method has already been used in transplanted patients in the presence of decompensated cirrhosis, veno-occlusive disease or portal hypertension. The success rate with different endovascular methods ranges from 68%-100% and the mortality and morbidity rates are between 0% and 11%, respectively[121].

Late PVT (PVT > 30 d): Two clinical presentations should be distinguished. Late PVT involving or not the superior mesenteric vein and normal liver function tests develop *de novo* hepato-portal collaterals and cavernoma formation. In these cases, observation may be justified, because of the appropriate venous inflow from the splenic circulation[19]; Late PVT with symptomatic manifestations such as acute gastroesophageal bleeding or ascites that should be treated with percutaneous or transjugular transhepatic procedures. Regarding the transjugular experience, Lodhia *et al*[122] (2010) reported 3 cases of acute PVT occurring years following LT treated with an approach combining a TIPS and mechanical thrombectomy. To reduce the risk of periprocedural pulmonary emboli, the authors performed direct PV thrombolysis prior to placing the TIPS stent in order to allow time for clot dissolution[122]. Another possibility reported by Guckelberger *et al*[123] (1999) was described for cases of late PVT with complete recanalization using a systemic low dose recombinant tissue plasminogen activator (rt-PA). The authors reported their experience with late PVT 45 mo after LT and justified the use of systemic low dose rt-PA lysis continuously for 10 d, along with 25000 IU heparin per day to adjust the partial thromboplastin time to favorable administration[123]. In fact, although, streptokinase (SK) and urokinase (UK) have been shown to be largely effective for thrombolytic therapies, both are characterized by limited thrombolytic potencies and major clinical disadvantages compared to rt-PA[124]. While streptokinase has a high antigenicity, both SK and UK, unlike rt-PA, lack fibrin-specific action which results in systemic consumption of plasminogen and decreased thrombolytic efficacy. Furthermore, it may increase bleeding complications[124].

PVT is associated with poor survival without treatment, but in cases of prompt diagnosis and adequate management, the literature shows good results in terms of morbidity and mortality.

To conclude, PVT is a rare but serious complication when it occurs in the early post-operative period. Diagnosis is mandatory as soon as possible by DUS screening protocols or with suspicious clinico-biological findings including abnormal abdominal pain and/or elevated liver enzymes and unexpected decrease PT. Surgical thrombectomy is traditionally required in the early post-operative period, but percutaneous radiological intervention has progressively become the best therapeutic option with good outcomes and safety.

PVS

The true incidence of PVS after LT is not really known, and the only data reported in the literature concerning the incidence of venous complications is < 3%[91] (Table 7).

When PVS occurs, it can be present with graft failure or the complication of portal hypertension[125]. In practice, the majority of patients with PVS are asymptomatic and the diagnosis of stenosis is an incidental finding detected on routine screening ultrasound. Conversely, when the patients are symptomatic, they may present with signs of portal hypertension, which include upper gastrointestinal tract bleeding from gastroesophageal varices, ascites and splenomegaly. Abnormal liver function tests are not constant, and are therefore not a reliable sign for PVS diagnosis[91].

Regarding the risk factors of PVS, similar to PVT, it is well-established that the major concern is surgical technical errors[91]. Classically, the portal anastomosis is end-to-end and is usually simple in OLT, though a tapered anastomosis may be required when a significant size mismatch exists between the recipient and the donor, which constitutes a risk factor of stenosis. It explains in part why the pediatric population represents a population highly at risk to PVS[91]. In most cases, early PVS is the consequence of a surgical mistake due to technical difficulties in the anastomosis and could

evolve into an early thrombosis if not treated promptly. In contrast, it is assumed that late PVS is secondary to fibrosis or intimal hyperplasia[126]. Schneider *et al*[125] (2011) reported some cases of PVS after neoadjuvant radiotherapy for cholangiocarcinoma, and highlighted radiotherapy as a predisposing factor in venous complications; 21% of the patients who received a LT following the Mayo protocol for cholangiocarcinoma developed PVCs[125,127].

Concerning a positive diagnosis, although DUS is the first screening morphological tool to use, its definition is still controversial because of the lack of definite and objective criteria. Moreover, DUS is sensitive for PVS but it is not specific. The PVS criteria for diagnosis include portal caliber size, velocities at the anastomotic site, as well as the preanastomotic and postanastomotic gradients. Recently Huang *et al*[107] (2010) reported a formula that can estimate the portal stenosis ratio in LDLT: They calculated the portal stenosis ratio (SR) = PRE-AS/PRE > 50% [anastomotic stenosis (AS); prestenotic stenosis (PRE)]; significant PVS was defined as a PVS with an SR > 50%. The portal velocity ratio (VR) was also calculated between AS and PRE, such that > 3:1 is defined as a significant VR value correlating with the SR evaluation. If these are confirmed, the patient should undergo contrast-enhanced CT to confirm the diagnosis[107]. Some authors consider the pressure gradient between the pre- and post-stenosis site. Wei *et al*[126] (2009) considered a gradient of > 5 mmHg to initiate treatment, while Shibata *et al*[128] (2005) used a significant gradient of > 3 mmHg. Other authors did not measure gradients if the stenosis was noted to be greater than 75% of the main portal vein diameter.

Surgical treatment, including anastomotic revision or retransplantation, is usually preferred for early portal inflow abnormalities following OLT[129]. In cases of asymptomatic patients with normal hepatic function test results, PVS may be solely observed with no therapeutic intervention[102]. In these particular cases, and in view of the possible evolution to PVT, it is reasonable to screen regularly by DUS to check for the patency of the PV. Moreover, in this condition, the use of anticoagulant therapy is still discussed and there is no international consensus or recommendation on this issue. In patients with clinical manifestations and radiological confirmation of significant stenosis, therapeutic intervention is mandatory to avoid graft loss, retransplantation and mortality. Interventional radiology has become widely recognized as the first choice for treatment for PVS after LT[103-105,111,125,126,128-132]. Regarding PVS management, it is possible to use the transhepatic access or transjugular access[133], but most authors choose a transhepatic approach, usually from the right side. Shibata *et al*[128] (2005) reported that a single balloon dilatation was sufficient to maintain patency in 77.7% of patients, with a mean follow-up of 24.8 mo. In some series, stent placement associated with PTA was used to prevent recurrence. However, problems related to stent placement have been reported by Zajko *et al*[130] (1994), *i.e.*, a thrombus that developed around the stent that could not be lysed, requiring retransplantation. However, Ko *et al*[129] (2007) reported on their experience in PVS management by percutaneous transhepatic primary stent placement after LDLT. In this series, technical and clinical success was obtained in 77.8% by using this method with a complication rate of 33% (including hemoperitoneum caused by blood oozing from the transhepatic tract and intrahepatic pseudoaneurysms)[129]. Finally, regarding the recurrence rate, this ranges between 0%-100%. Shibata *et al*[128] (2005) reported the most important series in the literature where the recurrence rate was 28.6%. Some authors recommend the use of anticoagulant therapy for the prevention of recurrent PVT[134]. Recently, Sanada *et al*[134] (2010) concluded that the use of three anticoagulant therapies, *i.e.*, low-molecular-weight heparin, warfarin and aspirin, significantly reduced the recurrence of thrombosis with a median follow-up of three months[134]. Additionally, some authors have coupled endovascular treatment with surgical PV access[106].

To conclude, PVS represents an uncommon venous complication following OLT. This condition is more specific to pediatric LT and LDLT. As described earlier, a DUS screening protocol is an important diagnostic tool to help the clinician because the majority of asymptomatic cases can progress until PVT if not promptly treated, with negative effects on the prognosis of the graft and ultimately patient survival. Currently, it is obvious that percutaneous transhepatic radiological intervention with stent placement is the method of choice to address this complication with a high rate of success and a low rate of recurrence and/or complications.

Caval vein complications

Currently, transplant outflow obstruction by kinking, stenosis or thrombosis of the inferior vena cava (IVC) or hepatic vein, especially in LDLT, are relatively uncommon complications following liver transplantation with an reported incidence of less than 3%[94,95] (Table 8 and Figure 5).

Clinical presentation ranges from lower limb edema, hepatomegaly, ascites, pleural effusions, Budd-Chiari syndrome, liver and renal failure to hypotension leading to allograft loss and multiorgan failure[4,89,135].

The main risk factor leading to caval anastomosis complications (CACs) is represented by technical errors in the connection of caval anastomoses, which lead to kinking or thrombosis in the early post-operative course. In the late post-operative period, chronic stenosis in the anastomotic area is the result of fibrosis, hyperplasia and/or extrinsic compression from the enlarged liver graft[2,136,137].

Diagnosis should be achieved by DUS, contrast-enhanced CT, and finally by cavography which allows for providing treatment.

Many techniques for caval anastomosis connection can avoid these complications, such as piggyback (PB) and subsequently modified-PB, first described by Starzl

Table 8 Caval anastomosis complication highlights

Summary of the clinical characteristics about CAC
The incidence of CAC is not known and is thought to be less than 3%
CAC is represented by stenosis, thrombosis and kinking depending on the type of caval anastomosis (cava resection or PB)
Clinical presentation of CAC ranges from lower limb edema, hepatomegaly, ascites, pleural effusions, Budd-Chiari syndrome, liver and renal failure, and hypotension, leading to allograft loss and even death
The main risk factor is a technical error in the creation of the anastomosis, which leads to kinking stenosis and thrombosis
Modified-PB with the three-hepatic vein seems to offer better outcomes because it has been demonstrated to be an efficient and safe method
Diagnosis tools include DUS, contrast-enhanced CT and cavography
Percutaneous radiological intervention is the method of choice *via* a transjugular approach or transhepatic approach if the anastomosis cannot be catheterized
It includes angioplasty by balloon dilatation and recurrences should be prevented by stent placement

CAC: Caval anastomosis complication; DUS: Doppler ultrasound; CT: Computed tomography; PB: Piggyback.

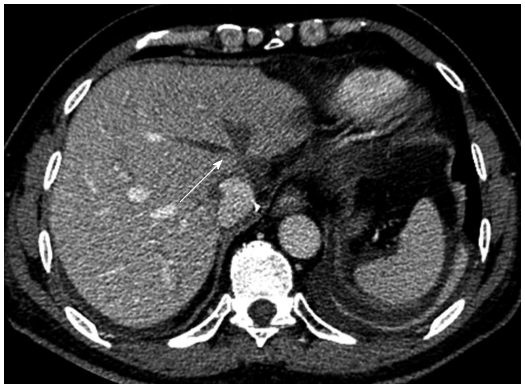

Figure 5 Contrast-enhanced-multidetector-row computed tomography-scan showing median and left thromboses hepatic veins following orthotopic liver transplantation (arrow).

et al[138] (1968). The method described by Starzl *et al*[138] (1968) consists of a complete resection of the recipient IVC and interposition of the donor intrahepatic part of the vena cava with two end-to-end anastomoses[138-144]. The preservation of the recipient IVC with the PB technique has been associated with an increased risk of suprahepatic IVC thrombosis or stenosis, leading to acute or chronic Budd-Chiari syndrome in 0% to 1.6%, venous congestion of the liver allograft in 1%, and with an increased incidence of post-transplant ascites[89,135]. To avoid such complications, techniques for optimizing outflow with the piggyback technique have been described; the main of these in undoubtfully the width of the caval anastomosis, while other authors have reported methods using either the two-vein or the three-vein technique for anastomosis with a low rate of CACs[89,94,135,145-149]. Finally, several studies have demonstrated the superiority of modified-PB with the three-hepatic vein technique, which should be routinely used in OLT because it is safe and efficient and involves few surgical complications[89,94,143]. Hepatic venous stenosis is specific to LDLT with an incidence of 2% to 4%, because of the different techniques of donor graft outflow venoplasty, leading to Budd-Chiari syndrome or outflow block syndrome after LDLT[150].

Therapeutic management of CACs depends on the time of the presentation and the delay following OLT. In the case of severe allograft dysfunction or multiorgan failure, retransplantation is always indicated. Beyond this particular situation, percutaneous radiological intervention is the method of choice, where mortality after interventional transplant salvage procedure is 11.1% as compared with 41.6% mortality for those patients managed by retransplantation[121,137]. Treatment can be performed by transjugular approach, but percutaneous transhepatic access may be necessary when the anastomosis cannot be catheterized from the jugular access. Angioplasty by balloon dilatation can restore anastomotic patency in almost 100% of cases, but recidive of stenosis is frequent and repeat angioplasties may be applied[137]. PTA associated with stent placement may be the better solution with a high rate of success ranging from 73% to 100% in the literature; this technique is safe and apparently durable[121,130,136,137,151-157].

To conclude, the incidence of CACs is very low, and particular attention should be paid to the caval anastomosis connection. Currently, modified-PB using the three-hepatic vein technique seems to show better outcomes. As with other VCs, prompt diagnosis and management are required if the patient is clinically symptomatic. The percutaneous endovascular method should be attempted to rescue the outflow patency, reserving surgical revision in unresolvable cases and ultimately retransplantation in patients presenting multiorgan failure.

CONCLUSION

VCs continue to be a major problem following transplantation with a relatively frequent incidence (7%). They carry a high rate of morbidity and mortality, especially if they occur in the immediate post-operative period (first month) and if diagnosed late. The only solution to reduce their gravity is to prevent it by controlling risk factors and, if this is not possible, to diagnose them as early as can be, even in asymptomatic or paucisymptomatic patients. Many transplant teams worldwide advocate the routine use of complementary explorations such as DUS and, if in doubt, a contrast-enhanced CT scan or classical arteriography, which is

the reference. Currently, if recognized promptly, and if there is no graft or multiorgan failure, endovascular treatment should be attempted first if a technical plateau is available, because this has demonstrated efficacious and safe outcomes. Conversely, if there are severe liver repercussions, the most efficient therapeutic procedure is an emergency retransplant which shows better outcomes in terms of efficacy and survival, but the organ shortage dramatically limits this therapeutic option.

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REVIEW

Selection of patients with hepatocellular carcinoma for liver transplantation: Past and future

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Abstract

The aim of liver transplantation (LT) for hepatocellular carcinoma (HCC) is to ensure a rate of disease-free survival similar to that of patients transplanted due to benign disease. Therefore, we are forced to adopt strict criteria when selecting candidates for LT and prioritizing patients on the waiting list (WL), to have clarified indications for bridging therapy for groups at risk for progression or recurrence, and to establish certain limits for downstaging therapies. Although the Milan criteria (MC) remain the standard and most employed criteria for indication of HCC patients for LT by far, in the coming years, criteria will be consolidated that take into account not only data regarding the size/volume and number of tumors but also their biology. This criteria will mainly include the alpha fetoprotein (AFP) values and, in view of their wide variability, any of the published logarithmic models for the selection of candidates for LT. Bridging therapy is necessary for HCC patients on the WL who meet the MC and have the possibility of experiencing a delay for LT greater than 6 mo or any of the known risk factors for recurrence. It is difficult to define single AFP values that would indicate bridging therapy (200, 300 or 400 ng/mL); therefore, it is preferable to rely on the criteria of a French AFP model score > 2. Other single indications for bridging therapy include a tumor diameter greater than 3 cm, more than one tumor, and having an AFP slope greater than 15 ng/mL per month or > 50 ng/mL for three months during strict monitoring while on the WL. When considering the inclusion of patients on the WL who do not meet the MC, it is mandatory to determine their eligibility for downstaging therapy prior to inclusion. The upper limit for this therapy could be one lesion up to 8 cm, 2-3 lesions with a total tumor diameter up to 8 cm, or a total tumor volume of 115 cm^3. Lastly, liver allocation and the prioritization of patients with HCC on

the WL should take into account the recently described HCC model for end-stage liver disease, which considers hepatic function, HCC size and the number and the log of AFP values. This formula has been calibrated with the survival data of non-HCC patients and produces a dynamic and more accurate assessment model.

Key words: Hepatocarcinoma; Liver transplantation; Alpha fetoprotein; Patient selection; Prioritization; Waiting list; Bridging therapy; Allocation; Downstaging

Core tip: This article aims to provide clinicians who treat patients with hepatocellular carcinoma, in whom liver transplantation may be indicated, with an actualized tool that considers a combination of morphological (size and number of tumors) and biological data (alpha fetoprotein value) and that facilitates the process of selecting candidates, predicts the indication of and response to neoadjuvant therapy prior to transplantation and also aids in the prioritization of patients once they are on the waiting list.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major global health problem. It is the sixth most common cancer worldwide[1] and the third most common cause of cancer death[2]. Without treatment, the 5-year survival rate is 10%-12%[3,4]. In the early stages, curative treatment includes resection, radiofrequency ablation and liver transplantation (LT). The latter technique remains the most effective treatment method in cases of early HCC because it jointly eliminates the tumor and the underlying disease and shows 1- and 5-year survival rates of 85% and 70%, respectively[5]. However, LT does not completely eliminate the possibility of recurrence, which is still a serious problem; therefore, it is discussed in this review.

DIAGNOSIS

In the last decade, great improvements in HCC diagnosis[6] have occurred, which are mainly based on imaging tests. In recent years, HCC has been diagnosed earlier[7], and due to the improvements in imaging tests, a progressive decline in the use of alpha fetoprotein (AFP) levels for the surveillance of HCC in cirrhotic patients[6,8,9] has occurred owing to their lack of appropriate sensitivity and specificity[8].

For lesions less than 1 cm, ultrasonography is repeated every three months, and for lesions larger than 1 cm, a typical image (arterial hypervascularity and venous delayed phase wash out) can be used to confirm the diagnosis[8] because this method is 100% specific, with a very high predictive value[10]. When a surveillance test is positive, a more definitive noninvasive imaging exam is recommended. Recent guidelines endorse multiphasic computerized tomography (CT) and magnetic resonance imaging (MRI) with hepatobiliary agents as first-line modalities for this purpose. Both modalities provide excellent sensitivity for nodular HCCs larger than 2 cm, modest sensitivity for 1-2-cm HCCs, and poor sensitivity for HCCs smaller than 1 cm. However, MRI is emerging worldwide as a leading method for the diagnosis and staging of HCC, and it is the most sensitive method for the detection of small HCCs[11]. However, the combination of dual-phase CT-angiography in the arterial and portal phase with positron emission tomography (PET) imaging using (18)F-fluorodeoxyglucose [(18)FDG] appears to be a sensitive method for the detection of HCC with the alternative presence of hypervascularity or hyperaccumulation of (18)FDG[12].

If the radiological pattern is not typical, the test should be repeated. If the result does not meet the criteria for HCC, a biopsy of the lesion can be performed while taking into account that a negative finding after a biopsy does not exclude HCC[1], and the possible complications of a biopsy such as hemorrhage and needle track tumoral implant should be considered[13]. Although in a recent, long retrospective series the incidence of HCC was only 0.2%[14], in a meta-analysis the incidence was 2.7% overall or 0.9% per year[15].

STAGING

The TNM classification, which is widely accepted for the staging of cancer, for HCC has a lower capacity to predict long-term survival[16]. For this reason, the Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy is most often used[9,17] because it includes information concerning the tumor, hepatic function and the general clinical status[18]. However, in spite of these facts, the TNM classification is used as the reference for pathological studies of surgical specimens.

SELECTION OF CANDIDATES WITH HCC FOR LT

The aim of LT for HCC is to obtain a level of disease-free survival (DFS) similar to that of patients who are transplanted for benign disease; therefore, we are obliged to adopt strict selection criteria for candidates, with the intention of obtaining the maximum survival with the minimum possible recurrence.

Table 1 Isolated biological criteria for the selection of candidates with hepatocellular carcinoma for liver transplantation

Ref.	Pretrasplant AFP levels (ng/mL)	Importance	
Figueras *et al*[19]	> 300	Factor for mortality	
Yao *et al*[16]	> 1.000	Reduced survival	
Bruix[20]	> 200	Significant worse outcomes	
Xu *et al*[21]	> 400	Higher tumor recurrence	
Mailey *et al*[22]	Low (≤ 20)	Mediun and high: Higher mortality	
	Mediun (20-399)		
	High (≥ 400)		
Muscari *et al*[23]		DFS	Recurrence
	Normal	71%	4%
	10-150	75%	10%
	150-500	57%	24%
	> 500	46%	62%
Chiao *et al*[24]	> 1.000	Reason for exclusion from the WL	
Hameed *et al*[25]			
Menon *et al*[26]	> 10.000	Reason for exclusion from the WL	

AFP: Alpha fetoprotein; DFS: Disease-free survival; WL: Waiting list.

Table 2 Selection criteria base on radiological/morphological tumor characteristics

Ref.	Parameters	Importance
Bismuth *et al*[30]	Up to 3 nodules Each < 3 cm	Best results
Mazzaferro *et al*[31]	Single lesion < 5 cm < 3 lesions, each < 3 cm No macrovascular invasion No extrahepatic disease	DFS > 75% Recurrence < 15%
Löhe *et al*[34]	Single tumor with size > 5 cm	Reduction in DFS
Yao *et al*[16]	Single lesion ≤ 6 cm 2-3 lesions each ≤ 4.5 cm Total tumor diameter ≤ 8 cm	DFS > 75% Recurrence < 15%
Mazzaferro[41]	Ordinates: *n* of tumors Abscissas: Tumor size	Progressive reduction of 5 yr survival
Mazzaferro *et al*[42]	Up to 7, as the sum of: Largest tumor in centimeter and *n* of tumors	71.2% 5 yr survival
Jang *et al*[46]	10 as the sum of: Largest tumor in cm and *n* of tumors	If >: Decreased DFS

DFS: Disease-free survival.

Isolated biological criteria for the selection and prognosis of patients with HCC for LT

More than a decade ago, several authors noted the importance of the isolated AFP value in predicting mortality and/or posttransplant recurrence. High AFP values may be a marker for vascular invasion or extra hepatic disease that has escaped detection by conventional imaging techniques. It has been observed that a pretransplant AFP level higher than 300 ng/mL is the only factor independently associated with mortality after LT[19], and a level higher than 1000 ng/mL is a significant predictor of reduced survival[16]. In general, HCC patients on the waiting list (WL) with a baseline serum level of AFP > 200 ng/mL display significantly worse outcomes[20]; however, several detrimental cut-off values for AFP levels have been reported recently. Xu *et al*[21] found that pre-transplant AFP levels > 400 ng/mL were associated with higher tumor recurrence. Mailey *et al*[22] classified patients into low (≤ 20 ng/mL), medium (20-399 ng/mL), or high (≥ 400 ng/mL) AFP level groups. In a multivariate analysis, the medium and high AFP groups were associated with higher mortality. Another study[23] correlated the DFS and 5-year recurrence rate to the AFP level. Normal AFP values between 10-150 ng/mL, those from 150-500 ng/mL and those > 500 reduce DFS from 71% to 57%, 46% and 28%, respectively, and increase the recurrence rate from 4% to 10%, 24% and 62%, respectively. Recently, it was shown once again that an AFP level > 1000 ng/mL is a reason for exclusion from the WL[24,25], confirming data reported in 2001[16]. However these data have not been taken into account by programs using expanded criteria that only consider an AFP level greater than 10000 ng/mL as a reason for exclusion[26]. This matter will be further examined when discussing the indications for downstaging of HCC prior to LT (Table 1).

In Japan, des-gamma carboxy prothrombin (DCP) is well established as a biomarker and is reported to correlate with post-LT recurrence of HCC[27,28]. We cannot predict whether new molecular markers of HCC such as PIVKA-Ⅱ, a protein induced by the absence of Vit K, will have widespread use, but Japanese studies suggest that it is correlated with microvascular invasion[29].

Selection criteria based on radiological/morphologic tumor characteristics

Some criteria include the number and size of the tumors and the tumor volume.

Criteria based on number and size: In 1993, Bismuth *et al*[30] noted that patients transplanted for HCC with up to 3 nodules (each < 3 cm) exhibited the best results. In 1996, the Milan criteria (MC)[31] set clear limits on the selection of HCC patients for LT, consisting of a single lesion < 5 cm or fewer than three lesions, each < 3 cm and without macrovascular invasion or extrahepatic disease, which resulted in 5-year DFS > 75% and a recurrence rate < 15%[31]. Since that time, these standard selection criteria for LT due to HCC have been accepted worldwide[20,32,33]. Other authors have confirmed that a single tumor with a size > 5 cm causes a reduction in DFS[34]. The MC have received criticism because the radiological studies used for evaluations are not very accurate[35] and highly variable between centers. In addition, some authors have argued that these criteria are strict[20], with tumor size and tumor number cut-offs that are somewhat arbitrary and too restrictive, and that they deprive patients of the possible benefit of LT[36] and therefore should be extended[16,37,38] (Table 2).

Thus, in 2001 the so-called expanded criteria of the University of San Francisco, California (UCSF) were proposed by Yao *et al*[16], which set the limit for LT to a single lesion ≤ 6.5 cm in diameter or 2-3 lesions each ≤ 4.5 cm with a total maximum diameter ≤

Table 3 Selection criteria based on functional/radiological features of the tumor

Ref.	Parameters	Importance
Hiraoka *et al*[56]	Hyperintensity on gadoxetic acid-enhanced MRI	HCC with more malignant potential
Ferda *et al*[12]	Hipervascularity or hiperaccumulation of (18)FDG/PET/ with Dual-phase CT angiography (arterial/portal phase)	Distinguishing between welland Poorly differentiated HCC
Ochi *et al*[57]	High positivity in (18)FDG/PET/CT	Increase the risk of early recurrence
Kornberg *et al*[58]	mSUV	Reflects the existence of distant microsatellite
Kornberg[59]	Positivity in (18)FDG/PET/CT	Statistically significant lower survival post LT

CT: Computerized tomography; MRI: Magnetic resonance imaging; HCC: Hepatocellular carcinoma; PET: Positron emission tomography; LT: Liver transplantation; mSUV: Maximun standized uptake value; (18)FDG: (18)F-fluorodeoxyglucose.

8 cm, thus obtaining similar survival after LT to that obtained with the MC. These criteria were criticized because in this study, only 24% of the patients did not meet the MC[39], and because it was a retrospective study based on the histology of explants[40]. By that time, Mazzaferro[41] had introduced the concept of the Metroticket calculator, a system of orderly Cartesian ordinates (number of tumors) and abscissa (tumor size) in which the progressive reduction of 5-year survival is graphically represented as these parameters increase, leading to the expression "the longer the trip, the higher the price". In 2009, Mazzaferro *et al*[42] found that a total tumor diameter greater than 7 cm resulted in an increase in the percentage of recurrence and proposed a new MC (the so-called up-to-seven), using seven as the sum of the size of the largest tumor (in centimeter) and the number of tumors, which yielded 5-year overall survival of 71.2%. Many groups have validated these criteria[43,44], but after 5 years, they have not been accepted as widely as the MC. Other authors have made similar suggestions[45]; however, others have placed this limit at 10 cm, which results in a decrease in DFS[46]. This value should be universally accepted as the upper limit[26]. The expanded criteria require further validation because recurrence could be less often reported, increasing the risk of vascular invasion, microsatellites and poorly differentiated tumors[35,47,48].

Morphological criteria based on the total tumor volume: Toso *et al*[37] calculated the total tumor volume (TTV) as the sum of the volumes of all tumors using the formula $(4/3)\,\pi\,r^3$, where r is the maximum radius of each tumor. The radiological accuracy of this formula was greater, and based on the risk of recurrence, a threshold of 115 cm^3 was established, which allowed the selection of more patients for LT with results similar to those of the MC and UCSF criteria[37]. According to this mathematical formula, the largest tumor has the maximum importance. As a result, the possibility of correct staging increases because larger tumors are evaluated more accurately than smaller ones.

Expansion of the MC may be justified in regions with less organ shortage, but this will require demonstrating high survival rates for the newly eligible patients[49]. Regional variation in survival does not facilitate a national policy[50], but it is undeniable that in the USA, 97% of patients transplanted for HCC meet the MC[51], and although this number has changed somewhat recently, the number of inclusions for patients for LT that do not meet the MC is still less than 5%[52]. It should be mentioned that until very recently, the criteria used in the United Kingdom for LT for HCC considered a maximum tumor diameter up to 15 cm (up to 5 tumors all $\leqslant$ 3 cm), which is well beyond the limit of the MC and UCSF criteria[26].

Selection criteria based on functional/radiological features of the tumor: Dynamic MRI may constitute a non-invasive and promising method to assess the biology of HCC due to its greater avidity of contrast uptake, which implies a higher degree of microscopic vascular invasion and greater aggressiveness[53,54]. Tumors that are heterogeneously hyperintense in the hepatobiliary phase on gadoxetic acid-enhanced MRI have more malignant potential than other types of HCC[55]. Other authors[56] have used 18F-fluorodeoxyglucose positron emission tomography/computed tomography (18F-FDG PET/CT) not only for detection[12] but also as a prognostic factor, which distinguishes between well and poorly differentiated HCC[12]. High positivity of HCC increases the risk of early recurrence after curative resection[56], and the maximum standardized uptake value (mSUV) of 18F-FDG PET/CT reflects the existence of distant microsatellites; therefore, it can be a useful tool in the treatment protocol of HCC[57]. In a comparison of two groups of transplanted patients who did not meet the MC, other authors[58] found that patients with positive PET findings had significantly lower survival than PET negative patients (Table 3).

Combined morphological and biological tumor parameters: Adequate patient selection should be based on tumor biology assessed *via* serum or pathological parameters rather than on the macro morphology of HCC[59]. In fact, the aggressiveness of a tumor can be determined by a higher histological grade and greater microscopic vascular invasion, and a biopsy can used to predict DFS. The Toronto criteria[60] select patients with HCC for LT who do not meet the MC by biopsy exclusion of poorly differentiated tumors, resulting in 5-year overall survival (OS) and DFS values of 70% and 66%, respectively, which are similar to those of the MC (72% and 70%, respectively). However, there is little correlation between the biopsy and

Table 4 Combined morphological/biological selection criteria

Ref.	Parameters	Importance
DuBay *et al*[60]	Liver tumor biopsy	Excluding poorly differentiated tumors
Toso *et al*[52]	TTV > 115 cm^3	Reduced survival at 3 yr (< 50%)
	AFP > 400 ng/mL	Limit for indication for LT
Lai *et al*[62]	AFP > 400 ng/mL	Strongest predictor for recurrence
	Total tumor diameter > 8 cm	
Duvoux *et al*[63]	Model combining log10 AFP, tumor size and *n* of tumors: Score > or < 2	Score greater that 2 predict a market increase in 5 yr risk of recurrence and decreased survival
Berry *et al*[66]	AFP < 15 or > 15 ng/mL	AFP levels predicts post-transplant survival independently of MC

TTV: Total tumor volume; AFP: Alpha fetoprotein; MC: Milan criteria; LT: Liver transplantation.

histology of an explant due to tumor heterogeneity and because, in multifocal disease, the dominant lesion is not always the most biologically representative. For these reasons, currently, the biopsy has a limited role in pre-LT staging[61] (Table 4).

In 2009, Toso *et al*[52] found that only the TTV and AFP levels predicted survival and established a composite score with a TTV > 115 cm^3 or AFP > 400 ng/mL as limits for indication for transplantation because patients with greater values for these parameters had 3-year survival rates < 50%.

Using a multivariate analysis, Lai *et al*[62] found that an AFP level > 400 ng/mL and a total tumor diameter > 8 cm were the strongest predictors for recurrence.

Recently, Duvoux *et al*[63] generated an improved prognostic model for predicting recurrence in LT candidates with HCC. A prognostic score was developed and validated prospectively. The AFP level independently predicted tumor recurrence and was correlated with vascular invasion and differentiation. A model combining the log10 value of the AFP, tumor size and number of tumors was highly predictive of tumor recurrence and death. Using a simplified version of the model with untransformed AFP values, a cut-off value of 2 was identified. In the validation cohort, a score greater than 2 predicted a marked increase in 5-year risk of recurrence and decreased survival. Among patients who exceeded the MC, a score of 2 or lower identified a subgroup of patients with AFP levels less than 100 ng/mL and a low 5-year risk of recurrence. In contrast, for patients who met the MC, a score greater than 2 identified a subgroup of patients with AFP levels greater than 1000 ng/mL and a high risk of recurrence. We will refer to this as the French model.

Our group[64], based on our previous experience with LT for patients with HCC and cirrhosis, has performed an analysis of the risk factors for HCC relapse and applied the French AFP model to LT for HCC and cirrhosis patients who met the MC[65]. We were able to confirm the predictive value for tumor relapse of the French AFP model both pre- and postoperatively.

Berry *et al*[66] established that the AFP level, rather than the tumor burden, was most strongly associated with posttransplant survival. Thus, patients with HCC and AFP levels < 15 ng/mL at the time of transplantation did not exhibit excess posttransplant mortality; increases in AFP (16-65 ng/mL; 66-320 ng/mL and > 320 ng/mL) result in progressively worse posttransplant mortality than similar increases in recipients without HCC. Patients who did not meet the MC showed excellent survival if their AFP level was < 15 ng/mL. In contrast, patients who met the MC exhibited poor survival if their serum AFP level was substantially elevated (serum AFP $\geqslant$ 66 ng/mL). AFP changes while on the WL closely corresponded to changes in posttransplant mortality. Not only the absolute serum AFP level but also changes in this level strongly predicted posttransplant survival independently of tumor burden.

These models, combining data related to the tumor (size and number of tumors) with preoperative levels of AFP, had previously been studied by Japanese authors[67] in living-donor liver transplant (LDLT) patients (Table 5). In these models, a value of 1 to 4 points (p) was assigned to each of the following parameters: tumor size: $\leqslant$ 3 cm (1 p), 3.1-5 cm (2 p), 5.1-6.5 cm (3 p), > 6.5 cm (4 p); number of tumors: 1 (1 p), 2-3 (2 p), 4-5 (3 p), > 5-6 nodules (4 p); AFP: $\leqslant$ 20 ng/mL (1 p), 20.1-200 ng/mL (2 p), 200.1-1000 ng/mL (3 p), and > 1000 ng/mL (4 p). Candidates with 3-6 total points were "transplantable" and those with 7-12 points were "non-transplantable". In Japan and other Asian countries, due to the severe organ shortage, LDLT comprises the majority of LT[68]. Each center has developed and proposed expanded selection criteria based on institutional and regional experience, which vary from the model of Tokyo University[68], which only considers morphological tumor parameters, *i.e.*, up to 5 nodules with a maximum diameter $\leqslant$ 5 cm, without taking into account any biological markers. The Kyoto group[69] considers patients with less than 10 nodules, all less than 5 cm, with a DCP level < 400 mAU/mL, and the Kyushu group[70] also use extended criteria without limiting the number of nodules but require a maximum tumor diameter less than 5 cm and DCP levels under 300 mAU/mL.

ORGAN ALLOCATION FOR LT

The allocation of organs for LT follows criteria of prioritization that have varied throughout the history

Table 5 Japanese combined morphological/biological selection criteria for living-donor liver transplant

Ref.	Parameters					Importance: Limits for LDLT
	Value	1p	2p	3p	4p	
Yang *et al*[67]	T size (cm)	⩽ 3	3.1-5	5.1-6.5	> 6.5	Patients with 3-6 points are transplantable
	n of tumors	1	2-3	4-5	> 5 or 6	Those with 7-12 points are not transplantable
	AFP (ng/mL)	< 20	20-200	200.1-1.000	< 1.000	
Akamatsu *et al*[68]	Up to 5 nodules Maximum diameter ⩽ 5					Upper limit for LDLT
Kaido *et al*[69]	Less that 10 nodules, all < 5 cm DCP < 400 mAu/mL					Upper limit for LDLT
Shirabe *et al*[70]	*n* of nodules: No limit Maximun diameter: < 5 cm DCP < 300 mAu/mL					Upper limit for LDLT

AFP: Alpha fetoprotein; DCP: Des-gamma carboxy prothrombin; LDLT: Living-donor liver transplant.

of LT, from prioritization of the more serious patients based on the Child-Turcotte-Pugh score and the time of inclusion on the WL to the more recent model for end-stage liver disease (MELD) score. However, because this method does not consider the risk of neoplastic growth while on the WL, HCC patients are prioritized based on their exception points and the MELD exception, with the goal of obtaining similar WL mortality for neoplastic and non-neoplastic patients. Exception points are assigned every 3 mo[36] because progression of HCC can produce a 15% increase in mortality[71]. Paradoxically, several years later, it was found that the likelihood of undergoing transplantation was higher for HCC candidates than for other patients[72], which produced a clear disadvantage for non-HCC patients[73]. For this reason, the "HCC-MELD" equation (1.27/MELD - 0.51/logAFP + 4.59) has been proposed[74], which takes into account hepatic function and the log of the AFP value, and has been calibrated to the survival of non-HCC patients. This formula gives additional points to patients with HCC, not arbitrarily, but based on a calculation of the benefits of transplantation, in a manner similar to that for patients without HCC. Other authors[73], with a similar aim, have studied and validated a new and promising model for allocation of patients using a large cohort in the United States and United Kingdom that includes: HCC size, HCC number, AFP value, and the classic MELD score calculated according to the following formula: New MELD = -37.8 + 1.9 × MELD + 5.9 (if HCC number ⩾ 2) + 5.9 (if AFP level > 400 ng/mL) + 21.2 (if HCC size > 1 cm). This new model provides a dynamic and more accurate assessment of dropout than the use of the MELD exception, showing a distribution similar to that of the MELD for non-HCC patients. Both scores could be used in parallel for the management of WL patients with and without HCC.

NEOADJUVANT TREATMENT OF PATIENTS ON THE WL (BRIDGING AND DOWNSTAGING TREATMENTS)

HCC patients who meet the MC and are included on the WL should be monitored every 3 mo by CT/MRI and AFP level evaluation for the identification of those at high risk of dropout[75]. AFP progression while on the WL[66], and more specifically an AFP increase of > 15 ng/mL per month, is the most relevant preoperative prognostic factor for low OS and DFS[76]. For patients with changes in tumor size and/or an increase of in the AFP level > 50 ng/mL, locoregional therapy (LRT) or removal of the patient from the WL should be performed, if necessary[77].

Bridging therapy

Bridging therapy is used for patients with HCC who meet the MC and are included on the WL but have the possibility of a delay in LT > 6 mo. Its purpose[78] is to prevent tumor progression[79], reduce the recurrence of HCC after LT and increase posttransplant survival. As the waiting time for LT has progressively increased[79], treatment of HCC in patients awaiting LT has become routine[80]. Bridging is not indicated for tumors that meet the current MC, except for those with a diameter greater than 3 cm or patients with more than 1 tumor, because these patients are more likely to have recurrence after LT[81].

The most employed method of LRT for bridging therapy is percutaneous ablation[1], which is frequently performed by radiofrequency (RF) and less often performed by ethanolization (ET) or surgery. ET and RF have similar effectiveness for tumors less than 2 cm, but with increased tumor size, RF is more effective and shows similar results to surgery. In lesions > 3 cm, ET failures increase; therefore, it is rarely used as bridging therapy[82,83].

Patients with small solitary tumors and very well preserved liver function are the best candidates for surgical resection[1], but tumor recurrence complicates 70% of cases at 5 years[6]. Certain favorable locations, such as peripheral tumors and left hepatic lobe location, may allow laparoscopic resection, which avoids the greater complexity of transplantation after laparotomic surgery. Resection may offer improved local tumor control and allows full microscopic analysis, with subsequent study of its biological aggressiveness, which

could lead to subsequent elective LT. Subsequent tumor recurrence after resection is an absolute indication for LT; this so-called salvage transplantation was first described by Majno *et al*[84] in 2000. This procedure requires fewer donors and allows better management of the WL.

Downstaging

Downstaging[78,79] is used to convert tumors that initially do not meet the transplant criteria, usually intermediate multinodular asymptomatic tumors (stage B of the BCLC)[6], into tumors that meet the MC (the most frequent endpoint), UCSF criteria or the up-to-seven criteria, with the aim of including the patients on the WL once the tumor has decreased in size. Tumors with more favorable histology are more likely to respond to treatment and exhibit a good outcome after LT[85]. The eligibility criteria for downstaging should have an upper limit, which can be set as follows[85]: (1) one lesion > 5 cm and up to 8 cm; (2) two to three lesions with at least one lesion > 3 cm and not exceeding 5 cm, with a total tumor diameter up to 8 cm; or (3) four to five lesions with none > 3 cm, and a total tumor diameter up to 8 cm.

The LRT technique depends on each center, and the response is evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) or the modified RECIST (mRECIST)[86], which we will further discuss later. Once the treatment is completed, it is mandatory to follow the "ablate and wait policy"[81], with close monitoring for at least 3 mo before inclusion on the WL[50,85] to evaluate the tumor's behavior and exclude aggressive tumors from LT; therefore, a total of six months will elapse until transplantation[81].

Some authors[87] have attempted to perform a meta-analysis of HCC downstaging, which has been impossible due to many factors such as the great variability of the inclusion criteria protocols[79], variability of post-treatment response assessment and absence of histological information on tumor biology[87]. At the moment, there is no evidence that patients submitted to downstaging followed by LT have a worse prognosis than those who initially meet the MC. Therefore, we must assume that those patients should be eligible for LT, as if they had been from the start[87], and will show an excellent posttransplantation outcome[85], reaching 5-year survival rates comparable to those of patients who meet the MC or UCSF criteria and do not require downstaging[75,88].

Trans-arterial chemoembolization (TACE) is the form of LRT most often used for downstaging[75], followed by RF ablation[89]. Chemoembolization improves the survival of stringently selected patients with unresectable HCC[90]. Posttransplant survival has shown a marked benefit in response to TACE, but this benefit was only seen in patients whose disease meets, but does not exceed, the MC[91]. TACE can reduce the percentage of posttransplant recurrence (17% with treatment *vs* 36% without treatment)[92], and it is possible to verify its effectiveness using (18)FDG PET/CT to compare the SUV before and after treatment[93].

At the present time, there is no evidence demonstrating the superiority of one form of LRT over another, but merging the techniques of drug eluting beads-TACE and trans-arterial radio-embolization with Yttrium-90 and external bean conformal radiotherapy[78] is generally better tolerated than conventional techniques.

Response criteria following downstaging with LRT

The efficacy of neo-adjuvant treatments should be evaluated[79] by the rate of dropout from the WL and, methodologically, with a 3-mo interval mRECIST[86] reassessment that considers not only the reduction in size, but the amount of tumor necrosis and the disappearance of any intratumoral arterial enhancement in conjunction with the initial and post-treatment AFP levels.

Patients presenting with an AFP level > 1000 ng/mL submitted to downstaging are a special problem because such high levels predict a greater risk of tumor recurrence and are considered the only factor in treatment failure[85].

In these cases, a stable decrease in the AFP level to < 500 ng/mL is necessary in subsequent determinations until LT to consider the downstaging effective[50,94]. However, other authors[48] state that the level should be < 400 ng/mL because levels > 400 ng/mL in the immediate pretransplant period are a unique risk factor for recurrence after LRT[36]. This is because patients who did not show a reduction of the AFP level to $\leqslant$ 400 after downstaging had less intent-to-treat survival, and only the last pretransplant AFP value, not the original value (even if it was originally > 1000 ng/mL) or changes in the AFP level, independently predicted posttransplant survival[95]. Others have set the level to 100 ng/mL[96], but in general, the mean AFP levels are higher in patients who do not achieve successful downstaging[97]. AFP levels are considered to play an important role in monitoring the response and/or tumor progression after LRT[25,98].

Combined radiological and biological modifications permit documentation of the response to LRT in patients waiting for LT and are essential elements for further refining the selection criteria for potential liver recipients with HCC[94]. An AFP level $\geqslant$ 100 ng/mL, a maximum tumor size $\geqslant$ 7 cm and a lack of complete necrosis at LT after TACE were found to be independent predictors of HCC recurrence[46]. However, patients with maximum tumor size < 7 cm who achieve complete necrosis together with AFP levels < 100 ng/mL at LT may be the best candidates for LT following downstaging[46].

In addition, an AFP slope > 15 ng/mL per month and mRECIST progression are unique independent risk factors for HCC recurrence and patient death regardless of whether the patient meets the MC[94].

CONCLUSION

Although the MC remain by far the standard and the most employed inclusion criteria for LT for HCC, in the

coming years, criteria will be consolidated that take into account not only data regarding the size/volume and number of tumors but also their biology, including AFP value and some of its published logarithmic models. Additionally, the AFP value will be considered in the allocation and prioritization of patients in the WL with the aforementioned new reform of the MELD-HCC system. Furthermore, the number of tumors, their volume and AFP levels will be important determinants for bridging and downstaging therapy and to evaluate the patient response. AFP values > 1000 ng/mL must be considered a sign of a bad prognosis and a questionable indication for LT unless the value can be reduced to < 400 ng/mL. Organ scarcity and the probability of recurrence following LT for HCC necessitate that all of these facts should be taken into account.

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MINIREVIEWS

Treatment strategies for chronic hepatitis C prior to and following liver transplantation

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Abstract

Hepatitis C virus (HCV)-related liver disease is the leading indication for liver transplantation (LT) worldwide. However, HCV is an independent predictor of lower survival following LT, and recurrence of HCV post-LT is virtually universal. The historic standard of care during the interferon era of HCV therapy was expectant management-initiation of antiviral therapy in the setting of documented disease progression following LT. With the advent of new direct acting antiviral (DAA) therapies for HCV, the paradigm of expectant treatment for recurrent HCV infection post-LT is shifting. The safety, tolerability, and efficacy of DAAs, even among the sickest patients with advanced liver disease, enables treatment of HCV in the pre-transplant setting among LT waitlist registrants. Finally, emerging data are supportive of preemptive therapy with DAAs in liver transplant recipients as the preferred approach. Expectant management of HCV following LT can rarely be justified in the modern era of HCV therapy.

Key words: Hepatitis C virus; Liver transplantation; Direct acting antivirals; Sustained virologic response

Core tip: The historic standard of care during the interferon era of hepatitis C virus (HCV) therapy was expectant management-initiation of antiviral therapy in the setting of documented disease progression following

liver transplantation. With the advent of new direct acting antiviral (DAA) therapies for HCV, the paradigm of expectant treatment for recurrent HCV infection post-liver transplantation (LT) is shifting. The safety, tolerability, and efficacy of DAAs, even among the sickest patients with advanced liver disease, enables treatment of HCV in the pre-transplant setting among LT waitlist registrants. Emerging data support preemptive therapy with DAAs in liver transplant recipients as the preferred approach.

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INTRODUCTION

Hepatitis C virus (HCV) infection afflicts an estimated 180 million people worldwide, or nearly 3% of the global population[1,2]. HCV results in 8000 to 13000 deaths annually in the United States[3]. To date, HCV remains the leading indication for liver transplantation (LT) in developed nations and represents 33% of patients currently on the LT waitlist[3,4].

NATURAL HISTORY OF HCV INFECTION BEFORE LT

Among 70% to 75% of patients, acute HCV infection is asymptomatic. The remaining minority of patients develops systemic symptoms, including weakness, malaise, anorexia, and, rarely, jaundice. Eighty-five percent of patients with acute HCV infection do not clear the infection without treatment and instead develop chronic infection[5]. Progression to cirrhosis or hepatocellular carcinoma occurs in between 15% to 40% of patients with chronic HCV[1]. Accelerated development of cirrhosis and end-stage liver disease ensue under certain conditions. Rate of progression to cirrhosis is impacted by age at exposure - higher risk with HCV exposure at advanced age; route of transmission - blood transfusion portends greater risk than injection drug use; duration of infection; HCV genotype; and coexisting illnesses, including human immunodeficiency virus infection, hepatitis B virus (HBV) infection, and alcoholic liver disease[6-10].

TREATMENT OF HCV INFECTION BEFORE LT

Although 5-year survival among patients with compensated cirrhosis due HCV ranges from 84% to 91%, there is a 20% risk of decompensation and a 10% risk of HCC[11,12]. Attainment of sustained virologic response (SVR) is associated with lower rates of hepatic decompensation, HCC, and all-cause mortality[13]. Indeed, an international multicenter study demonstrated that patients with chronic HCV who achieve SVR have long-term survival comparable to that of the general population[14,15]. Moreover, recent data reveal improved long-term survival following LT among patients in whom HCV was eradicated prior to LT[16]. As a third of LT in the United States are performed for HCV-related liver disease[4] and HCV-positive recipients have worse outcomes following LT[17], attaining pre-transplant SVR may yield significant improvements in patient outcomes. In the interferon era, HCV therapy was instituted with caution in patients with advanced liver disease due to the potential risk of hepatic decompensation. Now, with the advent of safe, well-tolerated, and efficacious direct acting antivirals (DAAs), a paradigm shift toward pre-transplant treatment of HCV is warranted. The shortage of donor livers in the United States, which results in substantial liver transplant waitlist mortality and dropout[18], underscores the importance of treating HCV prior to LT. The significance of this shift is even greater in regions where the availability of LT is limited to only very sick patients[19]. Treatment of HCV pre-transplant stands not only to improve post-LT outcomes but also reduce the overall societal need for LT. Viral suppression in HBV has been shown to lead to regression of fibrosis[20,21]. Likewise, emerging data now reveals histological regression of fibrosis among patients with HCV who have achieved SVR[4]. As such, long-term virologic suppression of HCV may lead to disease reversal.

LT FOR HCV

LT is optimal therapy for decompensated cirrhosis due to chronic HCV, but HCV reinfection poses challenging management issues that may arise either early or late after transplantation[22,23].

DONOR LIVER ALLOCATION FOR LT

In 2002, the model for end-stage liver disease (MELD) score shown to predict LT waitlist mortality was implemented as an allocation criterion for donor livers[24]. The goal is to improve survival and quality of life among patients with end-stage liver disease. LT has proven to be effective at achieving these goals. The benefits of LT are most established for patients with MELD scores of at least 15 or higher[25]. The MELD score necessary to receive a donor liver varies widely by United Network for Organ Sharing region. While patients with MELD scores in the mid-20s receive offers in some regions, MELD scores in the high-30s are commonly needed in other regions. Because offers are allocated to patients with higher MELD scores, concern has emerged about the possibility of a so-called "MELD purgatory" with pre-transplant treatment of HCV. Concern exists that certain patients may have delayed progression of liver disease after achieving SVR without substantial reversal or improvement in quality of life[26]. Proponents of this view

contend that post-LT treatment of HCV would alleviate this concern. We should be cognizant of the fact that up to 3000 potential liver transplant candidates are removed from the waitlist annually in the United States - half develop contraindications for LT while the wait for a potential donor and the other half die from complications of end-stage liver disease[27]. Therefore, necessitating changes in allocation policies to reduce waitlist mortality[28]. Therefore, deferring antiviral therapy from pre- to post-LT phase may not be safe. Morbidity and mortality associated with LT are low, but should be ignored with emerging DAA data supporting instituting treatment in the pre-transplant phase. Furthermore, most experts agree that fibrosing cholestatic hepatitis and compensated recurrent HCV infection following LT demonstrates relatively lower efficacy with DAA therapy[29,30]. The concerns regarding the use of HCV-positive allografts have been alleviated with more recent data suggesting that transplant outcomes for recipients who accept HCV-positive donor allografts may be comparable with those who receive HCV-negative allografts[31]. Emerging treatments to eradicate HCV have further improved the course of HCV-positive individuals, with improved efficacy and reduced side-effects. HCV-positive donors constitute 4.8% of HCV-positive LT recipients[32]. The use HCV-positive donor in HCV-negative recipients with the availability of DAAs needs to be studied further. Lastly, if LT is imminent in a Child-Turcotte-Pugh class C patient with MELD score > 35 or hepatocellular carcinoma patient with exception MELD points - it may be pragmatic to wait and institute antiviral therapy following LT[33].

NATURAL HISTORY OF HCV INFECTION FOLLOWING LT

Studies demonstrate worse outcomes post-LT among patients with recurrent HCV infection compared to patients transplanted for other causes of cirrhosis[23,34]. The natural history of HCV infection in liver transplant recipients is typically accelerated, partially due to concomitant administration of post-LT immunosuppression. Up to 20% of HCV-infected patients develop cirrhosis by 5 years following LT[23]. Recurrent disease ranges from asymptomatic mild hepatitis to severe chronic hepatitis and cirrhosis. Reinfection with HCV post-LT is virtually universal, occurring in over 95% of cases[22].

PREEMPTIVE TREATMENT OF HCV FOLLOWING TRANSPLANTATION

Historically, preemptive use of antiviral therapy post-LT was not advisable because of the increased rate of acute allograft rejection associated with interferon therapy[35]. However, with the emergence of safe and efficacious DAAs, the previous concern of interferon-related immunomodulation with allograft rejection and poor tolerance due to anti-HCV therapy following LT is abating. None of the new DAAs have yet been approved by the United States Food and Drug Administration for use among patients following LT, but the powerful body of emerging literature suggests that approval may be expected in the near future[29,30]. Preemptive treatment of HCV in the post-LT setting may alleviate the need for re-transplantation.

EXPECTANT TREATMENT OF HCV FOLLOWING TRANSPLANTATION

Despite being the previous standard of care in the interferon era, expectant management of HCV does not seem to have a role for the vast majority of patients in the era of DAAs. Delaying HCV therapy post-LT is not advisable due to the rapid progression of HCV-related liver damage and promising data regarding the use of DAAs.

CONCLUSION

Advances in peri-transplant management of liver transplant recipients in the setting of chronic hepatitis C have resulted in long-term post-transplant survival rates approaching 90%[36]. Nevertheless, survival following LT remains lower among patients with HCV compared to those undergoing LT for liver disease related to other etiologies[17]. Attaining SVR pre-transplant reduces all-cause mortality, may decrease the need for LT, and may improve survival following LT[14,15]. The improvements in the efficacy of antiviral therapy against HCV infection with DAAs argue against the interferon-era paradigm of expectant use of antiviral therapy following LT. The decision between treating patients pre-transplant or preemptively in the early post-transplant setting should be individualized for each patient in the context of the regional waitlist trends and exception policies for LT. Despite advancements in LT, there remains a shortage of donor livers to meet the demands for LT in the United States Treatment of patients on the LT waiting list may ultimately decrease the number of patients needing LT and help address the imbalance in supply and demand.

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ORIGINAL ARTICLE

Observational Study

Adipokines, cytokines and body fat stores in hepatitis C virus liver steatosis

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Emilio González-Reimers, Javier López-Prieto, Geraldine Quintero-Platt, Ricardo Pelazas-González, M Remedios Alemán-Valls, Onán Pérez-Hernández, M José de-la-Vega-Prieto, M Angeles Gómez-Rodríguez, Candelaria Martín-González, Francisco Santolaria-Fernández, Servicio de Medicina Interna, Hospital Universitario de Canarias, Universidad de La Laguna, 38320 Canary Islands, Spain

Author contributions: González-Reimers E, López-Prieto J, Quintero-Platt G, Pelazas-González R, Alemán-Valls MR, Pérez-Hernández O, Martín-González C contributed to study conception and design; González-Reimers E, López-Prieto J, Quintero-Platt G, Alemán-Valls MR and Santolaria-Fernández F contributed to data acquisition, data analysis and interpretation, and writing of the article; López-Prieto J, Pelazas-González R and Pérez-Hernández O contributed to histomorphometrical analysis of the liver biopsies; de-la-Vega-Prieto MJ contributed to determination of cytokines and adipokines; Gómez-Rodríguez MA contributed to data acquisition and analysis of body composition by densitometry.

Institutional review board statement: The study was reviewed and approved by the Ethics Committee of the Hospital Universitario de Canarias (PI/07) and the Institutional Review Board from the third cycle studies of the University of la Laguna.

Informed consent statement: All the patients provided written informed consent before starting the study procedures.

Conflict-of-interest statement: The authors declare that there is no conflict of interest regarding this manuscript. No funding or institutional grants have been received for this study.

Data sharing statement: They are available upon request by emailing egonrey@ull.es. There is no written informed consent for data sharing, but data are anonymized.

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Abstract

AIM: To identify patients with or without liver steatosis and its severity in treatment-naïve patients affected by hepatitis C virus (HCV) infection.

METHODS: We included 56 HCV infected patients, and assessed the amount of liver fat by histomorphometry, and its relationships with fat and lean mass at different parts of the body (by densitometry), hormones [insulin, homeostatic model assessment (HOMA)], adipokines (resistin, adiponectin, leptin), and cytokines (tumor necrosis factor α, interleukin-6).

RESULTS: Although the intensity of liver steatosis is related to trunk fat mass and HOMA, 33% of patients showed no liver steatosis, and this finding was not related to body mass index or genotype. Besides trunk

fat mass, no other factor was related to the presence or not of liver steatosis, or to the intensity of it, by multivariate analysis. Lean mass was not related to liver steatosis. Adiponectin levels were lower among patients. No differences were observed in leptin and resistin.

CONCLUSION: Steatosis in HCV infection is common (67.2%), and closely related to trunk fat, and insulin resistance, but not with leg fat mass or adipokines.

Key words: Resistin; Adiponectin; Insulin resistance; Proinflammatory cytokines; Leptin; Hepatitis C virus; Liver steatosis

Core tip: Pathogenesis of liver steatosis in hepatitis C virus (HCV) infection is complex and is not fully understood. For unknown reasons some patients, despite having a high body mass index (BMI), do not develop liver steatosis, whereas others with normal BMI develop intense liver fat deposition. We analyse if body fat and lean mass composition, insulin resistance and adipokine profile may help to identify patients with or without liver steatosis and its severity in treatment-naïve HCV patients. Multivariate analysis showed that only trunk fat mass and insulin resistance were independently related to liver steatosis assessed on histomorphometrical grounds and its severity.

González-Reimers E, López-Prieto J, Quintero-Platt G, Pelazas-González R, Alemán-Valls MR, Pérez-Hernández O, de-la-Vega-Prieto MJ, Gómez-Rodríguez MA, Martín-González C, Santolaria-Fernández F. Adipokines, cytokines and body fat stores in hepatitis C virus liver steatosis. *World J Hepatol* 2016; 8(1): 74-82 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i1/74.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i1.74

INTRODUCTION

Non-alcoholic steatohepatitis is observed in several clinical conditions, especially diabetes and obesity. In steatohepatitis hepatocytes become laden with fat droplets that elicit an inflammatory response which may evolve to liver cirrhosis and hepatocarcinoma. In diabetes and obesity insulin deficiency and/or resistance lead to increased mobilization of fatty acids from adipose tissue to liver. In chronic hepatitis C virus (HCV) infection, steatosis and steatohepatitis are also observed and the pathogenesis is based on complex mechanisms: although HCV by itself especially genotype 3 may lead to liver steatosis, obesity and concomitant alcohol abuse are the main factors involved[1]. However, many HCV infected patients do not drink alcohol at all, but they may develop liver steatosis. Cytokine activation and increased lipid peroxidation may contribute both to liver steatosis and to the progression of simple liver steatosis to steatohepatitis[2].

The main source of liver fat accumulation is body fat stores[3]. In this scenario, fat tissue is not only the source of fatty acids, but also produces several proinflammatory cytokines which are of paramount importance in the progression of liver disease. However, adipose tissue is heterogeneous. For instance, trunk fat is associated with increased insulin resistance and an increased vascular risk[4], whereas leg fat exerts opposite effects[5], probably due to secretion of a different cytokine profile.

The association of liver steatosis with distribution of fat stores at different parts of the body in chronic HCV infection is not well known. This is an important issue, since the heterogeneous nature of fat tissue may lead to different adipokine secretion[6]. In fact, notable controversy exists regarding serum levels of different adipokines, such as adiponectin[7-9] or leptin[10,11] and their relationship with histological changes in chronic HCV infection. In a previous report which analysed a series of patients (different from those included in this study) we found that an increased waist circumference (> 102 cm for men and > 88 cm among women) was related to increased liver fat, but we also found that 38.8% of non-obese patients also showed intense fatty infiltration[12], a result in accordance with other researchers, who have reported fatty liver among lean individuals[13]. Conversely, some HCV infected patients do not show liver steatosis, regardless of their body mass index. The mechanisms that underlie the lack of association in some cases between liver fat and body fat stores are unclear.

On the other hand, in a recent Indian study in a cohort of patients with steatohepatitis, 13% were lean patients[14], and sarcopenia has been described as an independent risk factor for steatohepatitis[15]. In addition it has been shown that interleukin-6 (IL-6) a protean cytokine also produced by muscle[16] strongly modulates liver fat accumulation[17]. Therefore, given these observations, it is important to also analyse the relationship between lean mass and liver steatosis.

Based on these facts, in the present study we analyse the association of the degree of liver steatosis with fat and lean mass stores at different parts of the body, insulin resistance, and serum adipokine levels, in treatment-naïve patients affected by HCV infection. Since we have assessed liver steatosis on histological grounds, we also look for differences in cytokine and adipokine profile, fat and lean mass distribution among HCV patients who did not show liver steatosis and those who did, in order to shed light on the reasons why some HCV patients do not develop liver steatosis.

MATERIALS AND METHODS

Patients

We included 56 patients with (19 women) HCV infection, aged 41.54 ± 9.57 years. Diagnostic criteria for HCV infection were the following: (1) presence of anti-HCV and/or HCV RNA by reverse transcriptase polymerase

Table 1 Differences in biochemical variables, body mass index and total lean and total fat area between patients and controls

	Patients		Controls		
	n	X ± SD, median (IQ range)	*n*	X ± SD, median (IQ range)	
Insulin (μU/mL)	44	12.48 ± 15.65, 7.89 (4.63-14.32)	10	8.34 ± 4.34, 7.15 (5.08-10.63)	$Z = 0.43$; NS
Resistin (ng/mL)	44	4.97 ± 1.76, 4.90 (3.98-5.60)	10	4.28 ± 1.42, 4.97 (3.32-5.29)	$Z = 0.88$; NS
Adiponectin (ng/mL)	44	11.99 ± 8.30, 9.54 (6.04-17.16)	16	24.92 ± 21.84, 19.05 (13.53-21.58)	$Z = 3.18$; $P = 0.001$
Leptin (ng/mL)	44	12.25 ± 15.83, 4.23 (1.15-17.78)	10	18.41 ± 16.03, 12.89 (4.65-34.42)	$Z = 1.78$; NS
Tumor necrosis factor-α (pg/mL)	56	10.65 ± 4.14, 10.18 (7.15-13.08)	19	6.05 ± 1.90, 5.20 (4.40-8.00)	$Z = 4.56$; $P < 0.001$
Interleukin-6 (pg/mL)	53	4.28 ± 4.75, 2.0 (2.0-4.29)	19	5.90 ± 1.64, 5.0 (5.0-6.60)	$Z = 2.97$; $P = 0.003$
Body mass index (kg/m^2)	56	24.19 ± 3.44	19	25.20 ± 3.42	$t = 1.02$; NS
Total fat mass (g)	50	19929 ± 11944	19	21443 ± 6393	$t = 0.54$; NS
Total lean mass (g)	50	48284 ± 8848	19	50131 ± 15796	$t = 0.64$; NS

Comparisons were made using non-parametric tests, such as Mann-Whitney's *U* test (*Z*) or parametric ones (Student's *t*-test). NS: Not significant.

chain reaction; and (2) Histology consistent with HCV. Most patients (43) were infected by HCV type 1 genotype, 5 by type 3 genotype, and 8 by type 4. All patients were recruited before treatment for virus C hepatitis was administered, and none of them were active drinkers. Liver function was still preserved: Liver function tests were normal, and none of them showed ascites or encephalopathy.

Nutritional evaluation

After informed consent was obtained, 51 patients underwent assessment of fat and lean mass at different parts of the body, such as right and left arm, trunk, right and left leg, and total body, with a LUNAR PRODIGY ADVANCE device, General Electric, Piscataway, NJ, United States. We further calculated (using the protocol established by other authors[18]) the trunk fat/(right leg + left leg fat) index, as well as the indices fat mass/lean mass at each of the body compartments mentioned before. Body mass index [BMI, as weight (kg)/height (m)^2] was also recorded.

Biochemical assessment

Blood samples were taken at 8:00 am in fasting conditions. Routine laboratory evaluation was performed and these analyses included, among others, prothrombin activity, serum albumin and bilirubin. Samples were immediately frozen at -20 ℃. We determined the following parameters-IL-6, by chemiluminescent assay interassay variation coefficient ranging 5.3%-7.5%, recovery = 85%-104%, diagnostic products corporation (DPC), Los Angeles, CA, United States; tumour necrosis factor α (TNF-α) by immunometric chemiluminescent assay (intra-assay variation coefficient ranging 4%-6.5%, interassay variation coefficient ranging 2.6%-3.6%, recovery 92%-112%, DPC, Los Angeles, CA, United States). We also determined serum insulin, by immunoanalysis (Chemiflex); interobserver variation coefficient = 1.9%-5.2%; intraobserver variation coefficient = 1.7%-4.2%; sensitivity = 1 μU/mL; recovery = 91.1%-101.6%; (Architect system, Abbott, Wiesbaden Germany), serum resistin, by ELISA (sensitivity = 0.033 ng/mL; intra-assay variation coefficient = 2.8%-3.4%; interassay variation coefficient ranging 5.1%-6.9%, recovery = 85.2%-99.2%, Biovendor, Heidelberg, Germany), serum leptin, by ELISA (sensitivity = 0.2 ng/mL; intra-assay variation coefficient = 4.2%-7.6%; interassay variation coefficient ranging 4.4%-6.7%, recovery = 85.7%-98.0%, Biovendor, Heidelberg, Germany); serum adiponectin by ELISA (sensitivity = 26 ng/mL; intra-assay variation coefficient = 3.9%-5.9%; interassay variation coefficient ranging 6.3%-7%, recovery = 92.4%-102.9%, Biovendor, Heidelberg, Germany); insulin resistance was estimated by the homeostatic model assessment (HOMA).

Cytokine values were compared with those of a control group composed of 19 healthy hospital workers, seven of them women, aged 40.45 ± 3.57 years. As shown in Table 1, not all the variables were determined in all patients and controls.

All these data were recorded the day at which the patients underwent a liver biopsy before receiving active treatment against HCV infection.

Histological assessment

The degree of liver steatosis was determined using software based on histomorphometry (LEICAQWin, version 3.0, Wetzlar, Germany). The specimens were stained with haematoxylin-eosin and Masson trichromic and were viewed at 40 ×. This protocol has been previously described[12]. The proportion of fatty area to total area in specimens was recorded. The Knodell index and the total amount of fibrous tissue determined by histomorphometry (using Masson trichromic stain) were also measured.

The study protocol was approved by the local ethical committee of our Hospital. All patients included gave their informed consent prior to their inclusion in the study, and the study conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Statistics analysis

We tested for normal distribution using the Kolmogorov-Smirnov test. In order to compare means between two groups or between three or more groups, we used Student's *t* test and ANOVA, respectively. If the variables did not show a normal distribution, Mann-Whitney's *U* and Kruskall-Wallis tests were used to compare

Table 2 Anthropometric measurements in patients with marked or less marked liver steatosis

	Steatosis over the median		Steatosis below the median		
	n	X ± SD	*n*	X ± SD	
Left arm fat mass (g)	27	1345.04 ± 871.98	24	783.30 ± 577.62	t = 2.68; P = 0.01
Right arm fat mass (g)	27	1396.97 ± 1084.44	24	852.37 ± 827.60	t = 2.00; P = 0.05
Trunk fat mass (g)	27	12673.68 ± 6077.05	24	7939.57 ± 5027.19	t = 3.01; P = 0.004
Left leg fat mass (g)	27	3919.72 ± 2533.87	24	2683.13 ± 2018.77	t = 1.91; NS
Right leg fat mass (g)	27	3948.29 ± 2626.75	24	2805.25 ± 1905.64	t = 1.76; NS
Total body fat mass (g)	27	23981.22 ± 12381.84	24	15733.66 ± 9812.41	t = 2.61; P = 0.012
Left arm lean mass (g)	26	2769.64 ± 783.77	24	2899.09 ± 941.35	t = 0.53; NS
Right arm lean mass (g)	26	2749.02 ± 819.68	24	3064.65 ± 1649.64	t = 0.87; NS
Trunk lean mass (g)	26	24458.49 ± 4791.16	24	23122.30 ± 4155.06	t = 1.05; NS
Left leg lean mass (g)	26	7469.77 ± 1684.70	24	7011.75 ± 1945.39	t = 0.89; NS
Right leg lean mass (g)	26	7651.20 ± 1664.56	24	7404.65 ± 1444.64	t = 0.56; NS
Total lean mass (g)	26	48592.11 ± 9723.42	24	47309.62 ± 8352.70	t = 0.50; NS
Body mass index (kg/m^2)	28	25.55 ± 2.51	27	22.79 ± 3.76	t = 2.61; P = 0.012
Trunk fat/legs fat	27	1.85 ± 0.87	24	1.56 ± 0.42	t = 1.49; NS

NS: Not significant.

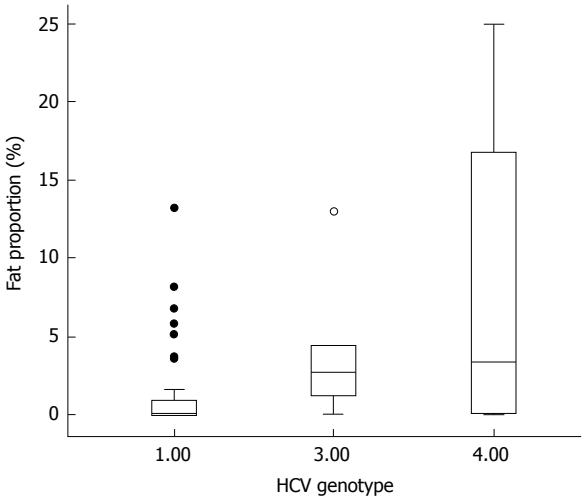

Figure 1 Fat amount among the three hepatitis C virus genotypes included in this study. Patients with genotype 1 (the most frequent) show significantly less amount of fat than patients affected by genotype 3 or 4 (solid circles are outliers, and hollow circle, extreme values). HCV: Hepatitis C virus.

means. Correlations between quantitative variables were established using Spearman's r and Pearson's r. The χ^2 test was used to compare qualitative variables. We performed stepwise multiple regression analysis to establish which parameters liver steatosis depends on. All statistical analyses were performed using SPSS software (Chicago, Ill., United States).

RESULTS

Liver steatosis was observed in 42 patients out of 56; in the remaining 14 patients, no steatosis at all was observed, and in 4 more, only very few small isolated fat droplets could be observed (fat amount < 0.05%). Median value of liver fat area was 0.20%, but 14 patients showed more than 5% of fat in their biopsies. Patients with genotype 1 showed significantly less steatosis than those with genotype 3 or 4 (Z = 2.17; P = 0.03; Figure 1). Indeed, as shown in Figure 1, patients with genotype 3 or 4 showed higher values of liver fat (fat proportion = 6.66% ± 8.42%) when compared with those with genotype 1 (fat proportion= 1.40% ± 2.78%). Only 1 (out of 5) genotype 3 patient showed no steatosis at all, compared with 13 (out of 51) affected by non-3 genotype infection, but this association was not statistically significant (χ^2 = 0.07). No differences in liver fat were observed when HIV-coinfected patients were compared with non-co-infected ones (Z = 0.40; P = 0.694). Seven patients were diabetics, but although they showed a trend to more intense liver steatosis (6.66% ± 9.68%) than non-diabetics (2.05% ± 3.97%), this difference was not significant (Z = 1.31; P > 0.20). None of the diabetics showed no fat in their livers, but association between diabetes/no diabetes and presence or not of liver steatosis was not significant (P = 0.17 by exact Fisher's test). No association was observed between viral load and proportion of liver fat.

Median proportion of fibrosis was 5.75% (interquartile range = 3.53%-8.88%). Twenty-one patients showed a Knodell index higher than 5, whereas 35 showed a Knodell index below 6.

Relationship of liver steatosis with nutritional status

Patients with marked steatosis (over the median) showed increased BMI and greater fat mass, especially at the trunk (t = 3.01, P = 0.004), as shown in Table 2. In addition to the finding of a significantly higher BMI among those with liver steatosis over the median (Table 2), we also found that patients with BMI over 25 kg/m^2 had significantly more liver fat (Z = 2.25; P = 0.031). Only 22 patients were overweight, and only 3 of them were obese (BMI > 30 kg/m^2). Three patients who were overweight showed no fat at all in their liver biopsies, *vs* 11 out of 33 with normal weight. This association was not statistically significant. Significant relationships were observed between fat parameters and liver steatosis, especially with trunk fat (r = 0.42; P = 0.002), right

Table 3 Biochemical variables in patients with steatosis over the median or below the median

	Steatosis over the median		Steatosis below the median		
	n	X ± SD, median (IQ range)	*n*	X ± SD, median (IQ range)	
Insulin (μU/mL)	24	15.30 ± 19.42, 11.20 (6.59-16.46)	20	9.09 ± 8.72, 7.30 (3.87-11.62)	$Z = 1.89$; $P = 0.059$
HOMA	24	1645.68 ± 2828.28, 1068 (644-1524)	20	825.01 ± 801.41, 645.5 (327.8-1082.0)	$Z = 2.15$; $P = 0.03$
Resistin (ng/mL)	24	4.66 ± 0.96, 4.87 (4.19-5.37)	20	5.34 ± 2.38, 5.03 (3.88-6.12)	$Z = 1.03$; NS
Adiponectin (ng/mL)	24	11.77 ± 6.92, 11.18 (5.45-17.62)	20	12.26 ± 9.90, 8.38 (6.04-16.65)	$Z = 0.21$; NS
Leptin (ng/mL)	24	10.85 ± 12.45, 6.23 (1.35-17.20)	20	13.92 ± 19.35, 2.72 (0.79-32.89)	$Z = 0.79$; NS
Tumor necrosis factor-α (pg/mL)	28	11.31 ± 4.90, 11.20 (6.84-14.18)	28	10.00 ± 3.17, 9.56 (7.19-12.75)	$Z = 1.00$; NS
Interleukin (pg/mL)	25	5.06 ± 5.17, 2.0 (2.0-5.94)	28	3.59 ± 4.31, 2.0 (2.0-2.5)	$Z = 1.14$; NS
Cholesterol (mg/dL)	28	167 ± 36.82	28	174.2 ± 45.56	$t = 0.65$; NS
LDL cholesterol (mg/dL)	27	95.04 ± 34.17	28	103.86 ± 36.48	$t = 1.01$; NS
HDL cholesterol (mg/dL)	28	46.71 ± 14.87	28	42.86 ± 13.82	$t = 0.92$; NS
Triglycerides (mg/dL)	28	136.25 ± 114.27	28	145.96 ± 93.04	$t = 0.35$; NS

Comparisons were made using non-parametric tests, such as Mann-Whitney's *U* test (*Z*). NS: Not significant.

arm fat (r = 0.31; $P = 0.029$), left arm fat (r = 0.30; $P = 0.033$), and total fat (r = 0.34; $P = 0.016$). The significant relationship between liver steatosis and trunk fat was observed both among women (r = 0.50; $P = 0.04$) and men (r = 0.41; $P = 0.016$). In a similar way, BMI was related to liver steatosis both among women (r = 0.53; $P = 0.02$) and men (r = 0.36; $P = 0.032$). However, while liver steatosis was related to arm and leg fat mass among both women and men, the correlations were not statistically significant, possibly due to the relatively low number of cases. No relationship was observed between parameters related to lean mass and liver steatosis, but when the indices fat mass/lean mass were compared with liver steatosis, the results were similar to those obtained with fat parameters (r = 0.39; $P = 0.006$ for the trunk, r = 0.34; $P = 0.017$ for the left arm, r = 0.32; $P = 0.026$ for the right arm, and r = 0.34; $P = 0.016$ for total fat). Remarkably, no association was observed when leg fat mass was compared with liver steatosis. The ratio trunk fat/legs fat was not significantly different among patients with liver steatosis below or above the median. A significant correlation was observed between liver steatosis and BMI (r = 0.41; $P = 0.002$).

Trunk fat was the only variable that was selected ($P = 0.011$) when a logistic regression analysis was done searching for the factors related to liver fat over or below the median values.

Similar results relative to fat mass at different parts of the body were observed when patients without liver steatosis (including those 4 with minimal steatosis) were compared with the remaining patients, although differences were less significant ($t = 2.73$, $P = 0.009$ for trunk fat, $t = 2.34$, $P = 0.023$ for left arm fat, $t = 2.31$; $P = 0.025$ for right arm fat) than when patients were classified according to the median values of liver fat. BMI was also significantly lower among those without liver steatosis ($t = 2.43$; $P = 0.023$). No differences at all were observed regarding lean mass variables. As with steatosis below or above the median, the only selected variable was trunk fat ($P = 0.015$) when a logistic regression was performed to discern which variables were independently related to the presence or absence of liver fat infiltration.

No associations were observed between the proportion of fibrosis in liver biopsy and any of the nutritional variables, but Knodell index was related both to fat mass variables (total fat, r = 0.37; $P = 0.007$; trunk fat, r = 0.32; $P = 0.024$); left arm and right arm fat, r = 0.47 and r = 0.45; respectively, $P < 0.001$; left leg and right leg, (r = 0.31 and r = 0.28, respectively, $P < 0.05$ in both cases), as well as to some lean mass variables (trunk lean mass, r = 0.35; $P = 0.012$; left leg lean mass, r = 0.30, $P = 0.034$).

Relationship of liver steatosis with insulin resistance and adipokines

No differences were observed in any of the adipokines, HOMA, insulin, TNF-α, or IL-6 among patients with or without liver steatosis. Only HOMA, out of these parameters, was significantly higher among patients with liver fat over the median compared with those with liver fat below the median (Z = 2.15; $P = 0.032$); a similar trend that was not statistically significant ($P = 0.059$) was observed with insulin (Tables 3 and 4, Figure 2).

Significant relationships were observed between liver steatosis (proportion of fat) and HOMA index (r = 0.30; $P = 0.046$). Serum insulin (r = 0.44; $P = 0.003$) and HOMA (r = 0.36; $P = 0.017$) were directly related to Knodell index, whereas no associations were observed between any of the adipokines and cytokines and the amount of fibrosis in the liver biopsies. Selecting only those patients with liver steatosis, a significant correlation was observed between IL-6 and amount of liver fat (r = 0.49; $P = 0.003$).

After introducing in a multiple regression analysis the fat variables which showed a significant relationship with liver steatosis in the univariate analysis, only trunk fat (beta = 0.37; $P = 0.026$) was independently related to the amount of liver fat. In a similar way, trunk fat was the only selected variable when a logistic regression analysis was done searching for the factors related to liver fat over or below the median values (Table 5).

Table 4 Correlations between body composition parameters and adipokines, proinflammatory cytokines and insulin resistance

	Leptin	Adipo-nectin	Insulin	HOMA	TNF-α	IL-6	Resistin
Trunk fat	ρ = 0.61, P < 0.001		ρ = 0.56, P < 0.001	ρ = 0.55, P < 0.001			
Left leg fat	ρ = 0.70, P < 0.001		ρ = 0.44, P = 0.005	ρ = 0.44, P = 0.005			
Right leg fat	ρ = 0.62, P < 0.001		ρ = 0.42, P = 0.006	ρ = 0.42, P = 0.006			
Right arm fat	ρ = 0.40, P = 0.011		ρ = 0.58 P < 0.001	ρ = 0.57 P < 0.001			
Left arm fat	ρ = 0.51, P < 0.001		ρ = 0.62, P < 0.001	ρ = 0.63, P < 0.001			
Total fat	ρ = 0.64, P < 0.001		ρ = 0.54, P < 0.001	ρ = 0.53, P < 0.001			
Total lean		ρ = -0.35, P = 0.032			ρ = -0.31, P = 0.029		
Left arm lean		ρ = -0.37, P = 0.02			ρ = -0.33, P = 0.021		
Right arm lean		ρ = -0.37, P = 0.02			ρ = -0.29, P = 0.04		
Left leg lean							
Trunk lean		ρ = -0.34, P = 0.021			ρ = -0.33, P = 0.021	ρ = -0.34, P = 0.018	
Right leg lean					ρ = -0.29, P = 0.039		
Total fat/total lean	ρ = 0.65, P < 0.001		ρ = -0.49, P = 0.001	ρ = 0.49, P = 0.001			
Trunk fat/trunk lean	ρ = 0.63, P < 0.001		ρ = -0.31, P = 0.036	ρ = 0.52, P < 0.001		ρ = 0.31, P = 0.036	
Right arm fat/ right arm lean	ρ = 0.60, P < 0.001		ρ = 0.55, P < 0.001	ρ = 0.53, P < 0.001			
Left arm fat/left arm lean	ρ = 0.69, P < 0.001		ρ = 0.58, P = 0.001	ρ = 0.57, P < 0.001			
Right leg fat/ right leg lean	ρ = 0.66, P < 0.001		ρ = 0.38, P = 0.016	ρ = 0.39, P = 0.014			
Left leg fat/left leg lean	ρ = 0.69, P < 0.001		ρ = 0.58, P < 0.001	ρ = 0.57, P < 0.001			
High density lipoprotein cholesterol		ρ = 0.56, P < 0.001					ρ = -0.41, P = 0.012
Low density lipoprotein cholesterol	ρ = 0.31, P = 0.046						

Only the significant relationships are provided (Spearman's ρ test). TNF-α: Tumor necrosis factor α; IL-6: Interleukin-6.

Table 5 Results of the logistic regression analysis performed in order to look for which parameters were independently associated with liver steatosis

		B	E.T.	Wald	Gl	Sig.	Exp (B)
Step 1	TrunK fat	0.000	0.000	6.157	1	0.013	1.000
	Constant	1.530	0.751	4.147	1	0.042	4.618

E.T.: Standard error; Gl: df (degrees of freedom); Sig.: Significance; Exp (B): Odd ratio.

Relationships of nutritional variables with insulin resistance and adipokines

Leptin, insulin and HOMA were strongly and directly related to fat parameters, as shown in Table 4 (r > 0.40 in all the cases; P < 0.006), but not to lean mass. On the contrary, adiponectin and TNF-α were inversely related to most of the lean mass parameters. Adiponectin was also inversely related to the trunk fat mass/leg fat mass index (r = -0.33; P = 0.037).

The fat/ lean indices were also strongly related to leptin, insulin and HOMA, and also, to IL-6, in this last case only with the trunk fat/trunk lean mass index. No associations were observed between serum resistin and nutritional parameters (Table 4).

DISCUSSION

We have found that liver steatosis is frequent among patients with HCV infection (67.86%), even surpassing the prevalence data (about 50%) reported by other authors[19]. This high proportion of patients with steatosis was observed despite a BMI that was not different - even slightly lower- than that of a control population. However, as expected, liver steatosis showed a significant relationship with BMI, but it is noteworthy that

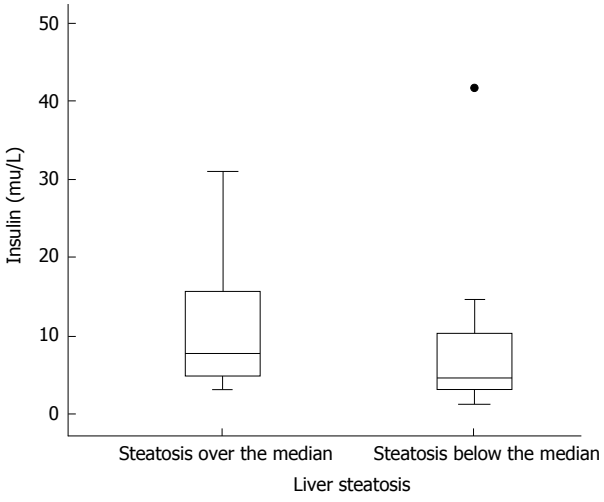

Figure 2 Serum insulin levels among patients with liver steatosis over the median (left) and below the median (right). Differences are not statistically significant, but there is a trend to higher values among patients with intense steatosis (P = 0.059). Solid circle represents an outlier.

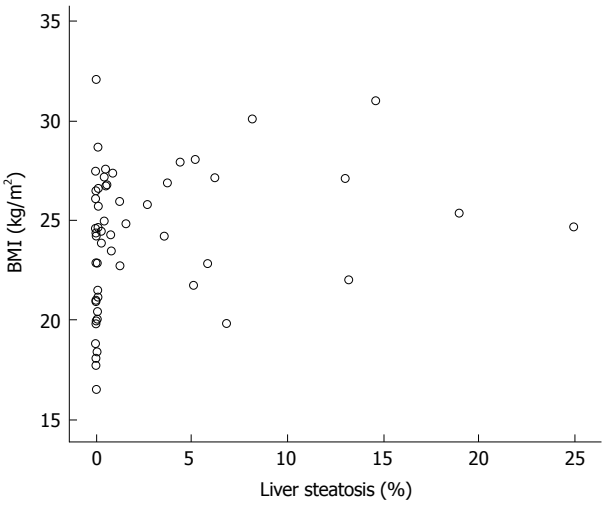

Figure 3 Scattergram showing the relation of body mass index with the amount of liver steatosis. Despite a significant relationship between both variables (ρ = 0.41; P = 0.002), as shown, some patients with BMI over 30 show no steatosis at all or only minimal amount of liver fat, in contrast with some others with BMI below 25 and marked steatosis in their livers. BMI: Body mass index.

some cases showed only minimal steatosis despite the fact that the patient was overweight. Some other cases showed considerable liver fat accumulation despite low BMI values (Figure 3), suggesting that factors other than BMI are involved in liver fat accumulation. This result is similar to that obtained by our group six years ago, in a different cohort of patients, in whom adiposity was assessed by waist circumference, triceps skinfold measurement, and BMI[12].

We have also shown that liver steatosis in HCV-infected patients is associated with trunk fat. This has been also reported by other authors[20,21], since, as mentioned above, it is generally accepted that trunk fat is associated with a more "noxious" adipokine secretion profile that is able to cause insulin resistance and a proinflammatory state. The opposite happens with peripheral fat. In this sense, we failed to find any relationship between liver steatosis and leg fat mass, as shown in Table 2. Therefore, in sharp contrast with trunk fat, which was clearly related to liver steatosis, liver fat accumulation seems to be independent of leg fat mass.

Regarding adipokines, adiponectin levels were significantly lower among patients than among controls, despite a similar BMI. Adiponectin was inversely related to lean mass, but not to fat mass or liver steatosis. However, it is important to highlight the inverse relationship between the trunk fat/leg fat ratio and adiponectin, fully in accordance with the observation of an inverse relationship between visceral fat and adiponectin levels in other settings[22]. Although there is little doubt about the protective role of adiponectin in steatohepatitis (it has been described that adiponectin antagonizes the effects of TNF-α[23]), in the present study, there seems to be no association between adiponectin levels and liver steatosis, despite the fact that their serum levels are lower in HCV patients in comparison to controls. This is not a universal finding. The studies on the levels of adiponectin in HCV-related steatohepatitis had been controversial[7-9,24-28]. It is also remarkable that we found, in accordance with the protective effect of adiponectin on vascular risk, a significant correlation between adiponectin and high density lipoprotein cholesterol (ρ = 0.56; P < 0.001), as other authors also did[29].

We also failed to find differences in resistin and leptin between patients and controls, or when these adipokines were compared among patients with intense or less intense steatosis. Leptin, a fat derived cytokine, may promote fibrogenesis through up-regulation of TGF-β[30], but also protects the liver from fat accumulation, by lowering the expression of SREBP-1[31]. These nearly opposite effects may explain, perhaps, disparate findings in relation to leptin levels in chronic HCV infection[32]. Indeed, there is also controversy regarding the levels of leptin in HCV-related steatohepatitis[10,11,33-35].

Hyperinsulinaemia decreases synthesis of apoB-100, thus preventing very low density lipoproteins formation and leading to liver steatosis. Moreover, transcription of lipoprotein lipase is decreased by TNF-α, leading to hypertriglyceridaemia[36]. Most of the results observed in this study sustain this hypothesis: We did find hyperinsulinemia and increased HOMA index in patients with more intense steatosis. This result is fully in accordance with the current knowledge, since insulin resistance leads to an ongoing lipolysis that overwhelms the liver capacity to metabolize them.

Genotype 3 infected patients usually show a more intense degree of steatosis, and it has been shown that it exerts a direct cytopathic effect on liver cell leading to steatosis[37]. Concordant with this, patients infected with genotype 3 showed a more intense liver steatosis than those genotype non-3 infected ones, but no significant differences were observed in nutritional anthropometric parameters among them. Also, although the number of patients infected with genotype 3 HCV was low, in one case no fat at all was observed in the liver, and this proportion was similar in HCV genotype non-3 patients. In fact, we have failed to find any difference in adipokine and/or cytokine profile between patients without fat and with fat in the liver. The only independent variable related to the intensity of liver steatosis or to the presence of liver steatosis was trunk fat. Lean mass parameters seem to play no role at all, and insulin resistance, assessed by HOMA, and IL-6 levels were also related to liver fat stores in the univariate analysis, being displaced by trunk fat mass in the multivariate analysis.

Therefore, we conclude that steatosis in chronic hepatitis C is a common event (67.86%), and is closely related to trunk fat, but not with leg fat mass; to insulin resistance, and to IL-6. The main factor involved is trunk fat, despite the normal BMI of the patients included in this study, and also despite the fact that at least 12 patients with BMI over 25 kg/m^2 showed no liver steatosis, or minimal amount of it, as shown in Figure 1. The reasons for this finding are unclear, and suggest that

factors other than BMI, HOMA or fat mass should be involved. The results here presented also do not support the hypothesis that lean mass plays a role in liver fat accumulation.

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COMMENTS

Background

Hepatitis C virus (HCV) infection is a common disease, ultimately leads to liver cirrhosis and hepatocarcinoma. Liver steatosis is an early finding in these patients. Mechanisms are poorly understood, although it is known that HCV genotype 3 may lead to steatosis. Possibly, trunk fat and some adipokines may be also involved.

Research frontiers

There is a lot of controversy regarding the association of main adipokines, such as adiponectin or leptin, with liver steatosis, and their role in the progression of simple steatosis to liver inflammation. In addition, although there is general agreement in the association between obesity and liver steatosis, the relationship between fat distribution at different body compartments is not well defined. Moreover, there are some studies that also suggest a role of lean mass in liver steatosis.

Innovations and breakthroughs

In this study the authors report that liver steatosis in chronic HCV infection is a common, but not universal event (67.86%). It is closely related to trunk fat and to interleukin (IL)-6, a cytokine that may be produced by trunk fat, but not with fat at the legs, and also to insulin resistance. However, there are still some unexplained results: The relationship between liver steatosis and trunk fat was observed despite the normal body mass index (BMI) of the patients included in this study, and also at least 12 patients with BMI over 25 kg/m^2 showed no liver steatosis, or minimal amount of it. In addition, their results also do not support the hypothesis that lean mass plays a role in liver fat accumulation.

Applications

This study provides new data relative to the association of liver steatosis with several adipokines and inflammatory cytokines in HCV-infected patients. As mentioned above there is considerable controversy regarding levels of some of these cytokines in HCV-infected patients, and even opposite results have been reported by several groups. In addition, this study underscores the role of trunk fat in liver steatosis, despite normal BMI, and does not support to the hypothesis that lean mass could play a role.

Terminology

Cytokines are small molecules with protean effects on inflammation and immune response, among many other effects on most organs. Tumor necrosis factor alpha is one of the first cytokines described, initially as the factor responsible for tumor-induced cachexia. IL-6 is a proinflammatory cytokine, that also bears an immunomodulatory effect. Adipokines are cytokines secreted by adipose tissue.

Peer-review

In this manuscript, the authors described about effects of adipokines, cytokines, and body fats on liver steatosis in hepatitis C patients. The key results are very interesting to the readers of HCV and other hepatic diseases.

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